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Enzymology in perchlorate rich, multi-extreme environments

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Abstract

The potential for life on Mars is one of the most interesting and yet elusive questions in modern science. The surface of Mars holds little prospect for biology due to the large daily temperature ranges, ionizing radiation, the presence of deleterious salts and the absence of liquid water, besides many other contributing factors. However, deep beneath the surface of Mars we may find environments which, while extreme in their own right, are free from some of the more destructive factors experienced on the Martian surface. The deep subsurface of Mars may hold liquid water environments, which would experience high environmental pressures due to their subterranean nature, while also experiencing extremely low temperatures, perhaps as low as -70 °C. In order for such an aqueous environment to remain liquid at such low temperatures, it would require the presence of saturating concentrations of perchlorate salts which have the ability to lower the freezing of water to temperatures around -80 °C. Such an environment provides us with three parameters, perchlorates, pressure, and temperature, against which we can determine the potential for proteinaceous biochemistry to exist in such an extreme environment. How each of these individual factors affect proteinaceous biochemistry is relatively well understood, but we know practically nothing about how these factors interact in combination to ultimately affect biochemistry in such a multi-extreme environment. This is explored throughout this thesis by investigating the effects of perchlorate salts, high pressures, and low temperatures on the activity and stability of the model enzyme α-chymotrypsin. Additionally a meta-analysis of cold adapted enzymes was conducted in order to facilitate a better understanding of the fundamental adaptations which allows enzymes to become more active at low temperatures. Through this research, I found that while perchlorate salts lower the enzyme activity of α-chymotrypsin, high pressures can rescue this lost activity. Furthermore, the perchlorate induced loss of enzyme activity is found to be temperature dependent, as I have shown that perchlorate salts can increase the activity of α-chymotrypsin at low temperatures. These results suggest that while perchlorate rich environments are generally deleterious towards proteinaceous biochemistry and life, the high pressures of deep subsurface environments may counteract some of the negative perchlorate effects, and that the perchlorate salts themselves may actually facilitate increased biochemical potential at low environmental temperatures. While this data does not suggest that perchlorate rich environments are necessarily habitable or inhabited, it does provide us with a mechanistic understanding of how biochemical adaptations could advantageously use physical parameters such as temperature and pressure in order to increase biomolecular perchlorate tolerance.
Lay Summary

When trying to understand the potential for life in environments beyond Earth, we first have to understand how life as we know it responds to such environments. Mars has become a particularly interesting focus of our scientific efforts due to the fact that we believe it was once looked very much like Earth. However, over billions of years, the surface of Mars has changed from being a potential Earth 2.0, to being the barren and desolate landscape which we now see when we look across the Martian horizon from the eyes of the robotic explorers we have sent to scout the land. From the data obtained by rovers and orbiting satellites around Mars, we now know that the surface of Mars is a harsh and unforgiving environment. For example, the temperature on the surface can vary wildly over the course of a day, from around 20 °C to as low as -60 °C. The surface is also continually bombarded by ionising radiation due to the thin atmosphere which Mars currently possesses, and additionally, Mars is covered in salts, including the iron oxides which give Mars its characteristic red hue. Perhaps though the most important feature of the Martian surface, is that it is completely devoid of liquid water. Whenever we look for life elsewhere, what we tend to look for first is the solvent, and in our case the only solvent we know of that can definitively support life is water. Mars’ surface water has long since either evaporated out into space, or has been locked away in the crust as hydrated salts.

While the Martian surface lacks aqueous environments, the deep subsurface may in actual fact contain bodies of liquid water. These could either take the form of deep groundwater, trapped beneath the frozen Martian cryosphere, or they could be subglacial lakes beneath the Martian polar ice caps. These subsurface environments would also experience other environmental factors such as high pressures and sub-zero temperatures. In order for the water in these environments to remain in a liquid state at sub-zero temperatures they would have to contain high concentrations of salts which can greatly depress the freezing point of water. In particular, these environments could contain high concentrations of perchlorate salts which are capable of reducing the freezing point of water to as low as -80 °C. However we do not know if such a harsh environment is habitable and therefore this thesis seeks to explore this question. Instead of looking at whether or not life can survive in perchlorate rich environments, I instead have looked at whether the biomolecular machinery which makes up life, can function under such conditions. To understand the ability for proteins to function in these conditions, I have used α-chymotrypsin, an enzyme found in animals which helps in the digestion of other proteins. In this thesis, the activity and stability of α-chymotrypsin is explored across perchlorate salt concentrations, high pressures, and at low
temperatures to aid in our understanding as to whether biochemistry, as we know it, can function in Martian environments.

The initial results showed that perchlorate salts reduced the activity and stability of α-chymotrypsin. This suggests that perchlorate salts are deleterious towards biochemistry and limits the potential for life in such environments. However, it was then important to examine the activity of α-chymotrypsin in the presence of perchlorates when combined with the other environmental factors such as high pressure and low temperatures. I found that high pressures actually have the ability to increase α-chymotrypsin’s activity, even in the presence of perchlorate salts. This shows that while perchlorate rich environments are bad for biology, if they are found deep underground, the high environmental pressures may be able to undo some of the negative perchlorate-induced effects. In order to understand how temperature may affect my enzyme, I completed a meta-analysis of temperature-adapted enzymes, to determine what were their main defining characteristics. It showed that a large gap between the optimum temperature for an enzyme’s activity and its melting temperature is a strong defining characteristic of cold adapted enzymes. Therefore for a protein to function at low Martian temperatures, it would need to have a flexible active site, yet be structurally sound enough to withstand the high concentrations of perchlorate salts. When the effect of temperature and perchlorates on α-chymotrypsin was considered, I found that perchlorate salts actually increase the activity of α-chymotrypsin at low temperatures, contrary to what I observed at room temperature. It was hypothesised that perchlorates lower the enzyme activity at room temperature by partially unfolding α-chymotrypsin, but this unfolding in fact makes α-chymotrypsin more flexible and thus allowing it to exhibit more activity at low temperatures in a similar fashion to cold adapted enzymes. When taken together, these results confirm the deleterious nature of perchlorate salts, but show that when they are examined in combination with other important environmental factors, their deleterious effects can be reduced, and are even sometimes beneficial for biochemistry. This suggests that perchlorate brines may be more habitable if they are found in the deep subsurface, with low environmental temperatures.
Declaration

I declare that this thesis has been composed by myself and that this body of work has not been submitted for any other degree or professional qualification. I further confirm that the work submitted is my own except where it is formed of publications produced through collaborative research. My own contribution to these published works and those of my co-authors are described below for each chapter.

The publication in Chapter 3 originally appeared in Astrobiology by Stewart Gault and Charles S. Cockell (supervisor). I, Stewart Gault, designed this study, conducted the experiments performed within, analysed the data, and produced the initial draft. Charles S. Cockell provided supervision for this publication. I and Charles S. Cockell both contributed to editing, data interpretation, response to reviewers and the editing and final formatting of the publication.

The publication in Chapter 4 originally appeared in Communications Biology by Stewart Gault, Michel W. Jaworek, Roland Winter and Charles S. Cockell (supervisor). I, Stewart Gault, designed this study, conducted the enzyme activity experiments, produced the first draft of the manuscript, and coordinated the co-author’s contributions to the manuscript. Michel W. Jaworek conducted the FTIR analysis and wrote FTIR relevant parts of the manuscript. All authors contributed to data interpretation, editing, response to reviewers and the final formatting of the publication.

The publication in Chapter 5 originally appeared in Bioscience Reports by Stewart Gault, Peter M. Higgins, Charles S. Cockell (supervisor) and Kaitlyn Gillies. I conceived the study, created the datasets, analysed the data, and produced the initial draft of the manuscript. I additionally acted as the supervisor for Kaitlyn Gillies, a Nuffield Summer Research student, who assisted in data gathering. Peter M. Higgins provided a python script in assistance with data analysis. I, Peter M. Higgins, and Charles S. Cockell contributed to the editing of the manuscript, response to reviewers and the final formatting of the publication.
The publication in Chapter 6 originally appeared in Scientific Reports by Stewart Gault, Michel W. Jaworek, Roland Winter, and Charles S. Cockell. I developed the theoretical premise for the work, designed the experiments, conducted the ambient pressure enzyme kinetics experiments, performed the kinetic thermodynamic analysis, produced the first draft of the manuscript, and coordinated with co-authors for their initial manuscript contributions. Michel W. Jaworek performed the high pressure kinetics experiments and the FTIR experiments and contributed relevant parts of the initial manuscript draft. All authors contributed to the data interpretation, editing, response to reviewers and final formatting of the publication.

Signed:

Stewart Gault
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Chapter 1

1. Introduction

1.1 Mars

For centuries, Mars has captured the imagination of scientists and the public alike for the alluring possibility that it may harbour life. In the late 19th century, Schiaparelli’s description of “canali” on the Martian surface led to speculation that they were created by a technologically advanced civilisation capable of engineering on a global scale. Such speculation finally gave way to the progress of science, and the dawn of Martian rovers and orbiters provided us with an altogether more bleak outlook for life’s prospects on Mars. Our robotic eyes have reported that the surface of Mars is arid, cold, rich in salts and constantly bombarded by radiation. These harsh conditions present considerable challenges to the habitability of the modern Martian surface, and chief amongst them is the absence of liquid water[1].

All life that we know of, is inextricably linked to the presence of liquid water. Not only is liquid water the solvent for biochemistry, but it is also one of the largest habitats for life on Earth. Therefore when we seek to find life on other planetary bodies in our solar system, we prioritise areas where liquid water may be present. On Mars, we face the problem that the average surface temperature is roughly 210 K (-60 °C), which is significantly lower than the freezing temperature of pure water at 273 K (0 °C). Therefore we do not expect to find stable liquid water on the Martian surface. However the discovery of perchlorate salts on Mars and deep subsurface environments may provide the right conditions for liquid water to exist in a stable state.

1.1.1 Perchlorate salts on Mars

In 2009, Hecht et al.[2] published a study detailing the discovery of perchlorate salts on Mars. They used the data from ion-selective electrodes as part of the wet chemistry lab on the Phoenix Mars Lander to describe the soluble chemistry at the landing site. These electrodes were initially intended to detect the presence of nitrates, but if the signal they detected originated from nitrates then the mass of nitrates would actually be greater than the mass of the sample. Thus perchlorates were deduced to be the source of the signal and their relative abundance was calculated to be ~0.5 %wt of the Martian soil at the Phoenix landing site. Since the 2009 detection, other groups and instrumentation have reported the presence of perchlorates on Mars[3].
The detection and widespread nature of perchlorate salts on Mars raises the central question of how they are formed? Perchlorate salts are relatively rare on Earth with them being found in typically dry environments such as the Atacama Desert[4,5] and the Antarctic Dry Valleys[6] and so it was a surprise to find them so widely distributed on Mars.

The precise mechanism of perchlorate formation on Mars has not yet been elucidated but models have been proposed. It has been suggested that ultraviolet (UV) irradiation could be the causative mechanism by which perchlorates are formed. One study[7] demonstrated that UV irradiation of sodium chloride on titanium dioxide under aqueous conditions could produce perchlorates according to the following half equation:

$$\text{Cl}^- + 4\text{H}_2\text{O} + 8\text{H}^+ \rightarrow \text{ClO}_4^- + 8\text{H}^+ \quad E_0 = 1.39\text{V}$$

However, this reaction requires an aqueous environment at the Martian surface, which, as discussed earlier, is not possible in the present-day. It could be argued that the perchlorate was formed during an ancient, wetter climate on Mars, but this suggests that there is no modern day perchlorate formation and therefore that ancient perchlorate has not become buried by dust over the course of geological time. The ancient nature of Martian perchlorates has been recently challenged by Martin et al. 2020[8], suggesting that the Martian perchlorates should at least be as young as the Amazonian era and alternative mechanisms allow for modern day perchlorate production on Mars. A further limitation of this study is that these reactions took place at standard room temperature, whereas on Mars temperatures regularly fall below 250 K. While this does not affect the fundamental thermodynamic feasibility of the reaction, the kinetic viability of the reaction becomes questionable, especially since water would mainly be present as ice instead of the liquid form necessary for this reaction.

Carrier and Kounaves (2015)[9] provided a mechanism by which UV irradiation could form perchlorates on modern Mars without the need for an aqueous environment. The authors show that UV irradiation can oxidise sodium chloride to perchlorate in the presence of silica, iron, aluminium, and titanium oxides. The authors highlighted the role of the silicate and metal oxides for their photocatalytic ability to produce $\text{O}_2^-$ radicals which are required for the production of perchlorates. This mechanism benefits from the lack of water in the reaction pathway but still falls into the potential kinetic trap of being conducted at room temperature.

In similar work, Zhao et al. (2018)[10] suggest photochemical production of perchlorate from chloride bearing evaporites with UV light. The authors took chloride bearing brines and evaporated them onto silica beads, thus mimicking an evaporite deposit you would expect to find on the surface of Mars. Upon UV irradiation the evaporites readily produced perchlorates. Again, this experiment was conducted at 25 °C, so it should be kinetically
much slower on Mars, but the reaction has the benefit of not requiring aqueous water. The authors conclude that two major pathways contribute to the accumulation of perchlorates on Mars, volcanic outgassing of chlorides reacting with oxidants in the atmosphere leading to deposition of perchlorates and additionally UV irradiation of evaporitic surfaces in contact with atmospheric oxidants.

The previous studies rely on surface mediated perchlorate formation, whereas the next studies to be considered suggest the possibility of perchlorate formation in the atmosphere. Catling et al.[5] suggest that the formation of perchloric acid in the atmosphere from hydroxyl radicals and chlorine trioxide followed by dry deposition on the surface could explain the presence of Martian perchlorates whilst also linking it back to a possible mechanism of perchlorate formation in the Atacama Desert. The reaction follows this general scheme (where M is a buffer gas, e.g. CO$_2$):

\[ \text{ClO}_3 + \text{OH} + M \rightarrow \text{HClO}_4 + M \]

This reaction is also dependent on UV irradiation. The scenario depicted here has been called into question by Smith et al.[11] who applied this model to Mars and found that the rate of perchlorate formation would be 7 orders of magnitude beneath what would be needed to account for the modern perchlorate observations on Mars.

A further study contemplating the origin of perchlorates on Mars does away with the reliance on UV irradiation and instead investigates the role that galactic cosmic rays may play in perchlorate production[12]. Their model relies on galactic cosmic rays penetrating the thin Martian atmosphere and producing secondary electrons on contact with the surface where reactive molecules sublime into the atmosphere and react to form perchlorate. Their reaction process requires the presence of chlorine dioxide being formed:

\[ \text{Cl} + \text{O}_2 + M \rightarrow \text{ClO}_2 + M \]

From chlorine dioxide the following reaction scheme is then possible and would form perchlorate:

\[ \text{H}_2\text{O} + h\nu \rightarrow \text{OH} + \text{H} \]
\[ \text{CO}_2 + h\nu \rightarrow \text{CO} + \text{O} \]
\[ \text{OCIO} + \text{O} + \text{CO}_2 \rightarrow \text{ClO}_3 + \text{CO}_2 \]
\[ \text{ClO}_3 + \text{OH} + \text{CO}_2 \rightarrow \text{HClO}_4 + \text{CO}_2 \]

Steele et al. (2018) suggest electrochemical origins of perchlorate[13] without the need of irradiation. This mechanism however is speculative and is based off the detection of perchlorate in meteorites and has not been demonstrated in Mars like conditions.
Ultimately it is likely that multiple mechanisms contribute to the formation of perchlorates on Mars. While debate exists as to which model is the major contributor, for all practical purposes the important factor is that we know that perchlorates are present on Mars and that their presence has important consequences for life.

The major consequence of the presence of perchlorate salts on Mars is with regards to the existence of liquid water. This is due to the extremely low eutectic points of perchlorate brines. For magnesium and calcium perchlorate, their respective eutectic temperatures are 204 and 198 K[14] which means that it may be possible to have liquid water habitats on Mars in regions which would normally be too cold to harbour an aqueous environment. These extremely low eutectic temperatures are only reached when a solution is saturated with perchlorate salts, and the Martian surface’s perchlorate concentration of 0.5 – 2 %wt[2] is too low to achieve the desired eutectic temperatures. Therefore, a mechanism of concentrating the perchlorates salts is needed, and at the Martian south pole we may have examples of where this has happened.

1.1.2 Deep aqueous perchlorate brine environments on Mars

Deep subsurface perchlorate rich brines were initially suggested as theoretical considerations for how deep and extensive the Martian groundwater may be beneath the cryosphere[15]. Clifford et al. (2010) suggested that the groundwater on Mars could exist up to depths of ~10 km beneath the Martian surface supposing that it was rich in perchlorate salts. As these depths are exceedingly beyond the sensing capabilities of rovers and orbiters, the case of deep Martian groundwater largely remains one of a theoretical nature as to whether it genuinely exists and as to what extent it is habitable.

In 2018 and 2021, evidence for the presence of liquid water beneath the Martian southern polar cap[16,17] was reported. This came from data obtained by ground penetrating radar onboard the MARSIS orbiter. The temperature at the base of the Martian south pole is predicted to in the region of 200 K, significantly lower than the standard freezing point of water. This led the authors to postulate that this water must be a highly concentrated perchlorate brine as perchlorates are the only known chemical species on Mars capable of depressing the freezing point of water to such low temperatures. This observation was not without its own controversy and debate. Other studies reported that the temperature beneath the Martian south pole would be lower than previously estimated[18] and that a local thermal anomaly would be required for the presence of liquid water.
Additionally others have argued that the reflections which were interpreted as water, can also be explained by volcanic clay materials[19]. The authors who originally reported the subglacial lake data have responded to these criticisms with data which suggests that clay minerals do not best represent the observed bright reflections and that perchlorate or chloride brines best fit the radar reflections[20]. However a question remains: if such a subterranean environment was present, would it even be habitable?

Deep aqueous environments on Mars would present unique environmental challenges which must be considered when investigating the habitability of such environments. These deep environments are largely protected from the more deleterious facets of the Martian surface such as the radiation, the wind, and the absence of available water. This, however, does not mean that deep aqueous environments on Mars are not without their own challenges to life. The exceedingly high concentration of perchlorate salts represents the first environmental challenge that life would have to surmount in order to survive in these waters. Perchlorate salts are widely regarded as being extremely deleterious to life as we know it. There is no known species on Earth that is capable of surviving in molar amounts of either magnesium or calcium perchlorate brines. We do see some species surviving in relatively high concentrations of sodium perchlorate, even up to 2.4 M[21]. However, as will be demonstrated in this thesis, sodium perchlorate is far less deleterious than its magnesium and calcium containing counterparts. As a result, we yet again do not know how life on Mars may adapt to tolerate and withstand such high concentrations of these salts. How life has come to tolerate other saturated salt solutions such as in sodium chloride brines or sulphate brines, may inform us as to some possible mechanisms of adaptation, while always bearing in mind that adaptation to “salinity” is not necessarily a universal response and that specific salts may require specific adaptations.

Secondly, these deep environments are extremely cold, in the range of 200 K cold. There is no known terrestrial life that can function optimally at such low temperatures. This is of course not surprising as there are no analogous environments here on Earth because any water at such temperatures would be frozen and thus no life has faced selection pressures promoting survival in such an extremely cold liquid environment. Fundamentally, knowledge is lacking on how life can respond or survive at such low temperatures, thus limiting our understanding of how any extant Martian life could do so. This is the first terra incognita that we are faced with in exploring the habitability of these deep aqueous environments.

The third environmental parameter examined in this thesis which is also present in deep subsurface environments is the high pressures experienced in such habitats. Pressure can largely be seen as the most benign of the three parameters explored as it rarely imposes limits to habitability. Later, I
discuss how the pressure ranges of terrestrial habitats is generally far from the enormous pressures required to actually unfold a protein and thus reduce habitability. While pressure rarely excludes the possibility of life, we do see examples of where it requires additional adaptation, specifically in the lipid membranes of deep sea organisms[22]. The cold temperatures of the ocean has led to organisms increasing the fluidity of their lipid membranes in order to ensure its proper functioning. In the deep oceans, the effect of low temperatures on lipid membranes is compounded by the ordering effects caused by high pressures on lipid membranes. In response to this, organisms from deep ocean environments exhibit lipid membranes that are even more fluid than those from shallower waters[22]. So while pressure is rarely the limiting factor in habitability, it can necessitate adaptation, and as seen in Chapter 4 it can in fact provide unexpected benefits to potential life in deep subsurface Martian environments.

Our lack of knowledge with regards to how low temperatures and high perchlorate concentrations ultimately affect habitability is compounded by the fact that we do not know how pressure, perchlorates, and temperature act in concert to affect the habitability of such environments. As others have shown it is important to consider all the extremes of an environment in combination as their interactions ultimately affect one another and how life can respond to multiple extremes[23], thus not only generating extremophiles, but polyextremophiles.

1.2 The extremophiles

Earth hosts many unique environments, in which life has had to adapt to each new environmental challenge it has faced in each new niche. As a result, all species are uniquely suited to exist in the environments in which we find them. Some environments are more "extreme" than others in their nature. These environments are characterised by parameters such as extremely high or low temperatures, high pressures or even extremes of pH. These environments are the exclusive domains of the extremophiles.

Extremophiles have adopted unique and specific adaptations which allow them to exist in their environments. In many cases, they can only survive in their extreme environments and if brought to more moderate conditions they will perish. They are therefore not only adapted to, but inextricably obligated to their niche.

The adaptive solutions which extremophiles have produced in response to extreme environments can be used to infer adaptations which may be possible in environments beyond Earth. As has already been described, Mars contains potential aqueous environments which would be subjected to extremes of salinity, pressure, and temperature. By studying how life on Earth has responded to these challenges, we can begin to understand what limits these environments place on biochemistry. Life adapted to these
conditions, halophiles, piezophiles and psychrophiles and their biochemical adaptations are examined in the following sections.

1.3 The biomolecules of life

1.3.1 Proteins

Proteins are the executors of biological function. Their roles in cells range from cell structure, communication, regulation, and enzymatic catalysis. These diverse and essential functions make them appropriate models for investigating the effects of extraterrestrial conditions on biomolecules and thereby assessing habitability. Exploring the effects of extreme environments to the basic units of biochemistry further expands our understanding of cellular responses to such extreme conditions and also informs us as to the habitability of environments beyond the confines of Earth.

Proteins are 3D globular macromolecules which exhibit four levels of structure. The protein primary structure is the exact sequence of amino acids which make up the overall protein. The secondary structure is the first level of folding in a protein once it exits a ribosome. The main secondary structural elements of protein folding are α-helices, β-sheets, loops and turns. These folding interactions are largely driven by hydrogen bond networks forming between the amino acids[24]. The protein tertiary structure is how these secondary structural elements then fold upon each other. The major driving force of this process is theorised to be the hydrophobic collapse[25] where hydrophobic amino acids are typically buried in the protein's core. This is due to interactions between water and charged amino acid residues being more favourable than between water and hydrophobic residues. This results in charged residues being predominantly exposed at the protein surface whilst the large, bulky hydrophobic residues are confined to the protein's interior. It is interesting to note that this phenomenon is reversed for membrane proteins whose exterior is exposed to the apolar acyl chains of phospholipids. Finally, protein quaternary structure is when two or more proteins come together to form complexes such as dimers, trimers and so on.

In this thesis, α-chymotrypsin was used as a model enzyme to study the effects of extreme environments on proteinaceous biochemistry. The activity that enzyme’s exhibit provides an additional parameter that can be explored in addition to protein structure and stability.

1.3.2 α-Chymotrypsin

α-Chymotrypsin is used throughout this thesis to investigate the effects of perchlorate salts on the activity and stability of proteinaceous enzymes in conjunction with variable temperatures and pressures. α-Chymotrypsin represents an archetypal serine protease with a deep history of scientific
research, therefore making it an ideal model enzyme for research. α-Chymotrypsin’s biological function is to cleave peptide bonds, allowing for the digestion of ingested proteins. However, unlike trypsin, α-chymotrypsin specifically cleaves the peptide bond after a bulky hydrophobic residue due to α-chymotrypsin’s altered dynamics[26]. α-Chymotrypsin’s mechanism of action, like other serine proteases, relies on what is known as the “catalytic triad”. The catalytic triad is composed of three amino acid residues within α-chymotrypsin’s active site, serine (hence serine protease), histidine and aspartate. The reaction mechanism is largely as follows: the aspartate residue attracts a hydrogen from an amide group on the histidine residue, the histidine now attracts the hydrogen from the serine hydroxyl group, thus greatly increasing the nucleophilicity of the serine’s reactive oxygen. This nucleophilic oxygen then attacks the carboxyl end of the peptide bond. This reaction breaks the peptide bond, releasing a portion of the protein, but the other portion of the protein substrate remains bound to the serine residue of α-chymotrypsin. It requires a water molecule to then enter the active site, become ionized and react with the histidine and the substrate carboxyl group that is bound to serine, thus releasing the substrate, and completing the catalytic cycle, ready for a new substrate to enter the active site. α-Chymotrypsin’s activity is aided by the presence of the “oxyanion hole” which dramatically stabilises the negatively charged transition state complex. This reveals the main mechanism by which enzymes are able to achieve astonishing reaction kinetics. It’s not because they contain the right reactive groups, but because their active site architecture greatly reduces the free energy required in order to achieve the transition state complex, and thus complete a reaction.

1.4 Effect of salts on biology

1.4.1 Halophiles: The salt lovers and their adaptations

The requirement of perchlorate salts for certain deep aqueous Martian environments necessitates that any life found within them must be extremely halophilic/ tolerant. Halophilicity as a concept generally applies to organisms which require dissolved NaCl for their optimum growth, however it can be broadly applied to describe the ability of an organism to tolerate any dissolved salts in its environment in a much more general sense. Halophilic adaptations to NaCl are extremely well characterised due to the salt’s prevalence on Earth and such adaptations may provide clues as to how life might adapt to tolerate the presence of perchlorate salts. Alternatively we may find that halophilic adaptations to NaCl are specific to that salt, and that adaptations to perchlorate salts may be unique in that respect also.

The first major challenge of living in a salty environment is regulating your water content, or osmoregulation. As water naturally diffuses down concentration gradients, organisms need mechanisms which allow them to
retain their intracellular water, otherwise they would shrivel and die. There are two mechanisms commonly employed by halophiles to facilitate osmoregulation, these are known as the “salt-in” and “salt-out” strategies. The salt-in strategy employed by microbes such as *Haloanaerobium praevalens* and *Salinibacter ruber* employs the intracellular accumulation of KCl to counterbalance the extracellular NaCl [27–29]. The salt-out strategy on the other hand uses the accumulation of organic compatible solutes such as betaine or ectoine to achieve halotolerance[30–32]. A general feature that is common to both strategies is that as the extracellular concentration of salt increases so too does the intracellular concentrations of salts or compatible solutes.

The second adaptation of halophiles to their salt rich environment is the acidification of their proteome[33–40]. This adaptation is, almost ironically, also required due to the high intracellular concentration of charged species as a result of the salting-in and out strategies described previously. For a protein to fold correctly in solution, it requires a critical amount of water surrounding it in order to drive the hydrophobic effect of protein folding. The intracellular accumulation of charged species binds water to various extents and thus lowers the water activity which can destabilise proteins[41–45]. Thus the acidification of the proteome increases the surface charge of proteins which in turn increases the hydrogen bond networks at the protein surface which aids peptide-water interactions. This is done by increasing the proportion of acidic residues such as glutamate and aspartate. This effect can be seen in the fact that the isoelectric points (pI) of halophilic proteomes is generally lower than those of non-halophiles[37]. A neat demonstration of how this effect works in reverse was shown by Li et. al. where they showed that the substitution of aspartate/glutamate for glycine was favoured in the evolution from halophiles to mesophiles in ubiquitin like proteins[46]. Proteins can also become adapted to high salt concentrations by reducing the hydrophobic patches on their surface without necessarily increasing the number of acidic residues[40]. It is also important to note that many halophilic proteins are not simply adapted to tolerate high salt concentrations, but in fact require them for optimal enzyme activity[41,42]. The propensity for acquiring negative charges on biomolecules in response to high salt concentrations is also seen in the lipid compositions of some halophiles. It has been observed that the lipid composition of halophiles tends to exhibit an increased number of negative charges through the use of glycerol and sulphate as the lipid head group[47,48].

**1.4.2 Effect of salts on proteins and enzymes**

Having considered the adaptations of halophilic proteomes to high salt concentrations, it is then pertinent to consider what effects salts actually have on protein structure, stability, and enzyme activity. The effects of salts on proteins seems, on the surface, to be a rather simple concept, but over a
century of work on the topic has revealed a surprisingly complicated underbelly. Studies on the effects of salts on proteins date back to the late 19th century with Franz Hofmeister who noted that salts could either increase or decrease the solubility of proteins[49]. He then ranked these salts on their ability to either increase or decrease the solubility of proteins, thus creating the famous Hofmeister series. In the modern literature we describe salts that reduce protein solubility as being “kosmotropic” (e.g. sulphates) and those which increase protein solubility as being “chaotropic” (e.g. perchlorates or thiocyanates). Kosmotropic salts are also associated with increasing a protein’s stability and enzymatic activity, whereas chaotropic salts tend to reduce a protein’s stability and enzymatic activity.

The effects that salts exert on proteins is easy to measure and observe, but the underlying cause of these effects has been a subject of scientific debate over recent decades. This is made even more difficult due to the fact that there are different explanations for salt effects depending on the concentration of said salt/ ion. Very low concentrations of ions such as phosphate or calcium can have dramatic effects on the activity of enzymes due to the fact that they can act as allosteric regulators of enzyme activity. This is generally due to the presence of pockets on an enzyme’s surface which have high binding specificity for these ions in order to function to the desired extent. Such effects can be extremely specific and therefore only require nanomolar amounts in order for their effect to be observed. At concentrations beneath 100mM electrostatic interactions dominate our interpretation for how salts interact with the bulk solvent, other salt ions, and proteins themselves. In this concentration range the effects of salts are largely dictated by how they affect the overall charge of the protein/ system. However at high salt concentrations, basically anything over 100mM, an electrostatic explanation of the results becomes less predictable and reliable and so the effects exerted by salts on proteins in these high concentration ranges are commonly grouped under the term “Hofmeister effects”. The initial explanation for Hofmeister effects was that salts affect the structure of “bulk water”, essentially suggesting that salts affect the hydrogen bonding network of the entire solvent[50]. This suggests that the altered hydrogen bond network of salty water is what affected the solubility and stability of proteins which were also in solution. This interpretation soon became questionable as our ability to measure the effects of salts in water improved. Evidence emerged which suggested that ions and salts in water do not significantly affect the structure of water beyond one or two hydration shells, thus directly contradicting the “bulk water” hypothesis. Recent experimental data and modelling shows that salts interact directly with proteins, and this forms the basis of our modern understanding of how salts affect proteins in solution[51]. The exact location and extent to which ions interact with the protein surface varies with ion charge and size.
Perchlorate salts are regarded as being particularly chaotropic in nature. This means that we typically expect them to reduce the activity of enzymes while also reducing protein stability[52]. Perchlorate salts exert these effects through their interactions with the protein surface. The perchlorate anion is large and has a low charge density, this means that it interacts poorly with water molecules and as such is normally described as being “weakly hydrated”, which is a general property of chaotropic anions. As the perchlorate anion is weakly hydrated, it is able to interact preferably with the surface of a protein[53]. The concept of preferential attraction versus exclusion is something which separates chaotropic anions from kosmotropic anions. Chaotropic anions preferentially interact with the protein surface and become locally concentrated compared to their concentration in the bulk solution, while the opposite is true of kosmotropic anions[54,55]. The perchlorate anion is specifically attracted to the α carbons of the protein backbone due to their slight δ+ charge but in general they are forming nonspecific transient interactions with the protein surface. As to how this causes protein unfolding is less well understood. The current theory suggests that the interactions between perchlorate ions and the protein alter the protein-water hydrogen bond network, thereby reducing the strength of intramolecular stabilising interactions whilst also increasing the solubility of hydrophobic residues which causes water penetration into the protein interior, thus leading to an unfolding event[51]. The extent to which chaotropic salts unfold proteins is largely concentration dependent and it’s typically this loss of macromolecular stability and structure which ultimately results in reduced enzyme activity in most cases.

1.5 High pressure biology

1.5.1 Life from the deep

As humans, we are accustomed to life at 1 bar of pressure, or even slightly less should we be from a mountainous region of the planet, and as such we have termed 1 bar of pressure to be normal. However, for the vast majority of life on our planet, 1 bar is a low pressure environment. With 70% of the Earth’s surface being composed of ocean, and an average depth of ~2000 metres, pressures up to 200 bar are exceedingly common on our planet. Venturing to the deepest parts of our ocean, the Marianas Trench at 10 km deep, we find life existing at 1 kbar of pressure. In addition to the deep aquatic habitats, the Earth’s crust may house a “dark biosphere” comprising the majority of the Earth’s biomass in deep subterranean environments at pressures well into the kbar range[56]. Life in these deep environments have either evolved to simply tolerate high pressures, such as piezotolerant species like diving animals, or have become obligately adapted to high pressures, such as some piezophiles. With high pressure environments necessitating biomolecular adaptations for survival, it is important to then
consider the effect that pressure has on proteinaceous biochemistry if we are to understand how it might affect life in deep perchlorate rich environments.

1.5.2 Effect of high pressures on proteins and enzymes

The effect of pressure on proteins and enzymes can largely be described in the context of Le Chatelier’s principle. If you increase the pressure of a system, the equilibrium point of the system’s constituent parts will move in order to occupy a lower volume. As the different conformations of proteins exhibit different volumes, increasing the pressure on a protein will cause a shift in the proportion of protein conformations, favouring those of a lower volume. At high enough pressures, proteins can undergo pressure induced denaturation[57], a process which is distinctly different from heat induced unfolding and is rarely associated with the complete loss of protein structure. Rather, pressure induced denaturation typically involves the elimination of void spaces in proteins by the penetration of water molecules into the protein interior[58]. The importance of water in pressure induced protein denaturation is highlighted by the fact that lyophilised or dried proteins exhibit pressure stabilities far in excess of when they are in solution. However, the pressure required to denature a protein is generally far in excess of the environmental pressures we see on Earth. As such, the most sensitive aspect of proteins with regards to pressure is their ability to polymerise, and this is where we see the bulk of protein adaptation towards pressure[59,60]. When two protein monomers (such as actin) polymerise, this results in the removal of water molecules from the site of polymerisation. This process normally increases the volume of the system, and as such, is disfavoured when the pressure is increased. Therefore the major adaptation of polymeric proteins to pressure is to either make the polymerisation process neutral with regards to pressure, or in fact make it a process of negative volume change, in which case pressure would then favour polymerisation. We see these adaptions in the cytoskeletal proteins of many deep sea animals and those which dive to depth as part of their lifestyle[59].

Applied to enzymes, pressure can either increase, decrease, or have no effect on the rate of catalysis, all of which depends on the volume changes throughout the reaction coordinate[61]. The volume difference of importance here is that between the enzyme-substrate complex (or any intermediate complexes) and that of the activated transition state complex. If the transition state complex exhibits a lower volume than the enzyme-substrate complex then pressure will favour the formation of the transition state complex and thus increase the rate of catalysis, and if the volume change is positive, pressure will decrease the enzymatic activity. Interestingly, the volume differences do not have to be large in order to see these effects. The activation volume of α-chymotrypsin is around -18 cm³/mol, a volume difference of roughly one single water molecule[62], and this small negative
volume change is enough to drastically increase the activity of α-chymotrypsin with increasing pressures.

1.6 Biology in the cold

1.6.1 Psychrophilic life

As Mars is a predominantly cold planet, we would expect that for any life to inhabit it, said life would need to tolerate low temperature environments. On Earth, cold environments represent a surprisingly large proportion of the available habitats. The ocean itself has an average temperature of ~4 °C, making it the largest low temperature habitat on Earth. In addition to the oceans, we find low temperature environments at both poles in the forms of frozen and ice covered environments such as cryopegs, ice veins, and the permafrost. These environments can exhibit blisteringly low temperatures, with Antarctica holding the record low of -88 °C. Yet despite these chilling temperatures, such environments can be teeming with life and are the rightful domain of psychrophiles, the cold lovers. Psychrophiles are organisms whose optimum growth temperature is below ~10 °C. While mammals such as whales, dolphins, and polar bears inhabit low temperature environments, they are not true psychrophiles as they maintain a steady body temperature of 37 °C. True psychrophiles have adapted their intracellular machinery to function at low temperatures, to the point where some microorganisms are obligate psychrophiles, meaning they wouldn’t survive at moderate temperatures.

One of the most typical psychrophilic adaptations is to the structure and content of the phospholipid bilayer[60]. Membranes require a certain degree of fluidity in order to function correctly, and this fluidity is strongly dependent on temperature. As the temperature is lowered, the fluidity of the membrane begins to decrease. Therefore, in order to maintain the required level of fluidity, psychrophiles have to increase the fluidity of their membranes. They do so by synthesising and incorporating unsaturated[63] or bulky branched[64] fatty acids into their phospholipid bilayers which reduce the number and strength of the Van der Waals interactions within the membrane, thus allowing the individual phospholipids to diffuse more easily throughout the membrane plane. The process of adapting membrane fluidity has been termed “homeoviscous adaptation”[65]. Beyond their lipid membranes, psychrophiles have also had to dramatically alter their proteins in order to function at low temperatures, the details of which are described below.

1.6.2 Effect of temperature on enzyme catalysis

Before considering the specific adaptations of psychrophilic enzymes, it’s important to understand the effect that temperature has on enzyme kinetics. As with standard chemical kinetics, enzymatic rates of reaction increase with temperature. Though, a major difference between chemical catalysis and
enzymatic catalysis is that after a certain point, increases in temperature will actually reduce the activity of an enzyme due to the heat-induced unfolding of the enzyme’s tertiary structure. Therefore enzyme’s have a point at which they exhibit maximal activity at their temperature optima, or $T_{opt}$.

The effect of temperature on reaction rates was largely described by Arrhenius by the following relationship:

$$k = A e^{-\frac{E_a}{RT}}$$

Where $k$ is the rate constant, $A$ is the preexponential factor, $E_a$ is the activation energy, $R$ is the gas constant and $T$ is temperature. As can be seen from the equation, the reaction rate increases exponentially with increasing temperature. In terms of enzyme kinetics, it is generally said that the rate doubles or halves with every 10°C change in temperature.

An alternative way to view the effect of temperature is through transition state theory, as done in this thesis by using the Eyring equation[66], shown below:

$$k_{cat} = \left(\frac{k_B T}{h}\right)e^{-\left(\frac{\Delta H^\ddagger}{R T}\right)+\left(\frac{\Delta S^\ddagger}{R}\right)}$$

Here $k_{cat}$ is the rate constant, $k_B$ is the Boltzmann constant, $h$ is the Planck constant, $\Delta H^\ddagger$ is the activation enthalpy, and $\Delta S^\ddagger$ is the entropy of activation. The difference between the two is that transition state theory posits the existence of an activated enzyme-substrate intermediate (ES$^\ddagger$) known as the transition state complex. The Eyring equation can then be used to elucidate the thermodynamic underpinnings of how temperature affects enzyme catalysis by calculating the enthalpy and entropy of activation ($\Delta H^\ddagger$, $\Delta S^\ddagger$).

1.6.3 Psychrophilic adaptations of enzymes

As previously described, enzymes lose activity exponentially with decreases in temperature. Therefore many mesophilic enzymes exhibit no or little activity at the temperatures in which psychrophiles thrive. This then begs the question as to how psychrophilic enzymes have become adapted to be active at these low temperatures.

In terms of enzyme kinetics, the major adaptations of psychrophilic enzymes is a higher $k_{cat}$ and a lower $K_M$ compared to mesophilic and thermophilic enzymes[67]. This can be achieved through a variety of adaptive mechanisms. A psychrophilic enzyme can increase its activity by having a larger active site[68]. This reduces the enzyme’s dependence on the diffusion of the substrate, but it also has the effect of making the enzyme less specific which can be the structural cause of the lower $K_M$ values. A larger active site can be achieved by replacing amino acids with bulky side chains[69], or in one example, using a metal ion to further open the active site[70].
Another distinct structural feature which psychrophilic enzymes use to increase their activity is their increased structural flexibility[71,72]. The increased flexibility of psychrophilic enzymes is a generally accepted adaptive feature, but it is not necessarily a universal feature of psychrophilic enzymes as evidenced by the comparison of the β-factors of homologous enzymes[73]. Evidence for increased psychrophilic flexibility and looser packing came from studies showing that psychrophilic enzymes were more susceptible to tryptic digests[74], and that fluorescence quenchers can more readily penetrate into the protein core. Additionally it has been shown that the introduction of flexibility can increase the low temperature activity of an otherwise thermophilic enzyme[75]. The increase in flexibility causes increased enzymatic activity due to the reduced number to stabilising interactions which need to be broken in order to facilitate catalysis. The concepts of looser enzyme structure and increased flexibility tie in incredibly well to the thermodynamics of psychrophilic enzyme catalysis.

As described previously, by examining the kinetics of enzymes across various temperatures and by using the Eyring equation, we can tease apart the thermodynamic differences between psychrophilic, mesophilic, and thermophilic enzymes. What we find is that psychrophilic enzymes exhibit a lower ΔH‡ and a more negative ΔS‡ compared to mesophilic and thermophilic enzymes, ultimately resulting in a lower ΔG‡[71]. The lower activation enthalpy stems from the fact that psychrophilic enzymes have to break fewer stabilising interactions in order to form the activated transition state complex[67] as result of their more flexible active sites. Thus, the lower requirement for activation energy allows psychrophilic enzymes to exhibit comparable activity to mesophilic enzymes at their respective physiological temperatures. However the enzymes pay an entropic price for the reduced activation enthalpy. As the enzyme is generally looser packed and is more flexible, it means that it can adopt a greater range of microstates and therefore when a psychrophilic enzyme forms the activated transition state complex there is a significantly greater reduction in entropy when compared to mesophilic or thermophilic enzymes[76]. This reveals two facts. Firstly, that there is a delicate balance between reducing the enthalpy required for catalysis and the entropic penalty you pay as a result in order to lower the Gibbs free energy of activation. Secondly, it reveals that the enzyme activity at low temperatures is largely dictated by the enthalpic terms, a feature which is highlighted in Chapter 6 of this thesis.

1.7 On the challenges of theoretical astrobiology from the biochemical perspective

The principal challenge to understanding the habitability of perchlorate-rich environments is that no life on Earth is specifically adapted to high concentrations of perchlorate salts. This is primarily due to the fact that aqueous environments concentrated with perchlorates are not naturally found
on Earth. While various studies have shown that certain species exhibit relatively high perchlorate tolerances[21,77], this tolerance is simply an exaptation of generally hardy species. Of the species on Earth that have evolved in the presence of perchlorates, the perchlorate reducing bacteria, they tolerate surprisingly low concentrations of perchlorate[78]. Additionally, their adaptation towards perchlorates seems more to be towards removing it from their local environment, than it is towards perchlorate tolerance. So while life on Earth has had approximately 3 billion years to evolve and adapt to molar amounts of salts such as NaCl, we really have no deep evolutionary history towards perchlorate adaptation. The problem is then further confounded when we consider the combined evolutionary challenges of perchlorate, low temperatures, and high pressures in a multi-extreme environment.

The second limit to understanding the potential for biochemistry in perchlorate rich brines is that we only have one example so far of what biochemistry actually is in terms of molecular composition. It may well transpire that our molecular constituents are simply incapable of folding and functioning effectively in high concentrations of perchlorate salts. This would bias us towards believing that such environments are strictly uninhabitable, at least to life as we know it. However, understanding the physiochemical interactions between perchlorate brines and abundant abiotic organic molecules may give us answers as to what chemical principles allow for a greater chance of biochemistry in perchlorate rich environments.
Chapter 2

Methods

2.1 UV/VIS Spectroscopy

When conducting enzymology experiments, we can use UV/VIS spectroscopy to determine the rates of reactions based on the changes in absorbance as a function of time. A UV/VIS spectrometer can measure the change in concentration of reactants/products by measuring their consumption/production. The change in concentration of the studied molecule is determined through the Beer-Lambert law which relates the absorption of the sample to the concentration through $A = \varepsilon CL$, where $A$ is the absorbance, $\varepsilon$ is the molar extinction coefficient, $C$ is the concentration and $L$ is the pathlength of the cuvette.

A general $\alpha$-chymotrypsin activity assay is conducted as follows:

Make 0.1M tris buffer with 0.01M CaCl$_2$ at pH 7.8, in addition to the desired salts at their respective concentration.

Make a 400 mM stock of substrate, then serially dilute into other desired concentrations in order to build an enzyme activity curve, these should be double the assay concentration as they will be diluted 1:2 during the assay. The assayed concentration here were 10, 20, 50, 75, 100, 150 and 200μM and hence their master stocks were double this concentration.

Make an initial stock of $\alpha$-chymotrypsin by adding 2mg of $\alpha$-chymotrypsin into 4ml of the desired Tris buffer.

Dilute enzyme mixture 1:2, then $A_{280}$ to determine the concentration of the stock.

Dilute enzyme stock to 40nM using more buffer.

Change the wavelength of the spectrophotometer to the correct wavelength. If BTEE is the substrate, then 256nm the absorbance peak of the product N-benzoyl-L-tyrosine. If using N-Succinyl-Phe-$p$-nitroanilide, then 410nm is the peak absorbance wavelength of the product $p$-nitroaniline.

Add 500 μl of enzyme to a UV compatible cuvette and place it into the spectrophotometer.

Add 500 μl of substrate with good mixing for accurate readings, hit start on the spec to begin the recording.

If using a spec that doesn’t have a computer with it, then record the absorbance at the start and again after 30 seconds. This will give you the
ΔA/S. To convert from ΔA/s to M/s you need to divide the ΔA/s by the molar extinction coefficient of the product. This is your rate per second in moles/s.

The low temperature spectrophotometer (located in the Swann building) follows largely the same steps as described above with a few extra considerations. The temperature within the spectrophotometer is controlled by a circulating water bath, the temperature of which is easily controlled by the manual controls on the water bath. When using the low temperature spectrophotometer it is important to equilibrate the substrate and enzyme at the assay temperature for a sufficient period of time in order to ensure that the assay components are at the correct temperature. As the software for the low temperature spectrophotometer is on a computer, it’s possible to do analysis through this software too. After finishing an assay the software will produce an absorbance trace across time. One can then select the kinetics tab which will produce a tangent to the curve between two user defined points. Once the tangent is selected the software will provide the ΔA/s, and if the molar extinction coefficient of 964 is already inputted to the software, then it can be automatically converted to M/s which is the rate to be recorded.

Once the user has rate data for all substrate concentrations a graph can then be constructed of substrate concentration vs rate. If using Graphpad Prism, once the data is input, select analyse, then non-linear analysis, then k_{cat}. When k_{cat} is selected the enzyme concentration must be input in the same concentration units as the substrate concentration. Doing so will produce a kinetic analysis which provides k_{cat}, K_M and from these the catalytic efficiency of k_{cat}/ K_M can be calculated.

The high pressure stopped flow spectrophotometer is based in the lab of Prof. Roland Winter at TU Dortmund and was produced by TgK Scientific. The high pressure spectrophotometer contains two syringes, one to be filled with enzyme and the other to be filled with the substrate. The system is then closed and brought to the desired pressure through the gas control panel. The wavelength of the spectrophotometer is adjusted as described previously to the desired value. Once all is ready, the reaction is initiated by a drive piston which injects a small volume of both syringes into the reaction vessel and starts recording the change in absorbance for the desired length of time.

2.2 Melting Curve SYPRO

To determine the melting point of α-chymotrypsin, differential scanning fluorimetry was used. Differential scanning fluorimetry can detect the melting point of a protein as the fluorescent dye used specifically binds to hydrophobic protein regions which become exposed upon unfolding. The binding of the dye can then be detected via fluorimetry through the increase in fluorescent intensity. This unfolding and binding typically produces a
sigmoidal curve, the midpoint of which is taken to be the melting temperature where the Gibbs free energy of folding is 0 and half the protein is assumed to folded and the other half assumed unfolded in a two-state transition.

These experiments were conducted using a Biometra TOptical real-time PCR machine which allowed for extremely precise control of the temperature gradient. The temperature ramp of the rtPCR was set at 1°C/min and from 5 to 74.5°C The fluorescent dye used was SYPRO which was excited at 490nm and its emissions were recorded at 580nm.

Initial experiments showed that α-chymotrypsin does not unfold in a manner detectable to the differential scanning fluorimeter at pH 7.8. Therefore in order to get a melting curve it was necessary to covalently modify the enzyme with an irreversible inhibitor Pefabloc SC. To do so, three milligrams of Pefabloc SC was added to a 40 μM stock of α-chymotrypsin for one hour.

After incubation, α-chymotrypsin is diluted to a working concentration of 10μM in Tris buffer containing the various assayed salts at a range of salt concentrations as described previously, and a SYPRO dye concentration of 5X (supplied at 5000X).

Pipette 50μl of each of these conditions into the 96 well PCR plate in triplicate, cover the plate with the film which helps prevent evaporation, then place the plate into the rt-PCR.

Once the data was collected, it was exported to Graphpad Prism for further analysis. A Boltzmann sigmoidal curve was fitted to the melting data and the midpoint of the inflection was taken as the melting temperature of α-chymotrypsin.

2.3 Circular Dichroism

Circular dichroism (CD) is a method of exploring the conformational structure of proteins. With CD you can determine the secondary structural composition of your protein if looking at the far-UV range, or you can get the fingerprint of your protein’s tertiary structure from its near-UV CD spectra.

Before using the CD, turn on the nitrogen generator the day before in order to ensure enough nitrogen has been produced to flush the chamber.

CD conducted on a Jasco J-815 spectropolarimeter with a 1cm path length quartz cuvette with the temperature maintained at 25 °C with a peltier block CDF-426S/15. Spectra represent the average of 10 scans with a scan rate of 10nm/min and a 1nm bandwidth between 260 and 320nm.

α-chymotrypsin concentration was maintained at 40 μM. Let the cuvette sit in the chamber for five minutes before starting the experiment so that it can be
flushed with nitrogen as failure to do so means oxygen might tarnish the mirrors within the CD.

To clean the cuvette between runs, fill the cuvette with the specific cleaner provided as it will remove any protein which may have stuck to the sides of the cuvettes and would contaminate future experiments. Once the cuvette is cleaned, use the compressed air canister to remove any residual water and moisture from the cuvette. Be careful not to spray too close to the walls and opening of the cuvette as doing so and spraying for too long can rapidly freeze the residual water due to the cooling effect of rapidly expanding gas. If this happens simply wait for the ice to thaw and try to remove it again with short, sharp bursts from the air canister.

2.4 HoTMuSiC + PoPMuSiC

HoTMuSiC and PoPMuSiC are browser based software for the prediction of changes to the melting temperature and free energy of folding of proteins upon amino acid substitutions. Both pieces of software implement statistical potentials to determine these thermodynamic changes. These statistical potentials account for factors such as the amino acid type, their volume, solvent accessibility, torsion angles and their geometric relationship to other amino acids.

As such, HoTMuSiC and PoPMuSiC require a protein databank (PDB) coordinate file in order to function. Therefore in order to create a database for HoTMuSiC and PoPMuSiC, the PDB was searched for proteins with the keywords “psychrophile”, “psychrophilic”, “thermophile”, and “thermophilic”. In addition to this, a literature search was conducted for the same keywords with regard to structural determination to help find more examples of temperature adapted enzymes which have had their structure determined. Once temperature adapted enzymes were found, the PDB was then searched for mesophilic versions, and when one was available it was included in the database.

To use the software, first make an account on https://soft.dezyme.com/. Then select either HoTMuSiC or PoPMuSiC. To use either software, enter the 4 figure PDB code, e.g. 2AYQ, then under “Select Mode”, choose systematic. The systematic mode means that the software will calculate the thermodynamic changes when each amino acid in your protein is replaced by each of the 19 other typical biological amino acids. When using HoTMuSiC, you can enter the T_m of your protein if it is known, doing so will increase the accuracy of the calculations.

After running the software two results files will be produced. One will show the average change at each amino acid position, whereas the other shows all
the individual substitution values at each amino acid position. For HoTMuSiC these values represent the $\Delta T_m$, where a negative value means a destabilising substitution and a positive value means the substitution is stabilising. For PoPMuSiC the values represent $\Delta \Delta G_r$, in this case positive values are destabilising whereas negative values are stabilising. You can then use either excel or a script to get the values into a useable format whereupon you can get the average change in the thermodynamic values across the entire protein.
Chapter 3

Perchlorate salts exert a dominant, deleterious effect on the structure, stability, and activity of α-chymotrypsin

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3.1 Introduction

In order to understand the potential for enzymatic biochemistry in perchlorate rich environments, it was first important to understand how my chosen model enzyme, α-chymotrypsin, behaved in the context of the major soluble ionic species we expect to find on Mars. Thus, in this paper, α-chymotrypsin’s activity, stability, and structure was assayed in the presence of Mars relevant salts. These salts included magnesium perchlorate (Mg(ClO₄)₂), sodium perchlorate (NaClO₄), calcium perchlorate (CaClO₄)₂, magnesium sulphate (MgSO₄) and magnesium chloride (MgCl₂). Magnesium, calcium, and sodium were the chosen as the metal cations to represent the perchlorates as they are expected to be the major cationic species present on Mars, as detected by the Phoenix Lander[2]. MgSO₄ and MgCl₂ were included as they are also expected to be major contributors to the soluble ionic chemistry of Mars[79,80] and thus it was important to assay them in conjunction with the perchlorate salts. As it is unlikely that any potential aqueous environments on Mars would contain only a single dissolved salt, it was thus also important to understand how the combined presence of various ionic species ultimately affected α-chymotrypsin. The salt concentrations assayed were as high as 1 M. While the average concentration of perchlorates on the Martian surface ranges between 0.5-1%wt, which corresponds to low mM concentrations, any evaporitic environments would be extremely concentrated, well into the molar range. Additionally, subsurface eutectic brines would necessitate molar concentrations of salts to sufficiently depress the freezing point of water to deep sub-zero temperatures. Therefore it was important to assess the functionality of biochemistry in concentrated brines to understand the habitability of extreme Martian environments.
Perchlorate Salts Exert a Dominant, Deleterious Effect on the Structure, Stability, and Activity of α-Chymotrypsin

Stewart Gault and Charles S. Cockell

Abstract

The presence of perchlorate ions on Mars raises the question of how these ions influence the biochemistry of any contaminant life introduced into the martian environment, or what selection pressures perchlorate ions exert on any environment that contains these ions, such as the Atacama Desert. In this study, we investigated the structure, stability, and enzyme activity of the model enzyme α-chymotrypsin in the presence of five Mars relevant salts, MgSO₄, MgCl₂, Mg(ClO₄)₂, Ca(ClO₄)₂, and NaClO₄. We found that all the perchlorate salts reduced the enzyme activity of α-chymotrypsin in a concentration-dependent manner, with Mg(ClO₄)₂ and Ca(ClO₄)₂ having the greatest effect. This observation extends to our structural studies, which show that 1 M Mg(ClO₄)₂ and Ca(ClO₄)₂ greatly alter the tertiary structural environment of α-chymotrypsin. We also found that all the perchlorate salts assayed reduced the melting temperature of α-chymotrypsin, whereas the sulfate and chloride salts were able to increase the protein melting temperature. We also demonstrated that a brine containing both perchlorate and sulfate ions exerts the same deleterious effects on α-chymotrypsin’s melting temperature and enzyme activity as that of a perchlorate-only brine. This suggests that the perchlorate salts exert a dominant, deleterious effect on protein biochemistry. These results indicate that although perchlorate salts are beneficial to the presence of liquid water due to low eutectic points, they also hamper the habitability of their own environment. Life in such brines would, therefore, have to adapt its cellular machinery to the perchlorate ion’s presence or find a way of excluding it from said machinery. Key Words: Perchlorate—Mars—Enzymology—Biochemistry—Protein—Astrobiology. Astrobiology 21, 405–412.

1. Introduction

The discovery of perchlorate ions on Mars by the Phoenix Mars Lander (Hecht et al., 2009) presents an environmental factor that may both help and hinder the habitability of Mars. On the one hand, perchlorate salts greatly depress the freezing point of water and so may increase the chance of liquid water on Mars (Chevrier et al., 2009). Further, perchlorate salts, which are oxidants, may also be involved in energy acquisition as seen on Earth in perchlorate-reducing bacteria (Coates and Achenbach, 2004). On the other hand, previous studies have shown the deleterious effects of perchlorate ions to microbial life (Laye and DasSarma, 2018; Stevens et al., 2019).

Perchlorate ions are found at the martian surface at ~0.5 wt % in the soil and may be concentrated in systems such as recurring slope lineae (Ojha et al., 2015) or in the subsurface, such as in the putative sub-glacial lake reported by Oroseei et al. (2018) or in deep groundwater (Clifford et al., 2010). Thus, perchlorate concentrations at the surface are in the range of millimoles, whereas environments in the deep subsurface may contain perchlorate concentrations that reach molar values.

To understand the habitability of environments containing perchlorate, it is important to investigate the effects that perchlorate salts have on life and its macromolecules. This information will allow us to assess the fate of biomolecules and organisms, for example from contamination on the martian surface. Studying how perchlorate salts affect the biological macromolecules produced by life on Earth may also offer insights into the adaptations that hypothetical martian life would have to evolve in response to perchlorate stress, and less speculatively, adaptations required in terrestrial environments, such as the Atacama Desert, that harbor perchlorates (Chong Diaz, 1994; Catling et al., 2010).

Mars also exhibits a rich solute chemistry beyond that of just the perchlorate ion. Multiple studies have suggested the presence of salts containing cations such as magnesium, sodium, and calcium, and anions such as chloride and sulfate (Squyres et al., 2004; Gendrin et al., 2005; Osterloo et al.,
2. Materials and Methods

Lyophilized \( \alpha \)-chymotrypsin from bovine pancreas, \( N \)-benzoyl-\( L \)-tyrosine ethyl ester (BTEE), \( \text{MgCl}_2 \), Tris HCl, and Pefabloc SC were purchased from Sigma Aldrich. \( \text{Mg(ClO}_4 \text{)}_2 \) and \( \text{NaClO}_4 \) were purchased from Alfa Aesar. \( \text{Ca(ClO}_4 \text{)}_2 \) was purchased from Acros Organics. \( \text{MgSO}_4 \) was purchased from Scientific Laboratory Supplies, and \( \text{CaCl}_2 \) was purchased from Fisher Scientific. SYPRO orange was purchased from ThermoFisher.

Bovine \( \alpha \)-chymotrypsin was used since it is a well-characterized and investigated enzyme whose response to a range of chemical conditions is well known (Warren and Cheatum, 1966; Blow et al., 1969; Broering and Bommarius, 2005). Thus, although its bovine origin might be considered of limited astrobiological relevance, its source is less important than the fact that it can be considered in the most generic terms to be a model protein catalyst with which to investigate ionic effects in any environment.

2.1. Mars-relevant salts

The salts investigated in this study were: \( \text{MgSO}_4 \), \( \text{MgCl}_2 \), \( \text{Mg(ClO}_4 \text{)}_2 \), \( \text{Ca(ClO}_4 \text{)}_2 \), and \( \text{NaClO}_4 \). These salts and their constitutive ions have been detected on the surface of Mars or theorized to be present in the subsurface (Squyres et al., 2004; Gendrin et al., 2005; Osterloo et al., 2008; Hecht et al., 2009; Clifford et al., 2010; Orosei et al., 2018). By selecting three salts with the magnesium cation and three salts with the perchlorate anion, it allows us to determine whether the observed results are due to the cation or anion, or to specific cation-anion pairs.

2.2. Enzyme activity assays of \( \alpha \)-chymotrypsin

Enzyme activity was measured with a Helios Gamma spectrophotometer. \( \alpha \)-Chymotrypsin stock was prepared with a Tris-HCl buffer (0.1 \( M \) Tris, 0.01 \( M \) \( \text{CaCl}_2 \)) at \( \text{pH} \) 7.8. \( \alpha \)-Chymotrypsin concentration was measured at 280 \( \text{nm} \) with an extinction coefficient of 51,000 \( M^{-1} \text{cm}^{-1} \) (Luong and Winter, 2015) and was maintained at 20 \( \mu M \) for all enzyme activity assays.

The BTEE concentrations used in the assays were 10, 20, 50, 75, 100, 150, and 200 \( \mu M \). The enzyme was allowed to equilibrate in the salt solutions for 5 min before the reaction was initiated with the addition of BTEE. The change in absorbance at 256 nm was measured for 2 min, and the rate was recorded as the average change in absorbance per second, \( \Delta A \text{s}^{-1} \). The change in absorbance was converted to molar concentration by using 964 \( M^{-1} \text{cm}^{-1} \) as the molar extinction coefficient for \( N \)-benzoyl-\( L \)-tyrosine (Dušeková et al., 2018), which is the product of the reaction with BTEE. The enzyme activity was measured in the presence of one of five salts \( \{\text{MgSO}_4, \text{MgCl}_2, \text{Mg(ClO}_4 \text{)}_2, \text{Ca(ClO}_4 \text{)}_2, \text{and NaClO}_4\} \) across a range of concentrations (0, 0.25, 0.5, 0.75 and 1 \( M \)). The data were plotted in GraphPad Prism with a least-squares fitting method according to the Michaelis-Menten equation:

\[
v = \frac{V_{\text{max}}[S]}{K_M + [S]}
\]

For the combined salt experiments the substrate concentration was 200 \( \mu M \) and the salts were maintained at 0.5 \( M \) for each respective salt.

2.3. Near-ultraviolet circular dichroism spectrum of \( \alpha \)-chymotrypsin

Circular dichroism (CD) spectra of \( \alpha \)-chymotrypsin were obtained by using a Jasco J-815 spectropolarimeter with a 1 cm path length quartz cuvette. The temperature was maintained at 25°C with a Peltier block CDF-426S/15. Spectra represent the average of 10 scans with a scan rate of 10 nm/min and a 1 nm bandwidth. Measurements were taken with an \( \alpha \)-chymotrypsin concentration of 40 \( \mu M \) in 0.1 \( M \) Tris buffer, pH 7.8, with no salt and 1 \( M \) of each of the salts.

2.4. Determination of \( \alpha \)-chymotrypsin’s melting temperature

To obtain a melting curve for \( \alpha \)-chymotrypsin, it was necessary to covalently modify the protein with the irreversible inhibitor Pefabloc SC. Three milligrams of Pefabloc SC was added to a 40 \( \mu M \) stock of \( \alpha \)-chymotrypsin and allowed to equilibrate for an hour. The enzyme concentration was maintained at 10 \( \mu M \) for all melting curve measurements. The same salts were used as previously described, at the same concentrations, except for 0.75 and 1 \( M \) \( \text{Mg(ClO}_4 \text{)}_2 \) and \( \text{Ca(ClO}_4 \text{)}_2 \), which did not produce measurable melting curves.

SYPRO Orange was added to all the melting curve assays at 5 \( \times \) concentration, and melting curves were obtained with a Biometra TOptical real-time PCR instrument by exciting at 490 nm and measuring the emission at 580 nm. The temperature ramp was set at 1°C/min and run between 5°C and 74.5°C. Fluorescent intensity increases as the protein unfolds before plateauing, whereupon a decrease in intensity occurs where protein aggregation is favored over SYPRO binding (Wright et al., 2017). The values where fluorescent intensity declined postmelt were excluded. The data were then fitted with a Boltzmann sigmoidal curve, and the midpoint of the slope was taken as \( T_m \).

3. Results

3.1. Effect of salts on \( \alpha \)-chymotrypsin enzymatic activity

Figure 1 shows the enzyme activity of \( \alpha \)-chymotrypsin in the assayed salts. As can be seen from Fig. 1A, \( \text{MgSO}_4 \) caused slightly reduced \( \alpha \)-chymotrypsin activity but not in
a concentration-dependent fashion. Figure 1B shows that MgCl₂ increased the activity of α-chymotrypsin across all concentrations tested.

Table 1 shows that MgSO₄ reduced both the $k_{cat}$ and $K_m$, whereas MgCl₂ increased both parameters. Despite these effects, the catalytic efficiency ($k_{cat}/K_m$) of α-chymotrypsin remained similar across all concentrations of both MgSO₄ and MgCl₂ with considerable overlap of the standard errors. Figure 1C and E show that Mg(ClO₄)₂ and Ca(ClO₄)₂ greatly reduced the enzyme activity of α-chymotrypsin in a concentration-dependent fashion. It must be noted that no enzyme activity was observed in the presence of 1 M Ca(ClO₄)₂ at BTEE concentrations beneath 75 μM. Therefore the Michaelis-Menten curve and the kinetic parameters in 1 M Ca(ClO₄)₂ should be seen to be representative and not descriptive. NaClO₄, Fig. 1D, caused a lesser reduction in α-chymotrypsin activity at each concentration.

Figure 1F shows the $k_{cat}/K_m$ of α-chymotrypsin in the presence of the three perchlorate salts [pink—Mg(ClO₄)₂, orange—Ca(ClO₄)₂, brown—NaClO₄] versus the concentration of perchlorate in solution. BTEE, N-benzoyl-l-tyrosine ethyl ester. Color images are available online.

3.2. Kosmotropic MgSO₄ cannot counteract the negative effects of chaotropic Mg(ClO₄)₂

In reality, any liquid water on Mars will not exist as a one-salt solution but is likely to exhibit a diverse range of ions in solution such as those described by Hecht et al. (2009). Some of these salts are likely to be kosmotropic, that is, order inducing, such as MgSO₄. To test the hypothesis that such kosmotropic salts could cancel out the disorganizing effects (chaotropic effects) of perchlorate salts, we assayed these salts in equimolar amounts. Figure 2 shows that 0.5 M MgSO₄ cannot undo the negative effects of 0.5 M Mg(ClO₄)₂. This suggests that even in a complex mixture of salts, there may be a dominant salt effect.

3.3. Mars-relevant perchlorates unfold α-chymotrypsin’s tertiary structure

To investigate the cause of the reduced α-chymotrypsin activity, near-ultraviolet (UV) CD spectra were obtained to compare the tertiary fingerprint of α-chymotrypsin in 1 M of each of the five studied salts. The CD spectra in Fig. 3 show a change in the tertiary environment of α-chymotrypsin in
Table 1. Catalytic Parameters $k_{cat}$, $K_m$ and $k_{cat}/K_m$ of $\alpha$-Chymotrypsin in the Assayed Salts at Each Concentration and in Buffer Alone

<table>
<thead>
<tr>
<th>Salt</th>
<th>[C]/M</th>
<th>$k_{cat}/s^{-1}$</th>
<th>$K_m/\mu M$</th>
<th>$k_{cat}/K_m/s^{-1}M^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td></td>
<td>55.48 ± 1.56</td>
<td>68.89 ± 4.82</td>
<td>8.05 x 10^5 ± 6.07 x 10^4</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.25</td>
<td>46.43 ± 2.63</td>
<td>56.59 ± 8.786</td>
<td>8.20 x 10^5 ± 1.36 x 10^5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>46.14 ± 3.89</td>
<td>58.93 ± 13.29</td>
<td>7.83 x 10^5 ± 1.89 x 10^5</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>52.88 ± 5.91</td>
<td>67.04 ± 18.73</td>
<td>7.89 x 10^5 ± 2.37 x 10^5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>50.99 ± 5.51</td>
<td>61.33 ± 17.25</td>
<td>8.31 x 10^5 ± 2.51 x 10^5</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>0.25</td>
<td>62.06 ± 5.18</td>
<td>81.17 ± 15.75</td>
<td>7.65 x 10^5 ± 1.61 x 10^5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>68.73 ± 5.66</td>
<td>97.43 ± 17.13</td>
<td>7.05 x 10^5 ± 1.37 x 10^5</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>70.82 ± 5.48</td>
<td>90.07 ± 15.45</td>
<td>8.76 x 10^5 ± 1.48 x 10^5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>60.18 ± 4.25</td>
<td>76.12 ± 12.91</td>
<td>7.91 x 10^5 ± 1.45 x 10^5</td>
</tr>
<tr>
<td>Mg(ClO$_4$)$_2$</td>
<td>0.25</td>
<td>41.38 ± 1.86</td>
<td>60.77 ± 7.11</td>
<td>6.81 x 10^5 ± 8.53 x 10^4</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>32.72 ± 1.31</td>
<td>54.31 ± 5.94</td>
<td>6.02 x 10^5 ± 7.02 x 10^4</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>19.10 ± 0.95</td>
<td>45.74 ± 6.76</td>
<td>4.18 x 10^5 ± 6.51 x 10^4</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>9.04 ± 1.20</td>
<td>38.57 ± 1.70</td>
<td>2.34 x 10^5 ± 3.28 x 10^4</td>
</tr>
<tr>
<td>NaClO$_4$</td>
<td>0.25</td>
<td>45.31 ± 2.10</td>
<td>61.54 ± 7.47</td>
<td>7.36 x 10^5 ± 9.57 x 10^4</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>47.61 ± 2.78</td>
<td>67.21 ± 9.75</td>
<td>7.08 x 10^5 ± 1.11 x 10^5</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>48.61 ± 2.61</td>
<td>66.21 ± 8.91</td>
<td>7.34 x 10^5 ± 1.06 x 10^5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>44.05 ± 1.58</td>
<td>61.40 ± 5.75</td>
<td>7.17 x 10^5 ± 7.19 x 10^4</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>22.59 ± 1.62</td>
<td>81.12 ± 18.16</td>
<td>2.78 x 10^5 ± 6.54 x 10^4</td>
</tr>
<tr>
<td>Ca(ClO$_4$)$_2$</td>
<td>0.25</td>
<td>37.89 ± 1.68</td>
<td>58.26 ± 6.72</td>
<td>6.50 x 10^5 ± 8.04 x 10^4</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>31.35 ± 2.15</td>
<td>56.35 ± 10.36</td>
<td>5.56 x 10^5 ± 1.09 x 10^5</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>11.53 ± 1.56</td>
<td>79.68 ± 25.34</td>
<td>1.45 x 10^5 ± 5.01 x 10^4</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.80 ± 1.25</td>
<td>52.67 ± 135.30</td>
<td>3.41 x 10^5 ± 9.09 x 10^4</td>
</tr>
</tbody>
</table>

1 M Mg(ClO$_4$)$_2$ and Ca(ClO$_4$)$_2$. This strongly suggests that 1 M Mg(ClO$_4$)$_2$ and Ca(ClO$_4$)$_2$ induces almost complete unfolding of the $\alpha$-chymotrypsin tertiary structure. Therefore, increasing Mg(ClO$_4$)$_2$ and Ca(ClO$_4$)$_2$ concentration may induce incremental unfolding of the $\alpha$-chymotrypsin tertiary structure, causing analogous changes to activity. The slight change in tertiary fingerprint produced by NaClO$_4$ may explain its lesser reduction of $\alpha$-chymotrypsin activity. Negligible deviation from the no-salt control was observed in the presence of MgCl$_2$ and MgSO$_4$. Without more specific structural analysis, it is impossible to say what tertiary structural changes occur as CD can only provide a general fingerprint of the tertiary environment.

3.4. Mars-relevant perchlorates lower the melting temperature of $\alpha$-chymotrypsin

Figure 4 shows the melting curves of $\alpha$-chymotrypsin in each of the five assayed salts. The results show that the perchlorate salts reduce the melting temperature of $\alpha$-chymotrypsin in a concentration-dependent manner. A reliable melting curve could not be obtained for Mg(ClO$_4$)$_2$ and Ca(ClO$_4$)$_2$ at 0.75 and 1 M as the fluorescent intensity was already maximal at 5°C and any further heating induced protein aggregation and the loss of SYPRO binding. Figure 4A also shows that MgSO$_4$ increases the melting temperature of $\alpha$-chymotrypsin in a concentration-dependent manner. MgCl$_2$ increased the melting temperature across all concentrations (except 1 M), but its maximum increase in T$_m$ was observed at 0.25 M.

Figure 4F shows that a solution of 0.25 M Mg(ClO$_4$)$_2$ and 0.75 M MgSO$_4$ reduces the melting temperature of $\alpha$-chymotrypsin to the same extent as a solution containing only 0.25 M Mg(ClO$_4$)$_2$, despite the fact that the 0.75 M MgSO$_4$ solution increased the T$_m$ by 5°C. The slightly lower T$_m$ in the combined salt melting curve may be due to the increased magnesium concentration in solution compared with 0.25 M Mg(ClO$_4$)$_2$ alone. This shows agreement with Fig. 2, where sulfate’s presence could not rescue enzyme activity, and likewise, here it is demonstrated that it cannot rescue the thermostability of $\alpha$-chymotrypsin in the presence of Mg(ClO$_4$)$_2$. Table 2 details the exact melting temperatures observed at each salt concentration.

![Figure 2](image-url)
4. Discussion

Mars is known to harbor a diversity of salts, including magnesium, sodium, calcium, sulfates, chlorides, and perchlorates. Any organisms on the martian surface or deep subsurface will be exposed to these salts at concentrations ranging from millimolar to saturated molar concentrations. In this work, we investigated the basic biochemical effects of Mars-relevant salts, specifically perchlorates, to enzyme activity. By studying the effects of perchlorate brines on biological macromolecules, we can begin to describe specific molecular responses that may dictate the habitability of martian environments.

Our results demonstrate that perchlorate salts reduce the enzyme activity of \( \alpha \)-chymotrypsin in a concentration-dependent manner. The most deleterious salt is \( \text{Ca(ClO}_4\text{)}_2 \), followed by \( \text{Mg(ClO}_4\text{)}_2 \) and \( \text{NaClO}_4 \). \( \text{MgSO}_4 \) decreased the \( k_{\text{cat}} \) and \( K_m \), whereas \( \text{MgCl}_2 \) increased both; however, for both salts the catalytic efficiency remained largely unchanged. These overall results compare well with those of previous studies, which also showed a reduction in \( \alpha \)-chymotrypsin activity in the presence of \( \text{NaClO}_4 \) (Warren and Cheatum, 1966; Endo et al., 2016; Dušeková et al., 2018). Interestingly, Dušeková et al. (2018) and Endo et al. (2016) showed a large increase in \( \alpha \)-chymotrypsin activity in the presence of \( \text{Na}_2\text{SO}_4 \) due to an increased \( k_{\text{cat}} \) and a decreased \( K_m \), resulting in a significant increase in catalytic efficiency. Here, we have shown that \( \text{MgSO}_4 \) has no comparable effect.

Due to the fact that no structural changes were observed in the presence of sulfate salts, it has been suggested that...
changes to substrate solubility are what ultimately cause the observed effects on enzyme activity in the presence of sulfates (Endo et al., 2016). We can further compare our results for MgCl₂ with those reported for other chloride salts. It has been known for decades that millimolar amounts of CaCl₂ increase the activity of α-chymotrypsin, hence its inclusion in our buffer (Green et al., 1952). Molar concentrations of CaCl₂ have been shown to increase α-chymotrypsin and chymotrypsin B, though this is extremely substrate dependent (Wu and Laskowski, 1956). In addition, molar amounts of NaCl have been shown to increase α-chymotrypsin activity, again in a substrate-dependent fashion (Endo et al., 2016; Dušeková et al., 2018).

The comparison of our results with those reported from other groups highlights the importance of the cation–anion pair in describing the effect of ions on enzyme activity and also the substrate and buffer specificity of these effects.

To explain our activity results in perchlorate solutions, we hypothesized that the perchlorate salts were having an effect on the tertiary structure of α-chymotrypsin, as might be expected from the predictions of the Hofmeister series and the effect of chaotrophic salts. The Hofmeister series places ions along a continuum based on their ability to either salt out (kosmotropes) or salt in (chaotropes) proteins. The chaotropes that salt in proteins do so by increasing the protein’s solubility and vice versa.

The near-UV CD spectra clearly show that Mg(ClO₄)₂ and Ca(ClO₄)₂ unfold the α-chymotrypsin tertiary structure, resulting in a significant decrease in enzymatic activity. The change in structure suggested by the α-chymotrypsin spectrum in the presence of NaClO₄ may be responsible for the reduction in activity observed when in the presence of NaClO₄. Dušeková et al. (2018) found no structural changes to explain their results, but they only focused on sodium salts. Endo et al. (2016) found comparable results to ours, as their near-UV spectrum of α-chymotrypsin in the presence of 1.5 M NaSCN, a chaotrope, produced a similar unfolding to what we observed with Mg(ClO₄)₂ and Ca(ClO₄)₂.

The melting curve experiments give an insight into the effect of our studied salts on the thermolability of α-chymotrypsin. One of the major features is that the perchlorates decreased the Tₘ of α-chymotrypsin whereas the MgSO₄ increased the Tₘ. With the perchlorate salts, there is an unexpected contrast between the melting curves and the kinetics assays. Namely, that in the melting curves it is Mg(ClO₄)₂ that produces the lowest α-chymotrypsin Tₘ at each respective salt concentration, whereas it was CaCl₂ that yielded the greatest reductions in enzyme activity. This may be due to the covalent modification of the α-chymotrypsin, with the PeFabloc inhibitor affecting the protein’s dynamics. Alternatively, there may be more activity-related site-specific interactions occurring with Ca(ClO₄)₂ than with Mg(ClO₄)₂.

The increased Tₘ due to the presence of MgSO₄ is comparable to that observed by Dušeková et al. (2018) with Na₂SO₄ but we observed no increase in enzyme activity whereas their study’s results were accompanied by a noticeable increase in enzyme activity.

It was impossible to obtain Tₘ measurements in 0.75 and 1 M Mg(ClO₄)₂ and Ca(ClO₄)₂. As 5°C was the lowest operational temperature, it suggests that at these perchlorate concentrations, the α-chymotrypsin may only be folded at sub-zero temperatures that are beyond our limit of detection. Alternatively, these concentrations may represent a “point of no return” for α-chymotrypsin’s ability to correctly fold no matter what the temperature is.

The MgCl₂ increased the Tₘ at all concentrations except 1 M and exhibited its highest Tₘ at 0.25 M. At higher concentrations, the MgCl₂ showed a stepwise reduction in Tₘ from its highpoint. The implications from the melting curves in mixed salt solutions are explored later in this discussion.

The conclusion to be deduced from these results is that perchlorate salts cause α-chymotrypsin to unfold, resulting in a loss of enzymatic activity. The concentration at which these salts exert their deleterious effect will vary among the specific salts, the specific proteins and will depend on factors such as size, charge, and structure of the protein. The conclusion to be deduced from these results is that perchlorate salts cause α-chymotrypsin to unfold, resulting in a loss of enzymatic activity. The concentration at which these salts exert their deleterious effect will vary among the specific salts, the specific proteins and will depend on factors such as size, charge, and structure of the protein (Lindman et al., 2006; Ferreira Machado et al., 2007). This is demonstrated in studies such as Warren and Cheatum (1966), who showed that 1 M NaClO₄ reduces different enzyme activities to different extents. Laye and DasSarma (2018) also noted that they could not detect β-galactosidase activity above 0.23 M Mg(ClO₄)₂ and that it exhibited 50% catalytic activity at 0.88 M NaClO₄, whereas Warren and Cheatum (1966) recorded β-galactosidase activity in 1 M NaClO₄ and we report here α-chymotrypsin activity in up to 1 M of perchlorate salts.

A further important consideration is that these experiments were conducted in buffers with controlled pHs and so, in the natural environment, pH may also contribute to the effects of salts on proteins, especially as the changing net charge of a protein’s surface with pH will affect how charged species interact with the protein.

To explain how the perchlorate salts are unfolding α-chymotrypsin, we must consider the interactions between the salts and the protein itself. For perchlorate salts, the most important interactions happen at the protein backbone (Robinson and Jencks, 1965; Nandi and Robinson, 1972; Von Hippel et al., 1973; Arakawa and Timasheff, 1982;
Collins, 2004; Rembert et al., 2012). The α-carbon of amino acids exhibits a slight δ+ charge due to the electron withdrawing effect of its neighboring nitrogen and carbonyl groups (Jungwirth and Cremer, 2014). This δ+ charge leads to favorable interactions with weakly hydrated ions such as the perchlorate anion. The strongly hydrated cations of magnesium and calcium are attracted to the carbonyl group of the peptide backbone (Jungwirth and Cremer, 2014).

The interaction of these salts with the peptide backbone increases the solvation of the peptide backbone, resulting in the “salting-in” phenomenon. As the salts increase the solvation of the peptide backbone, it begins to unfold the protein structure and in doing so exposes more peptide backbone to the salts and to the water. This then allows for the increased hydration and solvation of the peptide backbone, which is usually hidden in the collapsed hydrophobic core. This process causes the unfolding of proteins, as we have observed with the near-UV spectra shown in Fig. 3. The strength of the ionic interaction with the peptide backbone, which causes this effect, is significantly affected by the specific counter-ion present (Okur et al., 2017). Therefore when the literature describes an ion as chaotropic, its degree of chaotropicity is significantly affected by its counterion in solution. This is displayed in our results, for example, by the different decreases in enzyme activity caused by the various perchlorate salts. It is also of interest to note that our experiments were conducted at a pH 7.8, which is lower than the pI of α-chymotrypsin (8.75). In experiments beneath a protein’s pI, a reversed Hofmeister series is often observed (Finet et al., 2004; Boström et al., 2005), whereas we have observed a generally direct Hofmeister series in our results.

Although studying proteins in Mars-relevant brines directly reveals information about the potential for contamination of martian environments with life from Earth, it may also provide insights into potential martian biochemistry. This is not to say that we predict any martian life, or even other life to be proteinaceous, but we can gain generalized insights into what adaptations biochemistry must have to be able to cope with such salts, thus revealing whether the martian environment, in general, is habitable or not. For example, if we consider the electronegativities of key elements in life (CHNOPS), any life made of a polymer containing carbon atoms bound to or near nitrogen or oxygen will then result in the formation of a δ+ carbon due to the electron withdrawing effect of these elements, regardless of the other features of that polymer. Thus, the salts should still exert their effect on specific sites, even if these sites exist in a different structure to what we are accustomed to with Earth life. These data may also provide insights into required adaptations to biochemistry in environments where such salts exist on Earth, such as the Atacama desert; here, perchlorate ions reach ~0.6 wt% (Erikson, 1981).

An important contrast between studies of specific salt effects and of real environmental brines is that brines usually contain more than one salt. On Mars we would expect to find brines containing a mixture of both sulfate and perchlorate anions. In the Hofmeister series, sulfate and perchlorate sit at opposite ends of the series from one another with sulfates being kosmotropic and perchlorates being chaotropic (Okur et al., 2017). It then begs the question whether the perchlorate anion can exert its deleterious effect in the presence of the sulfate anion. The results in Fig. 2 show that the perchlorate anion was able to reduce α-chymotrypsin activity to the same extent even in the presence of the sulfate anion. This experiment suggests that there is a dominant chaotropic salt effect, even in the presence of kosmotropic salts.

This is further reinforced in the α-chymotrypsin melting experiments. We have shown that α-chymotrypsin in a brine containing 0.25 M Mg(ClO4)2 and 0.75 M MgSO4 exhibited a Tm (39.96°C) equivalent to that in a 0.25 M Mg(ClO4)2 solution (40.94°C) alone. This is despite the fact that 0.75 M MgSO4 increased the melting temperature of α-chymotrypsin by 5°C, thus further showing that there is no cumulative salt effect by, rather a dominant salt effect. In this case, the perchlorate dominates the ionic effect.

The results presented here, which demonstrate that perchlorate salts can reduce enzyme activity and unfold protein tertiary structure, may help us understand previous studies on the effects on habitability. Laye and DasSarma (2018) found that Halorubrum lacusprofundi exhibited half maximal growth in 0.3 M NaClO4 and in 0.1 M Mg(ClO4)2. Our results show that Mg(ClO4)2 affected protein folding to a much greater extent than did NaClO4, and so may help to explain the results of H. lacusprofundi growth in perchlorate brines. Stevens et al. (2019) also reported rapid loss of Sphingomonas desiccabilis viability in 3 M Mg(ClO4)2, which also may be attributed to the effect of perchlorate salts on proteins. Other factors that may determine perchlorate brine habitability might include osmotic stress, oxidative stress, lipid bilayer perturbation, and many other unexplored facets of perchlorate effects on biology.

5. Conclusion

The results presented here demonstrate that perchlorate salts have the ability to reduce the enzyme activity of α-chymotrypsin, unfold its tertiary structure, and increase its thermostability. The results also show that perchlorate salts exert these deleterious effects dominantly, despite the presence of kosmotropic ions such as sulfates. These results indicate that although perchlorate salts are beneficial for the presence of liquid water on Mars, they also reduce the prospect of habitability by limiting the potential for biochemistry within concentrated brines in the martian subsurface. These studies also elucidate the biochemical mechanisms of the deleterious effects of perchlorates.

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**Abbreviations Used**

BTEE = N-benzoyl-l-tyrosine ethyl ester  
CD = circular dichroism  
UV = ultraviolet
3.3 Conclusion

The research presented in this chapter was an exploration of the effects of perchlorate salts and other Mars relevant salts on the structure, stability, and activity of the model enzyme α-chymotrypsin.

It was found that perchlorate salts reduce the activity and structural stability of α-chymotrypsin in a concentration dependent manner. The deleterious nature of the perchlorates descended according to Ca>Mg>Na. The order of chaotropicity here largely fits with that reported in the literature, namely that magnesium and calcium salts exert larger effects than sodium salts. However it is important to note that the Hofmeister series is not a fixed ordering as ions regularly swap places with their neighbours. This is due to complex nature of features in which one can examine “salt effects” such as protein folding, flexibility, solubility, activity, binding and many other measurable facets of protein biochemistry. Thus it would be unexpected that salts would affect each of these facets to the same extent across all proteins despite variations in protein size, charge, structure, binding moieties, and function. Additionally the “Hofmeister effect” is a general descriptor of salt affects above ~250mM where theories of salt effects such as electrostatic interactions begin to become less reliable.

MgSO₄ and MgCl₂ had little effect on the enzyme activity of α-chymotrypsin, which was surprising considering that in other studies, Na₂SO₄ significantly increased α-chymotrypsin’s activity[81]. I did however observe that they both generally increased the melting point of α-chymotrypsin, most likely as a result of the kosmotropic nature of these salts.

Additionally I found that MgSO₄ did not offer any protection from perchlorate induced stress, either in terms of enzyme activity or stability, which suggested that the perchlorate effect was dominant. Thus, in an aqueous Martian environment, if there are multiple dissolved ionic species these results suggest that the perchlorate effect is strong enough to overshadow any protective effects and thus from the biochemical perspective, perchlorate may be the main chemical stress that must be tolerated on Mars.

There are important caveats and nuances though to be understood within the context of this study and others like it. Firstly, the effect of any chemical agent on the activity of an enzyme will be highly dependent on experimental factors such as the pH, buffer type, and the substrate used as shown by Dusekova et al[81].

A second factor is that there is limited scalability in terms of ascribing universal effects of a chemical agent such as perchlorates on enzymes based of the results from a single enzyme. Thankfully there are multiple studies which also describe perchlorate salts as being deleterious to a range
of enzymes, so in general it can be said that perchlorate salts are deleterious to proteinaceous biochemistry.

With the effects of perchlorate salts on α-chymotrypsin now established, with regards to my experimental conditions, it was then time to explore how these effects changed in the presence of other extremes.
Chapter 4

High pressures increase α-chymotrypsin enzyme activity under perchlorate stress

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4.1 Introduction

The previous research chapter showed that perchlorate salts exert a dominant, deleterious effect on the structure, stability, and activity of \( \alpha \)-chymotrypsin. However, these results were obtained at ambient pressure and temperature, significantly different from those we might expect to find in deep subsurface aqueous Martian environments. Thus the question remained as to how this relationship might change across temperatures and pressures.

To examine this question, I conducted high pressure enzyme kinetics experiments to understand how the activity of \( \alpha \)-chymotrypsin changes in the presence of perchlorates at elevated pressures.

The hypothesis I developed was that if perchlorate salts progressively unfold \( \alpha \)-chymotrypsin, and the partially unfolded states occupy a larger volume than the folded state, then increased pressures should favour a more natively folded compact state according to Le Chatelier’s principle, thus potentially restoring enzyme activity. If high environmental pressures could relieve perchlorate stress, then it may be possible that perchlorate rich environments are more habitable if they are subterranean in nature. As it’s already known that high pressures can increase enzyme activity, I was also testing whether this was still possible in the presence of perchlorate salts. If so, then a negative activation volume would still be a viable adaptation to pressures in perchlorate rich environments, and potentially a way of limiting perchlorate-induced activity losses.
High pressures increase α-chymotrypsin enzyme activity under perchlorate stress

Stewart Gault, Michel W. Jaworek, Roland Winter & Charles S. Cockell

Deep subsurface environments can harbour high concentrations of dissolved ions, yet we know little about how this shapes the conditions for life. We know even less about how the combined effects of high pressure influence the way in which ions constrain the possibilities for life. One such ion is perchlorate, which is found in extreme environments on Earth and pervasively on Mars. We investigated the interactions of high pressure and high perchlorate concentrations on enzymatic activity. We demonstrate that high pressures increase α-chymotrypsin enzyme activity even in the presence of high perchlorate concentrations. Perchlorate salts were shown to shift the folded α-chymotrypsin phase space to lower temperatures and pressures. The results presented here may suggest that high pressures increase the habitability of environments under perchlorate stress. Therefore, deep subsurface environments that combine these stressors, potentially including the subsurface of Mars, may be more habitable than previously thought.
When searching for habitable environments elsewhere in the cosmos, one criterion is to seek liquid water. This is well grounded in our understanding of the importance of an appropriate solvent for the processes of life to occur in ref. 1. Therefore on Mars, much attention has been paid to environments where evidence of liquid water has been observed or is predicted to exist2-4. However, the mere presence of liquid water does not make an environment habitable. How life responds to external physical and chemical factors such as temperature, pressure, pH, and salinity will also define the edges of habitability5,6.

On Mars, prime candidates for habitable environments are deep aqueous environments. These may take the form of the reported subglacial lake at the Martian south pole7, or the deep groundwater beneath the Martian cryosphere8. These cold environments have been hypothesised to contain high concentrations of perchlorate salts, following their detection at the surface9, due to their extremely low eutectic temperatures. Perchlorate salts have been shown to have deleterious effects on microbial life10-12, and so must be considered as a factor that will shape the habitability of these environments. In addition to perchlorate salts, sulphates are widely distributed across the Martian surface13 and so provide a useful comparison for Mars relevant salt effects on biochemistry. The Martian groundwater would experience pressures of ~100 MPa (1 kbar) at the base of the cryosphere at the Martian poles if it reached a depth of 10 km14. Thus, to be able to assess the habitability of subsurface environments, we must investigate the combination of strong ionic effects and high pressures. Though there is quite a lot known about the effects cellular organic osmolytes, such as trimethylamine-N-oxide, impose on organisms thriving in the deep sea under high-pressure stress up to the 1000 bar level10-12, the combined effects of high salt, low temperature, and high pressure on biochemical processes is still terra incognita.

Previous studies have demonstrated the deleterious effects of destabilising ions such as perchlorates on the activity of α-chymotrypsin (α-CT) and other enzymes7,13. Conversely, stabilising salts, such as sodium sulphate14, have been shown to increase the activity and structural stability of α-CT15. Our knowledge of how these molecules affect enzyme activity is largely restricted to ambient temperatures and pressures. Therefore, a greater understanding of how these effects change with pressure is yet to be achieved.

The effect of pressure on enzyme kinetics is largely dictated by Le Châtelier’s principle. If an enzymatic reaction exhibits a negative change in volume ($\Delta V < 0$), or negative activation volume ($\Delta V^\ddagger < 0$), the overall reaction yield and rate will increase in that direction. A negative volume change is achieved when the volume occupied by the enzyme-substrate complex (ES) is lower than that of the enzyme (E) and substrate (S) in solution. The same holds true for negative activation volumes, which occur when the activated transition state (TS) occupies a lower volume than the ES complex. Previous studies have shown that α-CT exhibits a negative activation volume and, as such, its activity increases with pressure16-18.

In this work, the effects of Mars relevant salts (MgSO$_4$ and Mg(ClO$_4$)$_2$) and high hydrostatic pressures exert on the activity and structural stability of α-CT, an archetype of a digestive enzyme, are explored. Assayed concentrations of MgSO$_4$ and Mg(ClO$_4$)$_2$ of 0.25 and 0.5 M allow for a comparison of the effects of both salts on our system as concentrations of Mg(ClO$_4$)$_2 > 1$ M induce unfolding of α-CT at room temperature. This work advances our understanding of how the interactions of high concentrations of these ions and high pressure influence the biochemistry and habitability of terrestrial and extraterrestrial environments.

**Results**

α-chymotrypsin activity in Mars salts. The effects of pressure and high concentrations of Mars relevant salts on enzymes has not been previously investigated. The Michaelis–Menten plots in Fig. 1 show the activity of α-CT in the absence and presence of MgSO$_4$ and Mg(ClO$_4$)$_2$. The activity of α-CT increases with pressure, as has been previously described16-18. Figure 2a, b show that the Michaelis constant, $K_M$, decreases slightly with increasing pressure, i.e., the affinity of the substrate increases slightly upon compression. The turnover number, $k_{cat}$, increases about 2–3 fold and the catalytic efficiency, $k_{cat}/K_M$, rises by a factor of 5 in all solutions upon compression from 1 to 2000 bar. Table S11 lists the kinetic parameters of $k_{cat}$, $K_M$ and $k_{cat}/K_M$ for α-CT in buffer in the absence and presence of MgSO$_4$ and Mg(ClO$_4$)$_2$. The data show that the $k_{cat}$ of α-CT is lower at each pressure step at both Mg(ClO$_4$)$_2$ concentrations than when in buffer alone. Despite the lower $k_{cat}$ values, the reduced $K_M$ values result in a greater catalytic efficiency ($k_{cat}/K_M$) for α-CT at 2000 bar in 0.25 M Mg(ClO$_4$)$_2$ compared to buffer, whereas the catalytic efficiency is reduced at 2000 bar in 0.5 M Mg(ClO$_4$)$_2$. The activity of α-CT is also increased in the presence of MgSO$_4$ at both concentrations with greater $k_{cat}$ values above 1 bar, lower $K_M$ values, and greater catalytic efficiency.

The change in the kinetic parameters, in both real numbers and as percentages, are presented in Fig. 2a–f. The greatest proportional change in $k_{cat}$ from 1 to 2000 bar is in the presence of 0.25 M Mg(ClO$_4$)$_2$ (+144%), the greatest proportional change in $K_M$ is in buffer (∼76.5%) and the greatest proportional change in $k_{cat}/K_M$ observed is in 0.25 M MgSO$_4$ (∼466%).

Figure 2g and Table S12 show the data for the activation volume, which were determined using Eq. (2) (see below). All $\Delta V^\ddagger$ values exhibit a negative value (order of magnitude, $\sim$11 cm$^3$ mol$^{-1}$), which is similar to that described in the literature19,20. Furthermore, the $\Delta V^\ddagger$ slightly increases with increasing substrate concentration (Table S13), reaching plateau values for substrate concentrations of about 2 mM, i.e., for concentrations where the enzyme is saturated with substrate. The activation volume for large substrate concentrations, where $v_0 \propto k_{cat}$ describes the difference in the volumes of the TS and the ES complex, i.e., $\Delta V^\ddagger = V^\ddagger - V_{ES}$, whereas measuring the activation volume at low substrate concentrations, where $v_0 \propto k_{cat}/K_M$, includes the volume change due to substrate binding, i.e., $\Delta V^\ddagger = V^\ddagger - V_{E=S}$21,22. The latter contribution seems to depend slightly on the particular solution conditions and amounts to about −4 to −6 cm$^3$ mol$^{-1}$, i.e., the ES substrate complex is slightly more compact relative to the partial volumes of enzyme and substrate.
probably due to partial desolvation upon substrate binding. $\Delta V^\neq$ values for all salt conditions reveal no significant differences compared to the neat buffer. Altogether, the TS is more compact than the ES complex, which might be due to a decrease of void volume and/or hydration of charges (electrostriction effect) in the TS.

The difference in the effect of both salts is largely attributable to the extent to which they preferentially interact with the peptide backbone (perchlorate) or are excluded from the peptide surface (sulphate)\(^{23}\). It is also worth noting that while this can be examined as an anion or cation effect, it is worth considering the whole salt due to the varied extent with which the ions pair with each other in water\(^{24}\).

Pressure–temperature stability of $\alpha$-chymotrypsin. To follow the temperature- and pressure-dependent unfolding process of $\alpha$-CT in detail, the secondary structural changes in the presence of perchlorate compared to neat buffer (+10 mM CaCl\(_2\)) were measured using Fourier-transform infrared (FTIR) spectroscopy, covering a temperature range from 20 to 70 °C and a pressure range from 1 bar to 10 kbar (1 GPa). Figure 3 displays the normalised temperature-dependent FTIR spectra of $\alpha$-CT (Fig. 3a, c, e) as well as the corresponding changes in secondary structural elements (Fig. 3b, d, f) for different concentrations of perchlorate. At ambient pressure and 20 °C, the amide I' band of $\alpha$-CT in neat buffer shows a broad band at 1638 cm\(^{-1}\), with aggregation bands appearing at 1617 cm\(^{-1}\) and 1684 cm\(^{-1}\) at high temperatures. The
secondary structure components obtained, which are derived from the curve fitting procedure (Fig. 3b), are in accord with earlier results reported by Meersman et al.25. At ambient pressure and 25 °C, α-CT shows high contents of intramolecular β-sheets (~43%) and turns and loops (~21%) and lower amounts of α-helices (~9%). These results are in rather good agreement with crystallographic data26. The minor differences between the X-ray diffraction and FTIR results (see Table SI 4) may be due to different absorption coefficients of the various secondary structure elements, which is not significant as only relative changes are essentially discussed here. Upon temperature-induced unfolding/denaturation, the intramolecular β-sheet content decreases (~−5%) and aggregation of the protein via formation of intermolecular β-sheets (~+5%) takes place in neat buffer (Fig. 3b).

Remarkably, in the presence of Mg(ClO₄)₂, no aggregation via formation of intermolecular β-sheets occurs upon unfolding at high temperatures. The intramolecular β-sheet content decreases and the α-helices & turns/loops content increases concomitantly. With increasing perchlorate concentration, the unfolding temperature, $T_u$, of α-CT decreases from 57.6 °C to 39.8 °C, i.e., the
Salt leads to a marked destabilization of the temperature stability of the protein. Further, the width of the unfolding transition region increases upon addition of perchlorate, pointing to a decreasing cooperativity of the transition.

Figure 4 depicts the normalised pressure-dependent FTIR spectra of α-CT (Fig. 4a, c, e) and the corresponding secondary structural changes (Fig. 4b, d, f) at different concentrations of perchlorate at 35 °C up to 10 kbar. With increasing pressure in neat buffer solution, the amide I’ band of α-CT shifts to higher wavenumbers and partial unfolding is observed at around 6700 bar. Regarding the relative changes of secondary structure elements, the α-helix & turns/loops content increases (∼+4%), while the percentage of intramolecular β-sheets decreases (∼−4%) concomitantly upon compression. In the presence of 0.25 M Mg(ClO₄)₂, the secondary structural changes are similar, and α-CT partially unfolds at a lower pressure (5600 bar, 35 °C) compared to the buffer condition. In summary, the enzyme is very pressure stable, even in the presence of 0.25 M of perchlorate. The temperature- and pressure-induced (partial) denaturation is irreversible for all conditions measured.
in the absence of Ca\(^{2+}\) for solution conditions different from ours. The protein unfolds, losing its enzymatic activity. As is clearly seen from the figure, increasing the perchlorate concentration reduces the pressure and temperature stability of the protein, shifting the boundary to lower values of temperature and pressure. However, the enzyme is stable in the whole p, T-range covered in the HPSF studies, and also in the presence of the two salts.

Contrary to the conformational entropy gain of the peptide chain, which is the main driving force for the temperature-induced unfolding of proteins, volume changes and compressibility play a decisive role for the pressure-induced unfolding process, where the system tends to occupy an overall smaller volume state at high pressure\(^{27,28}\). To gain a quantitative measure, the volume change of (partial) unfolding, \(\Delta V_{\text{unf}}\), was determined using Eq. (4). For the partial unfolding of \(\alpha\)-CT at 35 °C in the absence of Mg(ClO\(_4\))\(_2\), a value of \(\Delta V_{\text{unf}} = -41 \pm 7 \text{ cm}^3\text{ mol}^{-1}\) was obtained, which is consistent with the volume change of the precursor (chymotrypsinogen) of \(\alpha\)-CT\(^{29}\) and a typical value observed for unfolding of monomeric proteins\(^{28,30-32}\). At lower temperatures (25 °C) or higher perchlorate concentrations (0.5 M), smaller changes in the secondary structure elements are observed upon pressure-unfolded unfolding, which is reflected in smaller \(\Delta V_{\text{unf}}\) values.

The p, T-phase diagram of \(\alpha\)-CT in the absence and presence of Mg(ClO\(_4\))\(_2\), resulting from these temperature- and pressure-dependent FTIR measurements, is depicted in Fig. 5. At low temperatures and pressures, the native state of the protein is favoured. Crossing the transition line to the unfolded state, the native conformation loses its stability and the protein partially unfolds, losing its enzymatic activity. As is clearly seen from the figure, increasing the perchlorate concentration reduces the pressure and temperature stability of the protein, shifting the boundary to lower values of temperature and pressure. However, the enzyme is stable in the whole p, T-range covered in the HPSF studies, and also in the presence of the two salts.

Literature data on the phase diagram of the \(\alpha\)-CT only exists for solution conditions different from ours. The protein unfolds in the absence of Ca\(^{2+}\) at 4.9 kbar for \(T = 21\) °C and at 41 °C for ambient pressure conditions\(^{33}\). As expected, Ca\(^{2+}\) ions have a positive effect on the temperature and pressure stability of the protein, probably by electrostatic screening of negatively charged surface patterns of the protein. A stabilising effect of Ca\(^{2+}\) ions on \(\alpha\)-CT has been observed in other unfolding studies of proteins as well\(^{34,35}\). A marked destabilising effect of perchlorate, being a typical low-charge-density anion at the far end of the Hofmeister series of anions, at high concentrations, has been observed in lysozyme at pH 7 as well and is expected to be due to weak binding of the ClO\(_4^-\) anions to the protein\(^{36,37}\). This reduces the Gibbs energy associated with hydrating of the newly exposed interior of the protein upon unfolding, thereby destabilising the native protein’s fold. As has been shown recently by Dougan et al.\(^{38}\), the water structure also changes markedly at the high perchlorate concentrations mimicking conditions in Martian soil. They found that the tetrahedral structure of water is heavily perturbed, the effect being equivalent to pressurising pure water to pressures of the order of 20 kbar and more. Interestingly, the Mg\(^{2+}\) and ClO\(_4^-\) ions appear charge-ordered and bridged by water molecules under these conditions, thereby preventing ice formation at low temperatures.

**Discussion**

Little is known about the effects of high ion concentrations on cellular life or the combined effects of ions with high pressure. Yet these combinations can shape the habitability of deep subsurface environments on Earth and potentially elsewhere. Currently, one scientific question of great interest is the habitability of the Martian subsurface. Although the question of whether there is life on Mars remains speculative, we can ask questions about how physical and chemical conditions expected on Mars might theoretically influence its habitability. We can then use terrestrial organisms and biomolecules to explore the boundary space of habitability for known life under said Martian conditions. In this study, we used a well characterised enzyme to investigate the combined effects of perchlorate ions and high pressures as a general proxy for biomolecular stability under these extremes.

From the Michaelis–Menten plots it is demonstrable that increasing the hydrostatic pressure also increases the enzyme activity of \(\alpha\)-CT, even in the presence of high concentrations of the chaotropic salt Mg(ClO\(_4\))\(_2\). As \(\alpha\)-CT has a negative activation volume (\(\Delta V^* < 0\)), the increasing activity with pressure is largely attributed to Le Châtelier’s principle as previously described\(^{16-18}\).

From the kinetic parameters it is also shown that Mg(ClO\(_4\))\(_2\) reduces the activity of \(\alpha\)-CT at both concentrations, 0.25 and 0.5 M, and across all pressure conditions tested. This agrees with previous ambient pressure studies showing the same effect of perchlorate salts on \(\alpha\)-CT and other enzymes\(^{7,13}\). Furthermore, the observation that MgSO\(_4\) increases the activity of \(\alpha\)-CT agrees with previous studies which show that the sulphate ion can stabilise proteins\(^{14}\) and increase the activity of \(\alpha\)-CT\(^{15}\). This sulphate-induced effect is also shown to be substrate dependent as it was absent in a previous kinetic study using N-benzoyl-L-tyrosine ethyl ester as the substrate.

Different proportional increases in \(k_{\text{cat}}\) were observed in response to increasing hydrostatic pressure for the different salt conditions. From previous work at atmospheric pressure it has been shown that perchlorate salts reduce the structural stability of \(\alpha\)-CT. It may therefore be the case that high pressures can partially undo this negative structural effect, manifesting itself as the greater proportional increase in \(k_{\text{cat}}\). In essence, lesser salt-induced structural disruption at higher pressures may confer greater than expected activity increases than due to Le Châtelier’s principle alone. This suggests the existence of an advantageous salt-pressure-activity interplay.

The shift of the \(\alpha\)-CT pressure-temperature stability diagram to lower pressures and temperatures with increasing Mg(ClO\(_4\))\(_2\) concentration also suggests that the region of peak stability is concomitantly shifting to the left also. Therefore, in the presence of Mg(ClO\(_4\))\(_2\), the region of peak \(\alpha\)-CT activity and stability may be found at elevated pressures, and lower temperatures.

It is of interest to note that the pressure-temperature phase diagram of life resembles that of proteins, which is due to the
particular temperature dependence of the proteins’ specific heat of unfolding. Life and proteins both exhibit similar, curved pressure-temperature phase diagrams, whereas lipids and nucleic acids generally have linear pressure-temperature phase diagrams. If we were to imagine a z-axis emerging from Fig. 5 representing the (standard) free energy of unfolding ($\Delta G_f$), we would be able to see the protein stability phase space as a function of free energy also. In this phase diagram, $\alpha$-CT would exhibit a peak of stability (maximum of $\Delta G_f$) which descends down to the measured unfolding pressures and temperatures where $\Delta G_f = 0$. It would be interesting to know how the perchorlate salts affect the position of the region of peak stability. If it was found that the perchlorate salt moves the region of peak stability to higher than ambient pressures and lower temperatures, is it also moving the region of peak fitness for life? If so, it would suggest that life is more favourable in perchlorate brines if they also experience increased pressures and lower temperatures (a similar salinity-pressure-temperature-growth relationship has been described by Kaye and Barsoss). It is then of interest that such environments have either been potentially observed or theorised to be present on Mars. Therefore, whilst concentrated perchlorate brines reduce the habitability of an aqueous environment, high pressures and lower temperatures may counteract the deleterious perchlorate effect, thus increasing habitability. There is already tentative evidence showing that sub 0 °C temperatures confer greater survival of bacteria in perchlorate brines.

While the effects of low temperatures on our model enzyme in the presence of perchlorate salts remains unknown, we have shown that high pressures can still increase enzyme activity even in the presence of a destabilising salt predicted to be concentrated on Mars. Moreover, high pressures may confer greater structural stability, reducing the destabilising effects of perchlorate salts on proteins. It would be of particular interest to expand our understanding of the perchlorate-pressure-temperature effect on membrane proteins as, in addition to secreted biomolecules, they would be directly exposed to the perchlorate-rich environment. Thus, the effect that concentrated perchlorate salts exert on processes such as molecular transport and signalling may be of particular importance to habitability. Finally, similar to the case of the enzymes of extremely halophilic archaea, changes in amino acid composition under such high Mg(ClO$_4$)$_2$ concentrations, e.g., by changing the ratio of negatively to positively charged and hydrophobic residues, might help in optimising enzymatic activity under such harsh salt concentrations.

The enzyme activity was plotted in Origin 9.3 (OriginLab) and a least-squares fit fitting method was applied according to the Michaelis-Menten equation:

$$v = \frac{v_{max}}{K_m + [S]}$$

The Eyring-equation describes the pressure effect on the rate of the reaction:

$$\frac{\partial \ln(k_b)}{\partial p} = -\frac{\Delta^V}{RT}$$

FTIR: Sample preparation and secondary structure analysis. The enzyme α-chymotrypsin from bovine pancreas was purchased from Sigma-Aldrich as lyophilized powder. For H/D-exchange, the protein was dialysed against D$_2$O using Amicon Ultra (2 ml) centrifugation units with 10 kDa cut-off and subsequently lyophilised. For the pressure dependent FTIR spectroscopy studies, the measurements were carried out in 100 mM Tris(hydroxymethyl)aminomethane buffer with 10 mM CaCl$_2$ in the absence and in the presence of Mg(ClO$_4$)$_2$. For the temperature-dependent experiments, phosphate buffer was used. The pD-value of both buffers was adjusted to 7.8 (pH + 0.4 = pD) by adding DCl. All chemicals were used without further purification.

FTIR spectra were collected using a Nicolet 6700 (Thermo Fisher Scientific) equipped with a liquid-nitrogen cooled MCT-detector (HgCdTe), operated at -196 °C, in the wavenumber range between 4000 and 650 cm$^{-1}$. The sample chamber was continuously purged with CO$_2$-free and dry air. All measured spectra were averaged over 128 scans in a row at a spectral resolution of 2 cm$^{-1}$ and were processed with Happ-Genzel apodization by using Omnic 7.2 spectroscopic processing software. The equilibration time before each spectrum was recorded at each temperature was 15 min, for the pressure dependent studies, 5 min. The temperature IR cell consists of two CaF$_2$ windows separated by a mylar spacer of 50 μm thickness. The setup of the pressure system consists of a membrane-driven diamond anvil cell (VivoDac Diacell$^*$ with type Ila diamonds, which is connected to an automated pneumatic pressure control (Diacell$^*$ gIM Controller, Almasylab). To measure the pressure inside the cell, by adding BaSO$_4$, the pressure-sensitive stretching vibration of SO$_4^{2-}$ (∼983.5 cm$^{-1}$ ± 1 bar, 25 °C) was used as an internal pressure calibrator. For each measurement, a protein concentration of 5 wt.% was used and the temperature of the cell was regulated with an external, circulating water thermostat. Processing and analysis of the spectra was carried out with the Grams AI 8.0 software (Thermo Fisher Scientific). Depending on the sample, spectra of the buffer systems were subtracted from the spectra recorded and smoothed afterwards. Then, the area of the amide I band (1700–1600 cm$^{-1}$ which is essentially based on the conformation sensitive C=O stretching vibration of the peptide bonds) was normalised to 1. Due to small variations in molecular geometry and hydrogen bonding patterns, it is possible to analyse the secondary structural composition and conformational changes of proteins. The number of subbands and their positions were obtained via Fourier self-deconvolution (FSD) and 2nd derivative approaches. Eight subbands, similar to the results described in the literature were obtained and mixed Gaussian-Lorentzian functions were used to fit the peak areas in the amide I band region and to determine the relative changes in the population of secondary structure elements (curve fitting). Assuming a two-state unfolding process of the protein, a Boltzmann function can be fitted to the temperature- and pressure-dependent sigmoidal curve progression of the intensity changes:

$$I = \frac{I_1 - I_0}{1 + e^{-(\Delta f^0/T - \Delta f^\circ)}} + I_0$$

where $I_1$ and $I_0$ are the plateau values of the IR band intensities, $I_0$ of the folded and unfolded protein. The unfolding temperature, $T_u$, and unfolding pressure, $p_u$, were obtained from the inflection points of the sigmoidal curves. In addition, the thermodynamic parameters $\Delta H^\circ$ and $\Delta V^\circ$, i.e., the corresponding van’t Hoff enthalpy and volume changes, respectively, can be directly obtained from the fits of the temperature- and pressure-dependent FTIR data.

**Methods**

**Materials.** Lyophilised α-chymotrypsin from bovine pancreas, Tris(hydroxymethyl)aminomethane as well as the salts MgSO$_4$, Mg(ClO$_4$)$_2$ and CaCl$_2$ were obtained from Sigma-Aldrich, Germany.

**Measuring α-chymotrypsin activity.** Enzyme activity was measured using a high-pressure stopped flow system, IFS55, from Hi-Tech Scientifi...
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Author contributions
S.G. and M.W.J. conducted the experiments. R.W. and C.W.J. supervised the work. All authors contributed to data analysis and the writing of the paper.
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The authors declare no competing interests.

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4.3 Conclusion

In Chapter 3, I had shown that perchlorate salts were deleterious to \( \alpha \)-chymotrypsin’s activity, and in this chapter, I have shown that while this is still true, high pressures can effectively rescue the activity of \( \alpha \)-chymotrypsin in the presence of perchlorate salts.

\( \alpha \)-Chymotrypsin has a negative activation volume, which means that pressure should have increased its activity assuming that perchlorate salts did not significantly alter said activation volume. This follows from a simple Le Chatelier’s interpretation in that, increasing the pressure should shift the equilibrium states to those of smaller volumes. As the activated transition state complex has a smaller volume than the enzyme-substrate complex then the formation of the activated complex is promoted by increased pressure. However if the activity changes I observed were purely due to the effect of the negative activation volume, then one would expect that pressure should have affected all the conditions to the same extent. However, I observed that it was the 0.25M Mg(ClO\(_4\))\(_2\) condition which exhibited the greatest proportional increase in activity across pressure. I took this as evidence that at this low perchlorate concentration, pressure may be partially undoing the negative effects of the perchlorate salts i.e. returning the enzyme to a slightly more native state, thus contributing extra activity increases which manifests as a higher proportional increase in activity.

Additionally we hypothesised as to what effect perchlorate salts were having on the P/T phase diagram of \( \alpha \)-chymotrypsin. As was shown, Mg(ClO\(_4\))\(_2\) reduced the thermostability of \( \alpha \)-chymotrypsin as well as the onset of pressure induced unfolding. But the question remained as to what was happening to the region of peak \( \alpha \)-chymotrypsin stability, i.e. the point where the Gibbs free energy of folding, \( \Delta G_f \), is most negative. I hypothesised that the region of peak stability may be moving to lower temperatures and pressures which would suggest that proteins are actually more stable in perchlorate brines if they are found at low temperatures and high pressures, just like we expect to find in the Martian subsurface. Quantitative data for the region of peak stability was unavailable or unobtainable due to the lack of access to a high-pressure differential scanning calorimeter, which is typically used for thermodynamic determination of protein stability. Additionally, we could not thermodynamically analyse the unfolding of \( \alpha \)-chymotrypsin in the presence of Mg(ClO\(_4\))\(_2\) due to multiple peaks and the irreversibility of the process. As the unfolding process was irreversible and exhibited multiple peaks, it means that it does not meet the two-state transition assumption which is needed for a simple determination of the Gibbs free energy of folding. Therefore, under our conditions, a thermodynamic interpretation is not available beyond the point at which \( \Delta G_f = 0 \). It may be possible to get
thermodynamic data by changing the pH of the assay to around 3, but this was avoided as it takes us away from Mars like conditions and more importantly it does not match the pH of the activity assays and therefore an explicit link between activity and stability would not have been possible. Ideally these experiments would be repeated with an enzyme that unfolds through a two-state transition and thus its stability thermodynamics can be determined and related to its activity. As perchlorate salts reduce the freezing point of water, they may also allow for the temperature of cold denaturation to be experimentally determined, something which is normally prevented by the onset of ice formation in a DSC. This would be a fantastic opportunity to explore how accurate cold denaturation points are when inferred from the thermodynamics of a protein's high temperature melting point.

To gain a broader understanding of how perchlorates affect protein function at pressure, it would be interesting to investigate the pressure dependency of multimeric protein association. This would give us a better understanding of the limits to habitability in subsurface perchlorate brines and allow us to predict the necessary adaptations required to survive in such environments.

With the activity of α-chymotrypsin now understood in the presence of perchlorates and at elevated pressures, it was then time to explore how temperature affected this relationship.
Chapter 5

A meta-analysis of the activity, stability, and mutational characteristics of temperature-adapted enzymes

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5.1 Introduction

In order to understand how life on Mars may rise to the challenges of low temperatures, I had to understand how life as we know it has adapted to such environments.

There are a few defining characteristics of psychrophilic enzymes, namely, high(er) activity at low temperatures, low structural stability, susceptibility to chaotrope-induced unfolding, lower enthalpies of activation, and more negative entropies of activation.

In addition, psychrophilic enzymes are often described as being more flexible than mesophilic or thermophilic enzymes. It is often reported that psychrophilic enzymes exhibit a larger gap between their optimum temperature ($T_{\text{opt}}$) and their melting temperature ($T_m$) compared to mesophilic and thermophilic enzymes, a gap I have termed $T_g$. This however is normally described on a study by study basis and has never been shown to actually be a general feature of psychrophilic enzymes. As such, one of the main goals of this meta-analysis was to determine whether this larger $T_g$ is in fact a defining characteristic of psychrophilic enzymes. To obtain $T_{\text{opt}}$ and $T_m$ values, a literature search was conducted for psychrophilic and thermophilic enzymes with these reported characteristics. Once these were found then a mesophilic enzyme of the same or similar type was found for comparison, sometimes within the same study as the temperature adapted enzymes. Designed or randomly mutated enzymes were not included in the dataset as their characteristics did not arise due to natural evolution in a temperature defined environment. An enzyme was also only characterized as either psychrophilic or thermophilic if the organism it came from was definitively characterized as such, therefore any reconstructed enzymes from a metagenome were excluded from this analysis.

In addition to understanding the activity related characteristics of extremophilic enzymes, I also sought to understand the predicted effects of amino acid substitutions on the stability of extremophilic enzymes by using the predictive software HoTMuSiC and PoPMuSiC. These pieces of software use statistical potentials based off of protein structures to predict thermodynamic changes to protein stability. The software takes into consideration the volume, voids, torsion, bonds, and interactions within a protein to predict these changes. As they are structure-based software, they both require a protein to have a PDB code. Once a protein is entered into the software it can then calculate the changes to a protein’s melting temperature and folding free energy upon mutating each amino acid position with one of the other 19 major amino acids. This then allows us to predict whether
there’s a difference in effect between psychrophilic, mesophilic, and thermophilic.

In essence, by further understanding how psychrophiles on Earth have adapted their enzymes to low temperatures, I hoped it would reveal some mechanisms by which enzymes might become adapted to multi-extreme environments on Mars.
Research Article

A meta-analysis of the activity, stability, and mutational characteristics of temperature-adapted enzymes

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Understanding the characteristics that define temperature-adapted enzymes has been a major goal of extremophile enzymology in recent decades. In the present study, we explore these characteristics by comparing psychrophilic, mesophilic, and thermophilic enzymes. Through a meta-analysis of existing data, we show that psychrophilic enzymes exhibit a significantly larger gap (Tg) between their optimum and melting temperatures compared with mesophilic and thermophilic enzymes. These results suggest that Tg may be a useful indicator as to whether an enzyme is psychrophilic or not and that models of psychrophilic enzyme catalysis need to account for this gap. Additionally, by using predictive protein stability software, HoTMuSiC and PoPMuSiC, we show that the deleterious nature of amino acid substitutions to protein stability increases from psychrophiles to thermophiles. How this ultimately affects the mutational tolerance and evolutionary rate of temperature adapted organisms is currently unknown.

Introduction

Extremophiles on Earth have become adapted to both high and low ‘extreme’ environmental temperatures. In the process of evolving to survive in such environments, they have had to adapt their biomolecular machinery to function at extreme environmental temperatures [1–3]. As enzymes are the major facilitators of biological reactions, they represent an important window through which temperature adaptation of organisms can be understood. Temperature-adapted enzymes exhibit adaptations to both their activity and stability. Psychrophilic enzymes, those adapted to low temperature environments, exhibit greater activity at low temperatures compared with mesophilic and thermophilic enzymes [4]. Thermophilic enzymes on the other hand are adapted to be both active and stable at extremely high environmental temperatures, even upwards of 100°C [5–7]. These adaptations are achieved through specific changes to an enzyme’s amino acid composition [8–13], secondary structure [14], and the number and type of intramolecular bonds present in the enzyme [15–19]. In this study, thermophilic (from environments of ∼55–60°C) and hyperthermophilic (from environments >80°C) enzymes are grouped together.

Many studies of temperature-adapted enzymes focus on what may be considered the main physical characteristics of an enzyme: its optimum temperature (T°opt) and its melting temperature (T°m). Unsurprisingly, it is observed that psychrophilic enzymes exhibit a lower T°opt and T°m than their mesophilic and thermophilic homologues. However, it was also observed that some psychrophilic enzymes exhibited a T°opt that was far from their T°m [20]. Here, we term this difference between T°opt and T°m as an enzyme’s ‘temperature gap’ (Tg). It was initially suggested that this gap was due to the active site of psychrophilic enzymes being more thermolabile than the rest of the protein in order to have sufficient flexibility to achieve catalysis at low environmental temperatures [4,20,21]. However, alternative hypotheses have been
proposed to account for this, such as the equilibrium model [22], macromolecular rate theory [23–25], and the loss of temperature-sensitive enzyme–substrate interactions [26]. However, as most studies focus on one type of enzyme across a small sample of species, it is difficult to understand how representative this phenomenon is across many enzyme types. Therefore, the first aim of the present study is to determine whether a large $T_g$ can be characterised as a general feature of psychrophilic enzymes and to what extent we also see this phenomenon in mesophilic and thermophilic enzymes.

Another suggested characteristic of temperature-adapted organisms is that thermophiles exhibit particularly low mutational tolerance [27,28]. It has been suggested that the high temperatures of a thermophile's environment make it particularly constrained by temperature-sensitive mutations. However, it has also been suggested that microbial communities actually evolve faster in extreme environments [29], seemingly in contrast with the predictions made by Drake [27]. This raises the question as to whether mutations themselves have a greater effect on thermophilic enzyme stability, or do thermophiles simply live closer to their proteome's thermodynamic edge of stability than do mesophiles or psychrophiles? Thus, the second aim of the present study was to determine whether protein mutation software, PoPMuSiC [30] and HoTMuSiC [31], predicts a difference in effect to an enzyme's Gibbs free energy of folding ($\Delta\Delta G_f$) or melting temperature ($\Delta T_m$) upon mutation among psychrophiles, mesophiles, and thermophiles.

In the present study, it is shown through meta-analysis that the $T_{opt}$ and $T_m$ of an enzyme increases from psychrophiles to thermophiles, as is expected. It is also shown that, while most enzymes exhibit a $T_g$, the $T_g$ of psychrophilic enzymes is significantly larger than that of both mesophilic and thermophilic enzymes and in certain cases $T_g$ provides the best indication of whether an enzyme is psychrophilic or not. Additionally we show that the average amino acid substitution is more deleterious to thermophilic enzyme stability compared with psychrophilic enzymes, with a general increase in the deleterious effect from psychrophiles through to thermophiles. Owing to the small absolute predicted differences between the stability parameters for the temperature-adapted enzymes, it is unknown how this would affect the mutational tolerance of thermophiles compared with mesophiles and psychrophiles over evolutionary timescales.

**Methods**

**Dataset construction**

Two datasets were created for the present study. Dataset 1 contains the $T_{opt}$ and $T_m$ data for homologous temperature-adapted enzymes from psychrophiles, mesophiles, and thermophiles which were included following a literature search of published data. Dataset 1 also contains the calculated $T_g$. $T_g$ is defined here as the temperature gap between an enzyme's $T_m$, and its $T_{opt}$ and is calculated from the following equation:

$$T_g = T_m - T_{opt}$$

Dataset 2 contains the Protein Data Bank (PDB) IDs of homologous temperature-adapted enzymes from psychrophiles, mesophiles, and thermophiles which were found following a literature search or from searching through the PDB itself.

Each dataset had certain criteria which had to be met before data were entered into the dataset. For dataset 1, only wildtype enzymes were included. This meant that variants generated through random/targeted mutagenesis were excluded. This means that the data obtained for the studied enzymes result from their natural evolutionary history, whereas generated variants may have contained alterations which are not represented or permissible in the natural environment and as such may have affected the results. For an individual enzyme, the $T_m$ and $T_{opt}$ values were only taken from separate publications if it was clear that both studies were using the same enzyme from the same source organism. Reports of $T_{50}$ values were excluded as they primarily reflect the kinetic stability of an enzyme rather than the global stability which is inferred from $T_m$ measurements. Reports in which an enzyme's $T_m$ was lower than its $T_{opt}$ were excluded. Such reports were rare. Furthermore an enzyme was only included in dataset 1 if both $T_m$ and $T_{opt}$ could be obtained, otherwise $T_g$ could not be calculated. This has bearing for the thermophilic results as there were instances of thermophilic enzymes exhibiting high $T_{opt}$ values, however the $T_m$ values were experimentally unobtainable in the respective studies. These restrictions on data mean that the results presented here may represent a lower estimate of the mean $T_m$, $T_{opt}$, and $T_g$ of thermophilic enzymes.

Dataset 2 had similar entry requirements, such as only natural enzymes were included, and generated variants were excluded. As the mutational software used in the present study is structure based, a PDB ID was required for entry into dataset 2. Enzymes were taken as psychrophilic, mesophilic, and thermophilic based on how the source literature characterised them.
Predicting the effect of mutations to protein stability

In order to predict the effect of mutations on the stability of temperature-adapted enzymes, two pieces of software were used, HoTMuSiC and PoPMuSiC [30,31] (available at https://soft.dezyme.com/). Both pieces of software require a PDB ID as input. HoTMuSiC predicts the effect of a mutation to a protein’s melting temperature ($\Delta T_m$), therefore a positive value is interpreted as stabilising and a negative value is destabilising. PoPMuSiC predicts the effect of a mutation to a protein’s $\Delta G_f$ and so a negative value is stabilising, and a positive value is destabilising. For data analysis, the mean effect of mutations to the respective proteins was recorded. Together the two pieces of software report on different, but complementary parts of a protein’s temperature stability curve.

Statistics

Statistical analysis was performed on GraphPad Prism. The results were analysed for statistically significant differences using one-way ANOVAs followed by post-hoc Tukey’s multiple comparisons tests. If the group variances were found to be significantly different using a Bartlett’s test, then a Welch’s ANOVA was employed instead, followed by post-hoc Dunnett’s T3 multiple comparisons tests. This was implemented for the $T_g$ and $\Delta T_m$ data. The ANOVA results and post-hoc test results are provided as supplementary information.

Results

Enzyme activity and stability

The first hypothesis tested in the present study is to what extent can $T_{\text{opt}}$, $T_m$, and $T_g$ be described as defining characteristics of temperature-adapted enzymes. Figure 1 shows the $T_{\text{opt}}$ (A), $T_m$ (B), and $T_g$ (C) of enzymes from temperature-adapted organisms. The results displayed in Figure 1A show that the $T_{\text{opt}}$ of an enzyme increases with increasing environmental temperatures and that the $T_{\text{opt}}$ values were significantly different in pairwise comparisons ($P$-values, psychrophile-mesophile = 4.2 $\times$ $10^{-9}$, psychrophile-thermophile = 5 $\times$ $10^{-10}$, mesophile-thermophile = 1.4 $\times$ $10^{-5}$). The mean $T_{\text{opt}}$ values ($\pm$SEM) for psychrophilic, mesophilic, and thermophilic enzymes are 32.97 ($\pm$2.16), 55.03 ($\pm$2.52), and 78.03 ($\pm$2.25$^\circ$C respectively.

Similarly, Figure 1B shows that the $T_m$ of an enzyme increases from psychrophiles to thermophiles and that $T_m$ values were significantly different in pairwise comparisons ($P$-values, psychrophile-mesophile = 0.004, psychrophile-thermophile = 5 $\times$ $10^{-10}$, mesophile-thermophile = 5 $\times$ $10^{-10}$). The mean $T_m$ values for psychrophilic, mesophilic, and thermophilic enzymes are 55.02 ($\pm$2.25), 62.37 ($\pm$2.02), and 86.77 ($\pm$2.38$^\circ$C respectively.

The statistically significant difference between the means of both $T_{\text{opt}}$ and $T_m$ for all three groups of organisms suggests that, on average, $T_{\text{opt}}$ and $T_m$ can be described as defining characteristics of an enzyme from organisms adapted to different temperature conditions. Namely, that psychrophiles exhibit the lowest $T_{\text{opt}}$ and $T_m$ as they inhabit the lowest temperature environments, while the opposite is true for the thermophiles with the mesophiles exhibiting intermediate values.

Figure 1C shows that while all enzymes exhibited a $T_g$, it is only statistically significantly different when comparing psychrophilic enzymes to mesophilic or thermophilic enzymes ($P$-values = 0.000896 and 0.00276 respectively). There is no statistical difference between the $T_g$ of mesophilic enzymes and thermophilic enzymes ($P$-value = 0.765462). The mean $T_g$ for psychrophiles is 19.05 ($\pm$2.71)$^\circ$C whereas for mesophiles and thermophiles it is 7.34 ($\pm$1.26) and 8.74 ($\pm$0.99$^\circ$C, respectively. So while most enzymes exhibit a $T_g$, it is significantly greater in psychrophilic enzymes. These results suggest that a large $T_g$ may be considered as an indicative characteristic of psychrophilic enzymes in general, analogous to their canonical characteristics of a lower $T_{\text{opt}}$ and $T_m$.

Effect of mutations

The second hypothesis tested in the present study was that there was a difference in the effect of a mutation (specifically amino acid substitutions) to an enzyme’s $\Delta G_f$ or melting temperature ($\Delta T_m$) among psychrophiles, mesophiles, and thermophiles. Figure 2A shows a representative protein stability curve which could be produced with results from differential scanning calorimetry with the Gibbs free energy of folding on the y-axis and temperature on the x-axis. A protein’s stability curve shows a region of peak stability where $\Delta G_f$ is most negative, and also exhibits two melting points where the curve intersects the x-axis. On Figure 2A, the horizontal and vertical arrows represent the changes to a protein’s melting temperature and Gibbs free energy of folding predicted by HoTMuSiC and PoPMuSiC respectively.

Figure 2B shows the predicted $\Delta T_m$ to enzymes from psychrophiles, mesophiles, and thermophiles upon mutation by HoTMuSiC. The average $\Delta T_m$ was $-2.06$, $-2.23$, and $-2.51^\circ$C for psychrophiles, mesophiles, and thermophiles.
Figure 1. The activity and stability parameters of temperature-adapted enzymes

Panel (A) represents the optimum temperature for enzyme activity (Topt), while (B) shows the melting temperatures (Tm) of the individual enzymes. Panel (C) shows the temperature gap between Topt and Tm, denoted as Tg. The individual data points for psychrophiles are represented by circles, mesophiles by squares, and the thermophiles by triangles. All data points are plotted with the mean ± the SEM. * represent the statistical significance results from Tukey’s multiple comparisons tests for panels (A,B) and Dunnett’s T3 multiple comparisons tests for panel (C) (** = P<0.01, *** = P<0.001, **** = P<0.0001, ns = not significant).
Figure 2. The effects of mutations to temperature-adapted enzymes

Panel (A) shows a representative protein stability curve expressed as its Gibbs free energy of folding ($\Delta G_f$) across temperature. The stability curve exhibits two melting points where it crosses the x-axis, and a peak of stability where the curve has its most negative y value. Horizontal and vertical arrows represent the changes to protein stability predicted by HoTMuSiC and PoPMuSiC respectively ($\Delta T_m$ and $\Delta \Delta G_f$). Panel (B) shows the $\Delta T_m$ predicted by HoTMuSiC to enzymes from psychrophiles, mesophiles, and thermophiles as a result of single amino acid mutations. Panel (C) shows the $\Delta \Delta G_f$ predicted by PoPMuSiC to enzymes from psychrophiles, mesophiles, and thermophiles. All data points are plotted with the mean ± the SEM. * represent the statistical significance results from Dunnett’s T3 multiple comparisons tests for panel (B) and Tukey’s multiple comparisons tests for panel (C) (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, ns = not significant).
respectively. The results suggest that the average reduction in the melting temperature of an enzyme upon mutation increases from psychrophiles to thermophiles, which agrees with previous literature [32]. The only Dunnett’s T3 multiple comparisons test to produce a statistically significant result was between the psychrophiles and the thermophiles (P-value = 0.0019). The difference among the three categories is small in terms of absolute numbers, but as percentages they suggest that the average mutation to a thermophilic enzyme is ~10–25% more destabilising than those to their mesophilic and psychrophilic counterparts. To what extent such differences would have an effect over evolutionary timescales is currently unknown.

Figure 2C shows the predicted ΔΔGf to enzymes from psychrophiles, mesophiles and thermophiles upon mutation by PoPMuSiC. The average ΔΔGf was 1.058, 1.085, and 1.103 kcal.mol$^{-1}$ for psychrophiles, mesophiles, and thermophiles respectively. Similar to the ΔTm results, the average ΔΔGf upon mutation increases from psychrophiles through to the thermophiles. Post-hoc Tukey’s multiple comparisons tests showed statistically significant differences between psychrophiles-mesophiles and psychrophiles-thermophiles with P-values of 0.0451 and 0.000459, respectively.

From these results it is demonstrated that the average mutation to an enzyme not only lowers the melting temperature, but also reduces the thermodynamic stability, thus constricting the global folded phase space. Furthermore, it is shown that mutations are more deleterious to thermophilic enzymes than they are to mesophilic or psychrophilic enzymes.

**Discussion**

In this meta-analysis, we have collated and presented data which further expand our understanding of the defining characteristics of temperature-adapted enzymes. It was shown that the T$_{opt}$ and T$_m$ of enzymes increased with increasing environmental temperatures. In contrast, it was shown that an enzyme’s T$_g$, the gap between the optimum and melting temperature of an enzyme, is significantly larger in psychrophiles, and is in fact a defining characteristic of psychrophilic enzymes that could allow for the prediction of enzymatic psychrophilicity. Additionally it was shown that the average amino acid mutation is predicted to be more destabilising to thermophilic enzymes than it is to mesophilic or psychrophilic enzymes.

Our data allow for several important observations. There is a considerable overlap in the T$_m$ values for psychrophiles and mesophiles, suggesting that increased psychophilic enzyme activity at lower temperatures has not necessarily come at a cost to overall protein stability. This suggests that global protein stability is not a major constraint on psychrophilic enzyme adaptation and evolution. Conversely, thermophilic enzyme stability is more clearly an adaptive feature as seen from the larger difference between the thermophilic and mesophilic T$_m$ means.

The results show that not all psychophilic enzymes necessarily have psychophilic characteristics. This is perhaps best exemplified by one of the enzymes included in our dataset, the most stable psychrophilic glutathione reductase from an Arctic Sphingomonas with a T$_{opt}$ and T$_m$ of 60 and 84.6°C respectively [33], values typically associated with thermophilic enzymes. In this case, the large T$_g$ value of 24.6°C is the best predictive indicator that this enzyme came from a psychrophilic organism. Additionally, few psychophilic enzymes exhibit T$_{opt}$ values which would be considered similar to the expected environmental temperature of a psychrophile.

It should also be noted that the thermophilic T$_m$ and T$_{opt}$ (and consequently T$_g$) values represent a lower estimate of their true population. This is due to exclusion of studies which did not report both the T$_m$ and T$_{opt}$. This largely results from the limitations of circular dichroism apparatus and differential scanning calorimeters used in such studies, which prevent the measurement of high T$_m$ values. It raises a question as to whether there are thermophilic enzymes which are so thermostable that they resist melting until their carbon backbone begins to physically dissociate. The sample size of thermostable enzymes was further reduced due to the propensity to report T$_{50}$ measurements in the literature. This is understandable due to the considerable biotechnological interest in thermostable enzymes [1], where their kinetic stability at elevated temperatures is of more interest than the temperature at which global unfolding occurs.

These data also raise the question of the correlation between enzyme type and the size of T$_g$. Evidence for a correlation was seen with the luciferase enzymes included in our dataset. They exhibit high T$_g$ values in both psychrophiles and mesophiles. Our dataset contained four luciferase enzymes. The three psychophilic luciferase T$_g$ values were 56.4, 58.3, and 54.1°C with a mesophilic firefly luciferase exhibiting a T$_g$ of 15.8°C. Of additional interest is the observation that all three psychophilic luciferases were more thermostable than the mesophilic firefly luciferase, by as much as 31°C.

While our results show that a large T$_g$ is a defining characteristic of psychrophilic enzymes, they cannot elucidate the precise source of this phenomenon. We can however discuss the consequences of each hypothesis with regards to our analysis. Multiple explanations have been proposed to explain this observation such as, active
site unfolding [4,20,21], an equilibrium model [22], macromolecular rate theory [23–25], and the loss of specific temperature-sensitive enzyme–substrate interactions [26]. The initial explanation that the active site of α-amylase from the psychrophile *Pseudoalteromonas haloplanktis* is particularly thermolabile [21] possesses strong explanatory power and fits with observations that increased active site flexibility and dynamics are key to achieving greater enzymatic activity at low environmental temperatures [34–37]. Within the framework of this hypothesis, our results would suggest that, as a population, psychrophilic enzymes possess significantly more thermolabile active sites than do mesophiles or thermophiles compared with the stability of the whole enzymes. An equilibrium model interpretation of the data would suggest that psychrophilic enzymes reach the equilibrium temperature (*T*<sub>eq</sub>), the point at which half the enzyme is active, much before they reach their *T*<sub>m</sub>. This would suggest that the ratio of active to inactive enzyme forms (*E*<sub>act</sub>/*E*<sub>inact</sub>) is particularly temperature sensitive in psychrophiles and therefore results in a larger *T*<sub>g</sub>. The loss of temperature-sensitive enzyme–substrate interactions proposed by Sočan et al. [26] is largely a molecular level interpretation of the equilibrium model as they propose a ‘dead-end model’ where an inactive enzyme forms with increasing temperature. This would suggest that the interactions between substrates and psychrophilic enzymes is significantly weaker than those of mesophilic and thermophilic enzymes and therefore is the source of the large *T*<sub>g</sub> in psychrophilic enzymes. Macromolecular rate theory would predict that the change in heat capacity of activation (∆*C*<sub>p</sub>) is significantly lower in psychrophilic enzymes compared with mesophilic and thermophilic enzymes. This would cause a larger *T*<sub>g</sub> in psychrophiles due to the increasing curvature of the temperature-dependent activity profile as ∆*C*<sub>p</sub> is lowered. No single hypothesis may explain the *T*<sub>g</sub> phenomenon and diverse hypotheses may be applicable to different situations. It will require precise measurements on the molecular level to determine the true origin of *T*<sub>g</sub> for each enzyme.

The lower *T*<sub>g</sub> values for mesophilic and thermophilic enzymes may be useful for validating ancestrally reconstructed enzymes. Ancestral reconstruction tends to produce more thermostable enzymes [38,39], however there is a concerning that this may be an artifact due to biases in the reconstruction process [40]. Therefore based on our meta-analysis, if these ancestral enzymes were indeed more thermostable, then one should not expect to find that *T*<sub>g</sub> increases significantly when constructing an ancestral enzyme from the modern day mesophilic form.

The mutational data presented here are in strong agreement with the well-established observation that mutations are on average destabilising. The ∆*T*<sub>m</sub> values reported here are less destabilising than those presented in previous literature [32] which ranged from approx. −1.3 to −5°C, with thermophilic proteins predicted to experience more destabilising mutations. This may be due to the focus on enzymes in the present study, which may produce more stabilising mutations than the average non-enzymatic protein. This could be explained by the fact that the active site of an enzyme generally contributes little to stability, therefore mutating it tends to introduce stabilising interactions [41–44] or have more neutral effects. Our data does however point towards an increasing trend in this deleterious nature with increasing environmental temperatures. Therefore, studies regarding the trajectories and timescales of enzyme evolution may require varied weighting of mutational effects depending on the thermostability of the enzymes in question.

The observation that mutations are more deleterious to thermophilic enzymes agrees with the hypothesis put forward by Drake [27]. If there is a tight coupling between a thermophile's environmental temperature and its enzymes' temperature stabilities, then a difference in ∆*T*<sub>m</sub> of 0.5°C may be sufficient to make the average mutation particularly potent against thermophile survivability. So while thermophilic proteins may be more tolerant to mutations at ∼30°C compared with their mesophilic counterparts [45], the coupling of environmental temperature and *T*<sub>m</sub> would produce the phenomenon of lower mutational tolerance *in situ*. In contrast with Drake [27], Li et al. [29] have reported that microbial communities evolve faster in extreme environments. Drake reported that the *d*<sub>SN</sub>/d*<sub>IN</sub> (the non-synonymous/synonymous mutation ratio) for thermophiles was lower for thermophiles compared with mesophiles, 0.09 versus 0.14 respectively, suggesting thermophiles tolerate less mutation. However, Li et al. report that communities of thermophiles from hot springs have a higher *d*<sub>SN</sub>/d*<sub>IN</sub> than communities from the surface ocean, freshwater or soil (*d*<sub>SN</sub>/d*<sub>IN</sub> values of 0.126, 0.061, 0.087, and 0.087 respectively). Li et al. also reported higher relative evolutionary rates (rERs) for thermophilic communities compared to freshwater and soil communities. It is hard to directly compare the two studies though, as Drake [27] considered other mutation types such as chain terminations and indel mutations. On the other hand, Drake [27] examined two species of thermophiles, so it is difficult to extrapolate those results to all thermophiles, whereas Li et al. have reported data at the community level, making their work potentially more representative of thermophiles as a class of organism. The experimental determination of whether psychrophiles and mesophiles can tolerate higher mutational loads than thermophiles, while critical for answering this question, is limited by the long time-course required to culture and grow such organisms.
Conclusion
The aim of the present study was to further explore the characteristics of temperature-adapted enzymes. It was shown, in strong agreement with theory, that the $T_{\text{opt}}$ and $T_{m}$ increases with an organism’s environmental temperature. It was also shown that a large $T_g$ is a defining characteristic of psychrophilic enzymes and in certain cases is a better predictor of psychrophilicity than either $T_{\text{opt}}$ or $T_m$. The average effect of an amino acid mutation to temperature-adapted enzymes was also explored. It was found that the average $\Delta T_m$ and $\Delta \Delta G_f$ becomes more deleterious, with increasing environmental temperature. The difference in deleterious effect was small and the effect of this over evolutionary timescales is unknown.

Data Availability
The source data for all results are provided as Supplementary Data. Dataset 1 contains the $T_m$, $T_{\text{opt}}$ and $T_g$ values for each enzyme, their source organism, and their literature source. Dataset 2 contains the PDB IDs of all enzymes used in the mutation results with their average $\Delta T_m$ and $\Delta \Delta G_f$. Both datasets contain a summary table. The ANOVA results and post-hoc test results for each analysis are also provided as a supplementary data file.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution
Stewart Gault conceived the study and created the datasets. Stewart Gault, Peter M. Higgins, Charles S. Cockell and Kaitlyn Gillies contributed to data analysis, manuscript drafting and editing.

Abbreviations
PDB, Protein Data Bank; $T_g$, temperature gap; $T_m$, melting temperature; $T_{\text{opt}}$, optimum temperature; $\Delta \Delta G_f$, Gibbs free energy of folding.

References

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5.3 Conclusion

The aim of this research chapter was to understand the adaptations of temperature-adapted enzymes, specifically psychrophilic enzymes, and how these adaptations might apply to perchlorate rich environments on Mars.

I found that psychrophilic enzymes exhibit lower optimum temperatures and lower melting temperatures, as is expected of enzymes from low temperature environments. Additionally, I found that the temperature gap, $T_g$, was indeed significantly larger for psychrophilic enzymes. This was the first time that this has been shown to be a general feature of psychrophilic enzymes. What ultimately causes this large $T_g$ is up for debate as it can be explained by multiple models. I personally interpret the larger $T_g$ from the perspective of psychrophilic enzyme flexibility. By interpreting it in terms of psychrophilic enzyme flexibility, it allows for better comparisons with how perchlorate salts affect enzyme activity and allows for hypothesis development without the need of detailed structural and thermodynamic data. This became apparent in the development of the research presented in Chapter 6.

The mutation data suggested psychrophilic enzymes would experience the smallest reduction in protein stability after an amino acid substitution. The reason for this, and its consequences over evolutionary time however remain unclear. It could be that the weaker/ less stabilising interactions within psychrophilic enzymes means there’s less stability to be lost on mutation. Alternatively it’s that mesophilic and thermophilic enzymes have more stabilising interactions to lose upon mutation. What’s also unclear is how far the respective enzyme’s melting temperature is from their environmental temperatures. This is an important factor with regards to the evolutionary potential of these enzymes, because if a thermophilic enzyme’s melting temperature is significantly higher than the environmental temperature ($T_{env}$), then despite the fact their mutations are more deleterious, they would be able to tolerate more of them. Attempts to compare $T_m$ to $T_{env}$ were hampered by the fact that many psychrophilic and mesophilic enzymes came from organisms with a range of $T_{env}$ instead of a defined temperature, which is typically reported for thermophiles. Additionally, many thermophilic enzymes have no reported melting temperature in the literature as they remain stable up to the limit of detection through CD or DSC, so comparing thermophilic $T_m$ to $T_{env}$ might have at best produced a lower estimate for this gap.
Chapter 6

Perchlorate salts confer psychrophilic characteristics in α-chymotrypsin

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6.1 Introduction

The previous research chapters investigated the activity of α-chymotrypsin in the presence of perchlorate salts and at high hydrostatic pressures in order to understand the potential for enzyme activity in Martian deep subsurface environments. As these environments are extremely cold, theorised to be around -70 °C, understanding how the effect of perchlorate salts on enzyme activity might change with temperature is important.

After studying the structural and thermodynamic origins of psychrophilic enzymes’ increased activity at low temperatures, I hypothesised that while perchlorate salts lower the activity of α-chymotrypsin at ~24°C, they should in fact increase α-chymotrypsin’s activity at low temperature.

As psychrophilic enzymes are described as being more flexible due to reduced numbers of stabilising interactions, I hypothesised that perchlorate salts might make α-chymotrypsin more flexible by interacting with, and destabilising α-chymotrypsin’s intramolecular bonds. If this were true then the thermodynamics of α-chymotrypsin’s activity should change in the presence of perchlorate salts to mimic those of psychrophilic enzymes, namely the activation enthalpy should be lower, and the entropy of activation should also be more negative.

I found that perchlorate salts can indeed increase the low temperature activity of α-chymotrypsin. Thermodynamic analysis revealed that both the enthalpy and entropy of activation were both lowered in the presence of perchlorate salts.

The same low temperature activity analysis was attempted at high pressures to try to understand how perchlorate salts, pressure, and temperature interact to ultimately affect biomolecules such as α-chymotrypsin. However due to the limits of the spectrophotometer a rigorous analysis of all three extremes was not fully possible.
Perchlorate salts confer psychrophilic characteristics in α-chymotrypsin

Stewart Gault1,2*, Michel W. Jaworek2, Roland Winter2 & Charles S. Cockell1

Studies of salt effects on enzyme activity have typically been conducted at standard temperatures and pressures, thus missing effects which only become apparent under non-standard conditions. Here we show that perchlorate salts, which are found pervasively on Mars, increase the activity of α-chymotrypsin at low temperatures. The low temperature activation is facilitated by a reduced enthalpy of activation owing to the destabilising effects of perchlorate salts. By destabilising α-chymotrypsin, the perchlorate salts also cause an increasingly negative entropy of activation, which drives the reduction of enzyme activity at higher temperatures. We have also shown that α-chymotrypsin activity appears to exhibit an altered pressure response at low temperatures while also maintaining stability at high pressures and sub-zero temperatures. As the effects of perchlorate salts on the thermodynamics of α-chymotrypsin's activity closely resemble those of psychrophilic adaptations, it suggests that the presence of chaotropic molecules may be beneficial to life operating in low temperature environments.

Studies of the interactions between salts and biomolecules tend to rank their effects along the continuum of the Hofmeister series. The Hofmeister series for ions is ordered based on the ability of cations (K⁺ > Na⁺ > Li⁺ > Mg²⁺ > Ca²⁺) and anions (SO₄²⁻ > Cl⁻ > ClO₄⁻ > SCN⁻) at medium to high concentrations to decrease the solubility of proteins in water (‘salting-out’ effect)1. In general, anions appear to have a larger effect than cations. It has been proposed that salts higher up in the Hofmeister series increase the solubility of proteins in solution by weakening the strength of hydrophobic interactions (‘salting-in’ effect). Hence, protein stability should be disfavored with salts higher up in this series. Further, highly chaotropic anions, such as perchlorates (ClO₄⁻), which are found pervasively on Mars2, have been shown to act as a protein denaturant3 owing to their ability to interact with the surface of biomacromolecules4–8. They are also able to perturb the water structure and therefore are likely to perturb hydrogen bonding, including within that of water itself9–12.

Chaotropic salts are normally associated with deleterious phenomena such as reduced enzyme activity and protein stability3,13–16, whereas kosmotropic salts normally produce the opposite effect17,18. However, this representation is an oversimplification as it is possible to find examples where kosmotropic salts decrease enzyme activity19 and examples where highly chaotropic agents increase enzyme activity19–32. The cases of chaotropes increasing enzyme activity raises the question as to the mechanisms of this effect.

The activation of enzymes by chaotropic molecules is frequently attributed to increased enzyme flexibility as a result of structural destabilisation13–16. In a folding energy landscape picture, the addition of the chaotropic salt leads to the population of a conformational substate of the native-state ensemble of conformations of the enzyme which is more suitable for substrate binding and conversion. Increased conformational flexibility is also linked to the high activity of psychrophilic enzymes at low environmental temperatures39–38. For psychophilic enzymes, this increased conformational flexibility manifests thermodynamically as a lower activation enthalpy (ΔH‡) when compared to their mesophilic and thermophilic counterparts39. However, as psychophilic enzymes have more conformational freedom, it means that they suffer a larger entropic penalty when forming the activated transition state, thus resulting in more negative entropy of activation values (ΔS‡)39.

The effect of chaotropic salts on the fluorescent quenching of proteins is also strikingly similar to what is observed with psychrophilic enzymes19, in which an increase in quencher penetration into hydrophobic protein regions is observed. The interpretation is that chaotropic salts disturb protein structure and that psychophilic enzymes are more loosely packed, thus aiding quencher penetration. As psychophilic enzymes share many
similarities with non-psychrophilic enzymes exposed to chaotropic molecules, it raises the question as to whether they share similar underlying thermodynamic mechanisms. If chaotropic molecules lower enzyme activity at room temperature, but also reduce the enthalpy and entropy of activation, then we should hypothesise that this would result in increased enzyme activity at lower temperatures.

Here we use α-chymotrypsin as a model enzyme to explore the effects of perchlorate salts on enzyme activity across temperature. Bovine α-chymotrypsin is a standard serine protease which is mesophilic with regards to its temperature stability and has been shown to exhibit reduced activity and structural stability in the presence of chaotropic molecules such as perchlorate and thiocyanate salts\(^3,\)\(^13,\)\(^15,\)\(^17,\) whereas kosmotropic molecules such as NaSO\(_4\) has previously been shown to increase α-chymotrypsin’s activity and stability\(^17\). We show here that Mg(ClO\(_4\))\(_2\) and NaClO\(_4\) lower the enzyme activity of α-chymotrypsin at room temperature, but that at lower temperatures these salts become activators of enzyme activity. This low temperature activation is caused by an altered temperature dependency of enzyme activity as a result of lower Δ\(H^\ddagger\) and Δ\(S^\ddagger\). The results show that there is a balance between these thermodynamic parameters dictating when the low temperature activation occurs. We further show that α-chymotrypsin is pressure stable at sub-zero temperatures and that low temperatures alter the effect of pressure on the activity of α-chymotrypsin.

Materials and methods
Lyoophilised α-chymotrypsin from bovine pancreas, benzoyl-L-tyrosine ethyl ester (BTEE), D\(_2\)O, DCI, Tris HCl and glycine were obtained from Sigma-Aldrich. Mg(ClO\(_4\))\(_2\) and NaClO\(_4\) were obtained from Alfa Aesar. CaCl\(_2\) was obtained from Fisher Scientific.

Measuring α-chymotrypsin activity. Chymotrypsin’s activity was measured using BTEE as the substrate. Buffer consisted of 0.1 M Tris–HCl and 0.01 M CaCl\(_2\) adjusted to pH 7.8. Chymotrypsin concentration was maintained at 20 nM for all reactions. The concentration of the enzyme was determined using its absorbance at 280 nm with a molar extinction coefficient of 51,000 M\(^{-1}\) cm\(^{-1}\)\(^40\). The concentrations of BTEE assayed were 10, 20, 50, 75, 100, 150 and 200 μM. Activity was measured in the absence and presence of Mg(ClO\(_4\))\(_2\) (0.25 and 0.5 M), NaClO\(_4\) (1 M) and glycine (1 M). Salt concentrations were selected based on results from previous work\(^3\) in order to ensure sufficient enzyme activity at low temperatures to differentiate the signal from noise. Glycine has been shown to increase α-chymotrypsin activity\(^41\) and so was included in some experiments to assess how it affected the chaotropic effect. The production of benzoyl-l-tyrosine was measured at 256 nm with a Jasco V-730 spectrophotometer during the course of the reaction. The temperature of the cuvette holder and samples were controlled and adjusted using a circulating water bath. Enzyme activity was measured from 35 to 5 °C in 5 °C increments. Measurements below 5 °C were prevented by significant condensation within the spectrophotometer which affected absorbance traces. Product formation was measured for one minute and the initial linear portion of the absorbance trace was recorded as the rate. The enzyme activity was plotted in GraphPad Prism and a least squares fit fitting method was applied according to the Michaelis–Menten equation:

\[
v = \frac{v_{\text{max}} [S]}{K_M + [S]} \tag{1}
\]

where \(v_{\text{max}}\) is the maximal rate, \(K_M\) is the Michaelis constant (substrate concentration at \(v_{\text{max}}/2\)), and \([S]\) is the substrate concentration. The high-pressure stopped-flow system, HPSF-56, from Hi-Tech Scientific was used to investigate the enzyme activity under high pressure. The enzyme concentration and substrate concentration range used, and enzymatic analysis were the same as those used for the measurements at ambient pressure. The temperature was controlled and maintained at 6 ± 0.5 °C by a thermostat. The system and function of the high-pressure stopped-flow instrument has been described in detail elsewhere\(^41–43\). Glycine-containing conditions were excluded from these assays due to negligible activation when BTEE is the substrate at the perchlorate concentrations assayed. The high pressure activity results have limited accuracy, however, owing to technical limitations. The spectrophotometer used has a wavelength limit of 250 nm which is close to the peak absorption wavelength of the product at 256 nm. Additionally the low operational temperatures caused the pressure cell to begin to leak above 1.5 kbar, hence limiting our ability to probe higher pressures.

Thermodynamics of enzyme activity. The Gibbs free energy of activation (Δ\(G^\ddagger\)) was determined using transition-state theory through the following equation\(^42\):

\[
\Delta G^\ddagger = -RT\ln(hk_{\text{cat}}/k_BT) \tag{2}
\]

where \(R\) is the universal gas constant, \(h\) is the Planck constant, \(k_B\) is the Boltzmann constant, and \(T\) is the temperature in Kelvin. A plot of \(\ln(k_{\text{cat}}/T)\) vs. \(1/T\) allows for the determination of the enthalpies and entropies of activation (Δ\(H^\ddagger\), Δ\(S^\ddagger\)) from linear regressions and an expanded version of Eq. (2):

\[
k_{\text{cat}} = (k_BT/h)e^{-(\Delta H^\ddagger/RT) + (\Delta S^\ddagger/RT)} \tag{3}
\]

The slope of the linear regression is equivalent to − Δ\(H^\ddagger/R\) and the \(y\)-intercept of the regression gives Δ\(S^\ddagger\) by (Δ\(S^\ddagger/R\) + ln\((k_B/h)\).

Fourier-transform infrared (FTIR) spectroscopy. For pressure-dependent Fourier-transform infrared (FTIR) spectroscopy studies, the protein was dialysed against D\(_2\)O using Amicon Ultra centrifugation units with 10 kDa cut-off and subsequently lyophilized. The measurements were carried out in the same buffer conditions.
as for the HPSF measurements in the absence and in the presence of 0.25 M Mg(ClO₄)₂. A protein concentration of 5 wt% was used. The pD-value of both buffers was adjusted to 7.8 (pH + 0.4 = pD) by adding DCl. The temperature of the cell was regulated with an external, circulating water thermostat to −3 °C. The equipment and setup of the high-pressure system has been described elsewhere13. Spectra were processed and analysed with the Grams AI 8.0 software (Thermo Fisher Scientific) as follows: after buffer subtraction and smoothing, the area of the amide I' band was normalized to 1. The number of subbands and their positions for fitting were obtained via Fourier self-deconvolution (FSD) and 2nd derivative approaches. The amide I’ band region of α-chymotrypsin can be decomposed into eight subbands as already reported elsewhere45. To determine the relative changes in the population of secondary structure elements, mixed Gaussian–Lorentzian line shape functions were used to fit the peak areas in the amide I’ band region (curve fitting procedure)46. Assuming a two-state unfolding process of the protein, a Boltzmann function can be fitted to the temperature- and pressure-dependent sigmoidal curve progression of the intensity changes:

\[
I_f = \frac{I_f - I_u}{1 + e^{-\frac{(T - T_u)}{\Delta H_{vH,u} / R} + I_u}} + I_u
\]

\[
I = \frac{I_f - I_u}{1 + e^{-\frac{(p - p_u)}{\Delta V_{p,u} / R} + I_u}} + I_u
\]

\[
\Delta H_{vH,u} = \frac{\Delta H_{cal} - \Delta H_{vH}}{2}
\]

\[
\Delta V_{p,u} = \frac{\Delta V_{p,cal} - \Delta V_{p}}{2}
\]

\[
T_u and p_u are the plateau values of the IR band intensities of the folded and unfolded protein. The unfolding temperature, \(T_u\), and unfolding pressure, \(p_u\), were obtained from the inflection points of the sigmoidal curves.

**Differential scanning calorimetry.** The differential scanning calorimetric (DSC) experiments were conducted using a MicroCal (Northampton, MA, USA) VP-DSC system. While the sample cell was filled with the protein solution, the reference cell contained the corresponding buffer solution with a sample volume of around 0.5 mL. A protein concentration of 1 mg mL⁻¹ was used for the DSC experiments. The measurements were performed between 10 and 95 °C with a heating rate of 60 °C/h. The analysis of the DSC thermograms was carried out by the MicroCal Origin processing software. From the DSC measurements, the calorimetric enthalpy change, \(\Delta H_{cal}\), and the thermal denaturation temperature, \(T_m\), could be obtained. Three independent calorimetric measurements revealed a calorimetric enthalpy change of 791 ± 54 kJ/mol. The value may be compromised to some extent by the high-temperature aggregation of the protein. Here, only the determination of \(T_m\) and a rough estimate of \(\Delta H_{cal}\) is needed.

**Results**

**α-Chymotrypsin activity.** Figure 1 and Table 1 show that at 35 °C the activity, \(k_{cat}\), of α-chymotrypsin is reduced in the presence of all perchlorate-containing conditions. For example, 0.25 and 0.5 M Mg(ClO₄)₂ reduced the \(k_{cat}\) of α-chymotrypsin by ~10 and 35 s⁻¹ respectively at 35 °C compared to the buffer only condition.

As the temperature is lowered, the activities associated with different experimental conditions begin to converge, until eventually we observe increased α-chymotrypsin activity at 5 °C in the presence of perchlorate salts (except 0.5 M Mg(ClO₄)₂). Table 1 details the \(k_{cat}\) values for each condition at 5 °C. Additionally, the \(K_M\) of α-chymotrypsin was increased in the presence of perchlorate salts, with \(K_M\) gradually decreasing with increasingly lower temperatures across all conditions. The catalytic efficiency, \(k_{cat}/K_M\), was generally lower at 35 °C in the perchlorate-containing conditions, however at 5 °C some of the perchlorate-containing conditions exhibited increased catalytic efficiency compared to the buffer only condition. The \(k_{cat}, K_M\), and \(k_{cat}/K_M\) of α-chymotrypsin in all tested conditions and temperatures is shown in Tables S1, S2, and S3 respectively.
The effect of the compatible solute glycine on α-chymotrypsin activity is shown in Fig. 1 and Table 1. Our results show that glycine activates α-chymotrypsin to a much lesser extent when BTEE is the substrate, compared to when N-succinyl-l-phenylalanine-p-nitroaniline is used. The small activating effect of glycine may be caused by alterations to either enzyme or substrate hydration, therefore affecting the dehydration required for enzymatic catalysis. The activating effect of glycine was most evident in the 0.5 M Mg(ClO₄)₂ condition. In this scenario, glycine may be stabilising a subpopulation of α-chymotrypsin conformations which exhibit altered kinetic parameters compared to those present in the 0.5 M Mg(ClO₄)₂ condition. In general, glycine had the effect of increasing $k_{cat}$, decreasing $K_M$ and increasing $k_{cat}/K_M$ compared to the respective conditions without glycine.

The thermodynamic effects of perchlorate salts and glycine on α-chymotrypsin activity are shown in Table 2. In Table 2, we observe that the increased α-chymotrypsin activity at 5 °C in the presence of perchlorate salts is derived from the lower values of Δ$G^\ddagger$. This is shown schematically in Fig. 2 when comparing the free energy changes across α-chymotrypsin's reaction coordinate. The reaction coordinate of α-chymotrypsin at 308 and 278 K in buffer (black) and in the presence of perchlorate salts (red). Created in BioRender.com.

### Table 1. α-Chymotrypsin activity values. The turnover number ($k_{cat}$), Michaelis constant ($K_M$) and catalytic efficiency ($k_{cat}/K_M$) of α-chymotrypsin at 308 K (35 °C) and 278 K (5 °C) in the presence and absence of perchlorate salts and 1 M glycine. Kinetic parameters were determined from the Michaelis–Menten curves of n = 4 replicates.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>71.23</td>
<td>12.66</td>
<td>72.60</td>
</tr>
<tr>
<td></td>
<td>24.66</td>
<td>9.81 × 10⁵</td>
<td>5.13 × 10⁵</td>
</tr>
<tr>
<td>+ Glycine</td>
<td>74.99</td>
<td>14.17</td>
<td>67.41</td>
</tr>
<tr>
<td></td>
<td>12.80</td>
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<td>+ Glycine</td>
<td>67.47</td>
<td>15.02</td>
<td>91.49</td>
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<tr>
<td></td>
<td>27.49</td>
<td>7.37 × 10⁷</td>
<td>5.46 × 10⁷</td>
</tr>
<tr>
<td>+ Glycine</td>
<td>61.07</td>
<td>14.93</td>
<td>77.48</td>
</tr>
<tr>
<td></td>
<td>18.43</td>
<td>7.88 × 10⁷</td>
<td>8.10 × 10⁷</td>
</tr>
<tr>
<td>+ Glycine</td>
<td>61.76</td>
<td>15.47</td>
<td>121.30</td>
</tr>
<tr>
<td></td>
<td>34.37</td>
<td>5.09 × 10⁷</td>
<td>4.50 × 10⁷</td>
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<tr>
<td>+ Glycine</td>
<td>62.55</td>
<td>17.73</td>
<td>73.40</td>
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<tr>
<td></td>
<td>19.35</td>
<td>8.52 × 10⁷</td>
<td>9.16 × 10⁷</td>
</tr>
<tr>
<td>+ Glycine</td>
<td>36.58</td>
<td>10.54</td>
<td>54.05</td>
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<td></td>
<td>4.72</td>
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<td>42.43</td>
<td>15.65</td>
<td>83.04</td>
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<tr>
<td></td>
<td>25.67</td>
<td>5.11 × 10⁷</td>
<td>6.10 × 10⁷</td>
</tr>
</tbody>
</table>

### Table 2. α-Chymotrypsin thermodynamic parameters. The free energy of activation (Δ$G^\ddagger$) at 308 K (35 °C) and 278 K (5 °C) and the enthalpy and entropy of activation (Δ$H^\ddagger$, Δ$S^\ddagger$) in the presence and absence of perchlorate salts and 1 M glycine.

<table>
<thead>
<tr>
<th></th>
<th>Δ$G^\ddagger$ (kJ mol⁻¹)</th>
<th>Δ$H^\ddagger$ (kJ mol⁻¹)</th>
<th>Δ$S^\ddagger$ (J mol⁻¹ K⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>64.63</td>
<td>38.21</td>
<td>− 84.81</td>
</tr>
<tr>
<td>+ Glycine</td>
<td>64.50</td>
<td>35.54</td>
<td>− 94.12</td>
</tr>
<tr>
<td>1 M NaClO₄</td>
<td>64.77</td>
<td>32.73</td>
<td>− 104.01</td>
</tr>
<tr>
<td>+ Glycine</td>
<td>65.02</td>
<td>30.71</td>
<td>− 110.75</td>
</tr>
<tr>
<td>0.25 M Mg(ClO₄)₂</td>
<td>64.99</td>
<td>26.89</td>
<td>− 123.44</td>
</tr>
<tr>
<td>+ Glycine</td>
<td>64.96</td>
<td>27.33</td>
<td>− 121.22</td>
</tr>
<tr>
<td>0.5 M Mg(ClO₄)₂</td>
<td>66.34</td>
<td>26.13</td>
<td>− 129.72</td>
</tr>
<tr>
<td>+ Glycine</td>
<td>65.96</td>
<td>21.97</td>
<td>− 141.17</td>
</tr>
</tbody>
</table>
changes of α-chymotrypsin activity at 308 and 278 K. Perchlorate salts exert this effect on α-chymotrypsin by lowering ΔH‡, which is beneficial for enzyme activity, but as ΔS‡ is also lower in these conditions, the activating effect is only seen at lower temperatures where the TΔS‡ term has lesser bearing on ΔG‡. The extent to which ΔH‡ and ΔS‡ is lowered by perchlorate salts follows a standard Hofmeister series, with Mg(ClO₄)₂ having a greater effect than NaClO₄. It is also concentration dependent. The general thermodynamic effect of glycine was to further decrease both ΔH‡ and ΔS‡ in most conditions, thus allowing for further enzyme activation. The ΔG‡ of all conditions tested at each temperature is shown in Table S4.

We then attempted to analyse the relationship between temperature, pressure, and perchlorate salts on α-chymotrypsin activity in a high-pressure stopped-flow spectrophotometer (HPSF). The results are shown in Fig. 3. We found that, in the high pressure spectrophotometer (1–1500 bar), there was virtually no difference between the activity of α-chymotrypsin in absence and presence of 0.25 M Mg(ClO₄)₂ at 6.5 °C and that the enzyme activity remained constant across all pressures tested. The Michaelis–Menten curves for all enzyme activity measurements are shown in Figs. S1 and S2.

Low temperature pressure stability of α-chymotrypsin. To explore the pressure stability of the enzyme, i.e. the stability of the secondary structure elements of the protein at −3 °C, the amide I’ band was recorded over a pressure range from 1 bar to 10 kbar (Fig. 4). The amide I’ band of the enzyme is characterized by a broad band at 1638 cm⁻¹, which shows only minor changes upon compression up to 10 kbar. Changes of the secondary structural motifs as derived from the fitting procedure of the amide I’ band region (see Materials and Methods section for details) are accordingly very small (if there are any) and amount to a few percent (1–2%), only. The small shift of the amide I’ band to lower wavenumbers is essentially due to an elastic compression of the protein. In the presence of 0.25 M Mg(ClO₄)₂ no significant changes of the amide I’ band and the population of secondary structure elements could be observed at −3 °C. These results demonstrate that α-chymotrypsin exhibits a remarkable stability under these high-pressure, low-temperature conditions. At higher temperatures, however, for both solution conditions, partial unfolding of α-chymotrypsin has been observed at 6–8 kbar⁴².

p, T-stability phase diagram of α-chymotrypsin. The stability of a protein is a simultaneous function of temperature and pressure. The Gibbs free energy difference between the unfolded and native state, \( \Delta G = G_{\text{unfolded}} - G_{\text{native}} \) with respect to some reference temperature, \( T_0 \), and pressure, \( p_0 \), in a second-order Taylor expansion is given by⁴⁷:

\[
\Delta G = \Delta G_0 + \frac{\Delta \kappa' T^2}{2} (p-p_0)^2 + \Delta \alpha' (p-p_0) (T-T_0) - \Delta C_p \left[ T \left( \ln \frac{T}{T_0} - 1 \right) + T_0 \right] + \Delta V (p-p_0) - \Delta S (T-T_0)
\]

where \( \Delta \) denotes the change of the corresponding parameter upon unfolding, \( \kappa' \) is the isothermal compressibility factor, \( \kappa'_T = \frac{\partial V/\partial P}{T} \) = \( -V \kappa_T \), with \( \kappa_T \) being the coefficient of isothermal compressibility, \( \alpha' \) is the thermal expansivity factor, \( \alpha' = \frac{\partial V/\partial T}{T} = \frac{-(\partial S/\partial p)_T}{V} = V \alpha \), where \( \alpha \) is the coefficient of thermal expansion of the system, \( C_p \) is the heat capacity at constant pressure. The transition line, where the protein unfolds, is obtained by setting \( \Delta G = 0 \). As reference points, we have chosen \( p_0 = \) atmospheric pressure (1 bar = 10⁵ Pa) and \( T_0 = T_m \), the unfolding temperature of the protein at ambient pressure. Truncation of the Taylor series at the second-order terms means that the second derivatives of the Gibbs free energy difference (\( \Delta C_p, \Delta \kappa', \Delta \alpha' \)) do not change significantly with temperature and pressure. As can be seen from [Eq. (6)], for calculating \( \Delta G \), experimental data for all input parameters are required. For selected monomeric proteins, such as the protein staphylococcal nuclease (SNase), all thermodynamic input parameters have been determined experimentally⁴⁷, enabling us to successfully calculate \( \Delta G(p,T) \) and the corresponding \( p, T \)-stability diagram of the protein. For α-chymotrypsin, available experimental data for \( T_m \) and \( \Delta H \) have been used. For the remaining parameters, reasonable values have been assumed or were derived by the fitting procedure to the experimental data points: α-chymotrypsin in buffer solution: \( T_m = 329.1 \) K (FTIR data: 330.7 K, DSC data: 327.5 K), \( \Delta H = 800 \) kJ mol⁻¹ (DSC data: 791 ± 54 kJ mol⁻¹), \( \Delta V = -35 \) mL mol⁻¹, \( \Delta C_p = 3.5 \) kJ mol⁻¹ K⁻¹, \( \Delta \alpha = 7.5 \times 10^{-4} \) K⁻¹, \( \Delta \kappa' = 3.0 \times 10^{-7} \) bar⁻¹; α-chymotrypsin in 0.25 M Mg(ClO₄)₂: \( T_m = 318.6 \) K, \( \Delta H = 750 \) kJ mol⁻¹, the other parameters have been kept constant.)

![Figure 3. High pressure activity of α-chymotrypsin across pressure at 279 K. The high pressure enzyme activity of α-chymotrypsin expressed as \( k_{cat} \) at 279 K (6 °C) in buffer (pink) and in buffer containing 0.25 M Mg(ClO₄)₂ (orange). N=4 and error bars represent the standard error of the mean.](image-url)
Figure 5 shows the $p,T$-phase diagram for α-chymotrypsin in pure buffer and in 0.25 M Mg(ClO$_4$)$_2$, respectively (the two $T_m$-values at ambient pressure correspond to data from FTIR spectroscopy and DSC measurements, respectively). The lines show the fits to the experimental data using the parameters given above. Note that, owing to the many parameters involved, slightly different parameter sets will lead to similar fits. Further, as the unfolding process of α-chymotrypsin may not obey a simple two-state unfolding scenario at this pH value and solution conditions, van’t Hoff-derived enthalpy changes from spectroscopic data may differ significantly from the calorimetric ones. Further improving the fits to obtain better fits by assuming temperature and pressure dependent $\Delta C_p$, $\Delta \alpha$ and $\Delta \kappa$ values was omitted here. Hence the calculated stability curves may be regarded a rather approximate.

**Discussion**

Enzymes are adapted to operate across a range of chemical and physical parameters encountered in their environment. The effects of such parameters on enzyme activity and stability are normally studied in isolation. This, however, risks obscuring how environmental parameters collectively come together to affect enzymes and shape their evolution. Here we have shown that perchlorate salts alter the thermodynamic properties of α-chymotrypsin activity, and as a result, alters the temperature dependency of its activity. In essence, perchlorate salts impart psychrophilic characteristics unto bovine α-chymotrypsin.

We observed that α-chymotrypsin activity was increased at low temperatures in the presence of perchlorate salts and showed that this was caused by the salt lowering the activation enthalpy. As perchlorate salts destabilise enzymes, their disruptive effect reduces the number of weak stabilising interactions (e.g., H-bonds), thus increasing the conformational flexibility of the enzyme. The reduced number of stabilising interactions means that fewer bonds need to be broken in order to form the transition state complex, resulting in a lower activation enthalpy, which is beneficial for enzymatic catalysis. Previous studies on the activation of enzymes by chaotropic molecules have also suggested that such activation is caused by increased conformational flexibility, as evidenced by increased fluorescent quencher penetration and an increased susceptibility to tryptic digest. Furthermore, recent elastic incoherent neutron scattering experiments have shown that chaotropic agents, such as urea, increase the fast sub-nanosecond dynamics (mean-squared displacements) of protein atoms within the native fold. Certainly, if the perchlorate concentration is too high, partial, or full unfolding of the enzyme will take place, which will cause the enzyme activity to be lost.

Other mechanisms may contribute to the increase in enzyme activity. Owing to the chaotropic nature of the perchlorate anion at the high concentrations we tested, the hydrogen-bond network structure of the solvent H$_2$O
is perturbed\textsuperscript{9–12}, which might change the hydration properties of the reactants (and hence their activities), and, for example, help facilitate dehydration of the substrate and active site in the course of the reaction. Additionally, the extent to which \( \alpha \)-chymotrypsin is preferentially hydrated may differ across temperatures when in the presence of solutes such as perchlorate salts and glycine. As such, preferential hydration of \( \alpha \)-chymotrypsin at low temperatures may contribute to the increased enzymatic activity observed. However, as we lack direct data pertaining to the extent to which \( \alpha \)-chymotrypsin is preferentially hydrated across the various conditions assayed, we cannot directly ascribe the observations made here to preferential hydration. As a thermodynamic analysis is possible with the results obtained, we are obliged to interpret our results through the thermodynamic parameters and reference how such thermodynamic changes have been interpreted in previous studies of chaotropic salts and psychrophilic enzymes.

The increased conformational flexibility of \( \alpha \)-chymotrypsin induced by perchlorate salts does, however, come with a price, namely an increasingly negative entropy of activation. That is to say that in the presence of perchlorate salts, there is a greater loss of entropy in forming the highly ordered transition state complex from the enzyme–substrate complex. In fact, in a volumetric analysis of \( \alpha \)-chymotrypsin-catalysed peptide hydrolysis reactions, we could show that the transition state (ES\(^*\)) is rather compact and has a smaller partial molar volume compared to the ES complex\textsuperscript{41}. As the magnitude of the entropic effect on enzyme catalysis is temperature dependent, \(-T\Delta S^\ddagger\), we see different effects of perchlorate salts on \( \alpha \)-chymotrypsin activity at different temperatures. At higher temperatures when the entropic contribution is larger, we observe that perchlorate salts lower \( \alpha \)-chymotrypsin activity. However, when the temperature is lower, and the entropic contribution is much smaller, the benefit of the reduced activation enthalpy can be realised, resulting in increased enzyme activity.

The delicate balance between the enthalpic and entropic contributions is reflected in the Gibbs free energy of activation values at each temperature. At higher temperatures it was the buffer conditions which exhibited the lowest \( \Delta G^\ddagger \) values, whereas at lower temperatures it was the perchlorate salt-containing conditions which had the lowest \( \Delta G^\ddagger \). It is also an important reminder that, owing to the exponential dependence of \( k_{\text{cat}} \) on \( \Delta G^\ddagger \) [Eq. (2)], it only requires small changes to the \( \Delta G^\ddagger \) of an enzyme in order to facilitate large changes in activity.

The effect that we observed of perchlorate salts on \( \alpha \)-chymotrypsin’s thermodynamics, kinetics, and stability, are analogous to those seen when comparing the behaviour of psychrophilic enzymes to mesophilic enzymes. Namely, psychrophilic enzymes exhibit lower values of \( \Delta H^\ddagger \) and \( \Delta S^\ddagger \), increased low temperature activity, and reduced thermal stability compared to mesophilic enzymes\textsuperscript{39}.

Our results have some implications for natural environments. As the effects of chaotropic perchlorate salts mimic psychrophilic adaptations, the data show how, in theory, adaptation to the presence of perchlorate salts could also enable life to operate, or transition more easily into, low temperature conditions. We note the intriguing implications for Mars, a planet with pervasive perchlorate salts in its crust\textsuperscript{2}, which in the near surface and deep-subsurface, experiences low temperatures. Our data show that perchlorate salts and low temperatures complement each other in terms of the theoretically required biochemical adaptations, rather than representing two separate deleterious extremes, with implications for the habitability of high perchlorate-low temperature Martian environments. The concept that chaotropic molecules aid in low temperature survival has been proposed

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**Figure 5.** Pressure–temperature stability diagram of \( \alpha \)-chymotrypsin. The pressure–temperature stability diagram of \( \alpha \)-chymotrypsin in pure buffer (blue data points and dashed line) and in 0.25 M Mg(ClO\(_4\))\(_2\) (green data points and dashed line). Calculated data (dashed lines) were derived from the contours of the Gibbs free energy of unfolding at \( \Delta G = 0 \) [Eq. (6)]. Data points indicate measured values at different temperatures and pressure obtained from FTIR spectroscopy (at ambient and high pressure) or DSC (at 1 bar). Error bars show the maximum error and are derived from at least two independent measurements, \( N \geq 2 \).
Previously, here we have demonstrated a thermodynamic effect which may be the underlying reason for why chaotropic molecules support low temperature growth.

The phase diagram produced from the combination of DSC and FTIR shows that α-chymotrypsin is stable at all temperatures and pressures assayed, and that it remains stable at sub-zero temperatures. We had hoped to examine the thermodynamic phase diagram of α-chymotrypsin across temperature, pressure and ΔG conditions. However, the thermogram from α-chymotrypsin in the presence of perchlorate salts exhibited multiple peaks, so while the enzyme clearly unfolds, the irreversibility of the process prevented thermodynamic interpretation. This illustrates the technical challenges encountered when employing standard biophysical and biochemical techniques to biochemistry under extreme conditions. Surmounting such limitations will eventually allow for a detailed molecular and physical understanding of the biophysical limits to life.

In conclusion, it has been demonstrated for the first time that an enzyme can be activated at low temperatures by a chaotropic salt which has historically only been thought of as purely deleterious with regards to enzyme activity. We demonstrate that this effect is achieved through a reduction of the activationenthalpy, and that the temperature at which this activating effect becomes apparent is largely determined by the magnitude of the entropy of activation.

**Data availability**

The source data for the figures presented in this study have been included as a supplementary file.

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Author contributions

S.G. and M.W.J. conducted the experiments. R.W. and C.S.C. supervised the work. All authors contributed to data analysis and the writing and editing of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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6.3 Conclusion

This research chapter has shown that perchlorate salts can increase the low temperature activity of α-chymotrypsin, despite lowering its room temperature activity. Thermodynamically this is due to reduced activation enthalpy in the presence of perchlorate salts which means that less energy is required in order to facilitate catalysis. The reason why this doesn’t result in increased activity at room temperature is because the perchlorate salts also make the entropy of activation more negative. The temperature dependency of the entropic contribution (TΔS‡) is why the benefits of the reduced activation enthalpy can only be realised at low temperatures.

I also found that glycine did not activate α-chymotrypsin when BTEE is the substrate, which wasn’t necessarily surprising as Dusekova et al. [81] showed how substrate dependent activating effects can be for α-chymotrypsin.

This result shows us two things, firstly it’s not as simple to just say that perchlorate salts and chaotropes in general reduce enzyme activity, it depends on other physical parameters of the experiment. Secondly it suggests that perchlorate salts may assist in the adaptation towards low temperature activity by imparting some of the same thermodynamic characteristics which arise from natural selection of psychrophilicity. This highlights the importance of understanding these phenomena in the multi-extreme context.

Unfortunately, it was not possible to experimentally or computationally assay whether perchlorate salts affected the local flexibility of α-chymotrypsin, which my hypothesis suggested it does in order to increase low temperature activity. Computationally this idea could be tested by running a standard protein simulation solvated in a perchlorate brine and comparing the average flexibility and displacement of each residue against the same simulation but solvated in a pure water box. This would be a relatively simple simulation to run if we had reliable data for the behavior of perchlorates salts in a concentrated brine but unfortunately at the time of writing, the specific electrostatic interactions in such a solution have not been solved. Another hurdle to computationally testing the activity-flexibility hypothesis is that it is not possible to accurately simulate enzyme reactions. It would however be possible to simulate substrate docking in the α-chymotrypsin active site. This would allow us to explore whether the presence of perchlorate salts facilitates greater substrate binding at low temperatures compared to a buffer/water only condition.

The flexibility of α-chymotrypsin in the presence of perchlorates can only really be determined experimentally via indirect methods. For example, one could look at fluorescent quencher penetration into the protein core which
looks at how easily a molecule can diffuse into a protein, the easier said process is, the less compact and flexible/unfolded a protein is said to be. One could look at the loss of rotation of circularly polarized light as a proxy for protein flexibility, as a loss of polarization would suggest that aromatic residues can now move more freely. Using NMR to determine α-chymotrypsin structure in a perchlorate brine would allow us to determine any structural changes caused by the presence of perchlorates, but also the B-factors may give us an idea as to the flexibility of the residues in the α-chymotrypsin active site. The major problem with these experimental approaches is that while they may directly probe aspects of protein flexibility, they can only indirectly correlate that to activity. In other words, it’s a significant challenge to explicitly experimentally link protein flexibility and activity in this experimental scenario.

It would have been interesting to have examined α-chymotrypsin accurately at high perchlorate concentration, high pressures and low temperatures, however the unreliability of the high pressure results from this chapter prevent me from drawing any strong conclusions. In fact this highlights the challenges of research at the forefront of multi-extreme environments as standard lab equipment is rarely available or able to examine the multi-extreme space. Future research of this topic will require the development and creation of bespoke equipment specifically designed to answer these questions.

A further caveat of this chapter is that, while the temperatures assayed here are low in terms of molecular biology, they are not as low as the temperatures we expect to find on Mars. Probing the lowest temperatures will require equipment that can maintain such temperatures for months in order to assay activity. Additionally the challenge presented by cold denaturation of proteins may be an important limiting factor in our ability to probe these low temperatures.
Chapter 7

7. Conclusions

In the course of researching this thesis I wanted to achieve two things. Firstly I wanted to understand how perchlorate salts affect proteinaceous biochemistry through the lens of α-chymotrypsin as a model enzyme. Secondly, I wanted to determine whether there were unexpected benefits of the presence of perchlorates in order to slightly challenge the narrative that perchlorate salts were exclusively deleterious to all biology. By exploring these two facets, we can then better understand the potential for biochemistry and life in Martian deep subsurface environments.

In Chapter 3 I found that perchlorate salts, in keeping with the literature, were deleterious to the activity and stability of α-chymotrypsin. Not only were perchlorate salts deleterious, but were dominant in this regard. In other words salts such as MgSO₄, which stabilised α-chymotrypsin, could not undo the negative effects of perchlorates. This suggests that even if life found itself in a multi-ion environment, if perchlorate was present in similar concentrations to other ions (and was the most chaotropic ion present), then the perchlorate induced effects would dominate over other ionic effects. Therefore it is adaptations towards perchlorate salt tolerance which would be the most important facet for biochemistry in these environments, in terms of ionic tolerance.

It was then important to consider how other environmental factors might influence the relationship between perchlorate salts and biomolecules. As potential aqueous bodies on Mars have been reported in the subsurface, high pressures are an environmental factor which any life there would have to tolerate. Thus in Chapter 4 I investigated how the effects of perchlorate salts changed as the pressure was increased. I found that high pressures could increase enzyme activity in the presence of perchlorates, given that the activation volume was negative, but also that high pressures can potentially undo some of the perchlorate induced structural destabilisation of α-chymotrypsin. This suggested that perchlorate rich environments may be more be less detrimental to biochemistry if they were to be subterranean in nature due to the beneficial effects of pressure. This was argued for through α-chymotrypsin’s pressure, temperature stability diagram produced in Chapter 4. The boundaries of this phase diagram are set by the point at which the free energy of protein folding was zero for α-chymotrypsin, and as was shown, perchlorate salts reduced the temperature and pressure at which this boundary was found. However, what was not apparent from the phase diagram was the point at which α-chymotrypsin’s free energy of folding was
most negative, its point of peak stability. Unfortunately the determination of this point was impossible due to the nature of how α-chymotrypsin unfolds, which prevents a rigorous thermodynamic analysis. I hypothesise though that this region of peak stability also moves to lower temperatures and pressures along with the boundaries. This suggests that not only do high pressures favour protein folding in the presence of perchlorates, but that lower temperatures may also aid protein stability under such conditions.

Deep subsurface environments on Mars would not only experience high pressures, but should also be extremely cold, around -70°C. Therefore in order to understand the potential for biochemistry at such temperatures, I had to know how life on Earth has adapted to cold environments at the molecular level of the enzyme. To do so, I performed a meta-analysis of temperature-adapted enzymes which primarily sought to determine whether psychrophilic enzymes genuinely exhibited a large gap ($T_g$) between their $T_{opt}$ and their $T_m$. In doing so I was able to show that the $T_g$ of psychrophilic enzymes was indeed larger than that of mesophilic or thermophilic enzymes. This suggested that the active site of psychrophilic enzymes was indeed more thermostable/ flexible than the global protein. Therefore we might expect that an enzyme functioning in a low temperature, perchlorate rich environment should also have a flexible active site. This does raise one problem, there is an inverse relationship between protein flexibility and denaturant tolerance, i.e. the more flexible an enzyme is, the lower its ability to resist chemical-induced denaturation (such as by perchlorate salts). So the ideal enzyme in such an environment would be one whose active site is flexible enough to function optimally at low temperatures, but whose global structure is stable enough to resist high concentrations of perchlorate salts. It is interesting to note then that there doesn’t necessarily need to be a trade-off between low temperature activity, and global protein stability as was shown in the meta-analysis. Approaching this problem from the other angle, researchers have been able to increase the low temperature activity of thermophilic enzymes (which typically withstand more denaturant stress) by introducing flexibility to the active site while maintaining a high melting temperature. Therefore the ideal enzyme in our potential Martian environment would have the scaffolding of a thermophilic enzyme, but the active site of a psychrophilic enzyme, a real chimera from the extremes.

With the results of the meta-analysis in mind, I wondered whether perchlorate salts may in fact be imparting psychrophilic characteristics onto α-chymotrypsin by increasing the flexibility of its active site due to partial unfolding. In researching this, and detailed in Chapter 6, I found that perchlorate salts could increase the activity of α-chymotrypsin at low temperatures, and that the thermodynamic origin of this effect matched those exhibited by psychrophilic enzymes. This suggests that perchlorate salts may actually be beneficial to biochemistry at low temperatures due to the reduced
activation enthalpy required for enzyme catalysis. However, this is tempered by the severe entropic penalty you pay in order to reduce the activation enthalpy via perchlorates. So there is a limit to the thermodynamic benefits which perchlorates salts give to enzyme catalysis without the need for specific biological adaptation, but it may be possible for an interesting synergy to exist between adaptation and the presence of perchlorate salts with regards to low temperature activity. To further this research, it would be interesting to see how the low temperature activity of a thermostable enzyme changes in the presence of perchlorate salts. Additionally, using a thermostable enzyme whose active site architecture is designed for low temperature activity would test the hypothesis presented previously that the ideal enzyme in a perchlorate rich environment is one with a thermostable scaffold, but a psychrophilic active site.

Future work in this area would explore the stability and function of other biomolecules under the stresses of multi-extreme Martian environments. Already, our collaborations have studied the effects of perchlorate salts and pressure on model membrane stability[82], tRNA structure[83], and protein ligand binding[84]. These works reaffirmed the deleterious nature of perchlorate salts. In particular it was shown that lipid bilayers would not form spontaneously in the presence of high concentrations of perchlorate salts. Therefore we need to understand what it would take in order for lipid membranes to remain stable at high perchlorate concentrations, whether through the use of protein scaffolds or through the use of unique lipids and linkages. We also don’t yet understand the stability of DNA under perchlorate stress and whether high perchlorate concentrations would affect the abiotic rate of DNA damage and degradation. In terms of proteins, we still need to understand how global protein stability is affected by perchlorate salts. I have shown that perchlorate salts lower the temperature and pressure stability of α-chymotrypsin, but we don’t know whether this reflects a shrinking of the entire protein stability plane, or whether the entire stability region is moving to lower temperatures and pressures. Answering this question is vital to understanding the potential for proteinaceous biochemistry in multi-extreme Martian environments.

Additional future work would explore the cellular level effects of multi-extreme perchlorate rich environments. It’s one thing to understand how the biomolecules are affected by the environment and another to understand how the cellular system is affected by such environment especially since the system tends to be more sensitive. Initial experiments are needed to understand what it is which fundamentally causes cells to die under high perchlorate stress, i.e. is it protein unfolding or lipid perturbation etc. A multi-omics approach will be needed to answer this question, however as a concept it is made more challenging by the fact that the ultimate cause of cell death could vary from species to species. If this was the case then we are
limited to how much we can generally describe perchlorate stress, but if a specific cellular interaction was found to collapse first across a broad range of species then we could develop a theory driven understanding of the limits to habitability in perchlorate rich environments. As previously stated, life on Earth has not faced environments like those of the Martian subsurface over evolutionary timescales and so no life on Earth is explicitly adapted to high pressures and perchlorates and low temperatures. In lieu of natural adaptation, we can do artificial laboratory evolution in order to understand how the evolution of life is shaped by such conditions, albeit over much shorter timescales. One caveat to these experiments is that due to life’s evolutionary history, the building blocks and their organisation are largely “baked in”, so we would never be able to understand what evolution would do to life if it faced perchlorate rich environments from the inception of the first cells. Even with directed evolution, any putative life on Mars would have had billions of years to adapt to slowly increasing perchlorate concentrations and gradually lower temperatures whereas lab experiments would have at best a few decades in a well-funded and specialist lab to become adapted to these environments. Furthermore the choice of organism could drastically influence what potential adaptations manifest. For example it is possible to imagine that an organism might adapt to perchlorates by simply excluding it from its intracellular spaces whereas another organism could adapt its biochemical stability to withstand perchlorates and therefore benefit from the salt within its cytoplasm. Either adaptive strategy would demonstrate that life can adapt to perchlorates, but it would not necessarily tell us which is the one actually required for survival on Mars. Therefore, we are ultimately limited in what we can extract and interpret from adaptive laboratory experiments under these conditions, however this does not negate the worthiness of conducting them.

The work in this thesis explored the effects of perchlorate salts on proteinaceous biochemistry in order to understand the habitability of subterranean Martian environments. However, this work is purely theoretical if a cell in such an environment was physically incapable of executing its biochemistry, for example if its intracellular water content was frozen solid due to the low environmental temperatures. On Earth, the lower temperature limit for life is roughly -23°C due to the onset of intracellular vitrification. This is the process where the cell interior doesn’t freeze, but instead forms a glass like solid which inhibits diffusion and thus biochemistry ceases to function. Vitrification happens as the environment around a cell begins to freeze which concentrates the extracellular solutes generating an osmotic gradient across the cell membrane causing water to leave the cell. This process of continues until a critical intracellular water content is reached and the cell interior becomes glass like. As the temperature in the Martian subsurface is far below -23°C, one would expect any cells in such an environment to be vitrified, but what if perchlorate salts not only lowered the freezing point of water but the intracellular vitrification point also? To test this hypothesis I
travelled to France to work in the lab of Prof Fernanda Fonseca at University Paris-Saclay. I used a differential scanning calorimeter to detect the intracellular vitrification point of Bacillus Subtilis cells in the presence and absence of magnesium perchlorate. I was able to show that the Bacillus Subtilis glass transition temperature is lowered from ~ -26°C in pure water, to as low as -80°C in the presence of 2.5 M Mg(ClO_4)_2. This demonstrates that cells in concentrated perchlorate brines can remain in a liquid state even at extremely low temperatures, which is necessary for biochemical functionality in such an environment. While the Bacillus Subtilis cells were able to remain liquid, they were certainly not “alive” as they were not culturable at such perchlorate concentrations. Therefore, as mentioned previously, the next step in this line of research would be to find or engineer an organism which can tolerate molar amounts of Mg(ClO_4)_2 and with this organism it may be possible to demonstrate biochemical activity under conditions which we believe to be present in the Martian subsurface.

In conclusion, while we do not know whether life can survive in the harshness of the Martian subsurface, the work presented in this thesis has produced a body of work which taken steps towards understanding whether the biomolecules of life themselves could ever function in such environments. I found that perchlorate salts are deleterious towards α-chymotrypsin stability and activity, but that there exists beneficial synergistic interactions between perchlorates, high pressures, and low temperatures with regards to enzyme activity and stability. How far this synergistic relationship extends is still unknown and requires specialist equipment in order to really probe the boundaries of protein function under such extreme conditions. Should we find that the biomolecules of life can retain their function under Martian subsurface conditions, then it may just be a matter of time before we find that life too can operate under such extremes.
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