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Temperature dependent electrochemistry—a versatile tool for investigations of biology related topics

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Temperature dependent electrochemistry can be efficiently used to determine very different properties of the investigated system, such as thermodynamic parameters of redox processes (especially the entropy), the degeneration temperature of a protein or kinetic parameters, for instance activation energy. It can even be used in biotechnology for improved catalysis and detection of substances. This perspective describes a selection of different experiments that used temperature dependent electrochemistry in order to determine these different values or achieve an enhancement of biotechnological applications, respectively, and hence gives an overview of its versatile use in studies aimed at biological issues.

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Introduction

Temperature dependent electrochemistry is not a novel method. The first experiments were reported at the end of the 19th and beginning of the 20th centuries,[1–3] and even its use in biology is not new, with one of the first reports being published in 1979.[4] However, even though it is really a valuable method for the determination of various parameters (thermodynamics, kinetics, stability of proteins *etc.*) it has been used rather reluctantly over the years. The reason for this may be that the initial set-up looks a bit more complicated compared to electrochemistry at ambient temperature and that electrochemistry itself is nowadays not regarded as a very modern analytical method. But once the equipment is assembled, it can be used without much expenditure revealing interesting information about the investigated system that is often not, or not as easily, accessible with other methods. In fact, as will be demonstrated in the following discussion, temperature dependent electrochemistry allows the investigation and understanding of even subtle effects of active site properties in large enzymes down to the role of a single amino acid. Since the 1980s, the number of published studies using this method has increased slightly, but they are still not as common as one would assume, considering how much information can be obtained with a little demanding experimental set-up. Moreover, the number of groups resorting to it is notoriously small. This perspective is aimed at drawing attention to a very useful electrochemical method that has not been regarded as highly as it deserves by discussing a selection of very different results that were recently obtained from it.

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Discussion

How to reference the data

One problem inevitably associated with temperature dependent electrochemistry is the question of referencing. The reference electrodes are usually temperature dependent themselves. One should therefore try to keep for instance the reference electrode at a constant temperature while only the sample solution is heated or cooled. However, since the reference electrode must be in direct contact with the sample solution, there will always be at least a temperature gradient present in the experimental set-up. In general there are three possibilities to obtain more or less reliable values of the sample's redox potential at different temperatures: (1) using a metal electrode, for instance platinum along with an internal standard as the ferrocene/ferrocenium couple (Fc/Fc^+) for referencing. This should be employed only at room temperature and only after the sample has already been investigated over a temperature range assuming that the temperature effect on the solid metal is negligible. The individual data for different temperatures are then referenced to that value found at room temperature (25 °C) in relation to Fc/Fc^+. This is certainly the easiest and least demanding way to reference the data and suitable for comparative studies; (2) common non-isothermal cells: here the reference electrodes are kept at a substantial distance from the sample solution (Fig. 1) but are at the same time in contact with the working electrode by using glass tubes that end in capillary sized openings close to it. The electrolyte solution that establishes the contact is however exposed to two different temperatures, and

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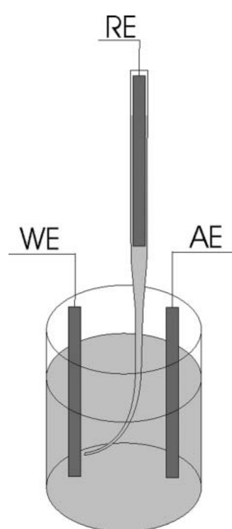

Fig. 1 Schematic of a non-isothermal cell with the reference electrode (RE) at ambient temperature and bulk solution, working electrode (WE) and auxiliary electrode (AE) at controlled temperature.

will unavoidably show a temperature gradient; and (3) a recently developed method of referencing uses heated working electrodes and by this, turns the experimental set-up of the common non-isothermal cells upside down. Here, the sample solution is kept at ambient temperature and only the working electrode, and of course the absorbed sample molecules and those in the proximity that are immediately exposed to the potential change, are heated. This is quite elegant because firstly the already present electricity is used to generate the needed heat and secondly only the molecules that will produce a signal in the experiment are directly exposed to the determined temperature. Using a wire electrode this could be heated directly. The other possibility is to use an inert heating element (for instance a ceramic chip) layered by the electrode metal (for instance gold) (Fig. 2). One example using the third method of referencing is a study with respect to the probably most often electrochemically analysed biomolecule cytochrome c (Fig. 3).[5] The electrochemical responses of cytochrome c are

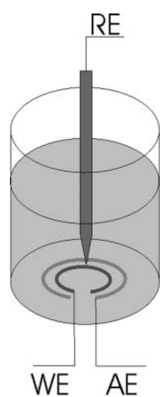

Fig. 2 Schematic of a "heated electrode" set-up with the reference electrode (RE), auxiliary electrode (AE) and the bulk solution at ambient temperature and the working electrode (WE) at controlled temperature. WE and AE are here part of a chip. The heated WE can be a simple wire electrode as well, although in this case the bulk solution would be affected to a larger extent.

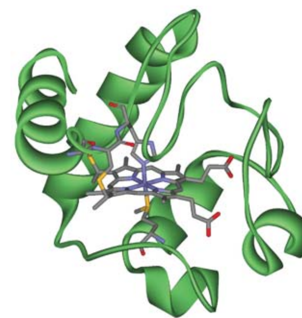

Fig. 3 Cytochrome c with its prosthetic group containing the redox active iron (II/III) with the equatorial porphyrin ligand heme c and histidine (top) and methionine (bottom) as axial ligands.[6]

very sensitive with respect to prior surface treatment or the used electrolyte and even its concentration. The large number of published results of electrochemical studies on cytochrome c are therefore far from unanimous. The redox active heme iron centre of cytochrome c is covalently bound to the protein and surrounded by hydrophobic amino acid residues. It is therefore shielded from the environment and only about 0.6% of the protein surface allows a redox interaction with the electrode. A further problem associated with some cytochrome c proteins is a substantial blocking of the electrode surface which causes the electrode response to decrease rapidly until reaching zero. An increase in temperature in this case could delay the blocking to a certain extent due to a more rapid movement of the molecules. Voss *et al.* used a heated electrode chip to obtain temperature dependent electrochemical responses of the most commonly used horse heart cytochrome c (cyt.Fe(II)c → cyt.Fe(III)c + 1 e^-) to determine the denaturation temperature from the absorbed molecules and those that are near the surface while avoiding denaturation of the bulk solution. They observed no signal in the monitored potential range at ambient temperature, but above 37 °C a signal evolved at around 0.05 mV *vs.* SCE and its current (peak height) increased gradually up to the highest temperature of 78 °C. This signal was attributed to a denaturation of the protein allowing increasingly direct access to the redox active iron site. They could therefore determine the denaturation temperature of cytochrome c to be 37 °C by using their newly developed electrically heated electrochemical sensor and a very small amount of sample solution that did not even have to be changed throughout the experiment. They suggest that by using pulsed heating techniques it should be possible to reach temperatures even above the boiling point of the solvent which would, in general, dramatically increase the accessible temperature ranges not only for biological experiments.

Obtaining thermodynamic data

Since the free energy of a redox process and its potential are related *via* eqn (1), both entropy and enthalpy of this redox process can be determined using temperature dependent electrochemistry and eqn (2) and (3), respectively, at constant pressure (n = number of transferred electrons; F = Faraday constant).[7]

$$\Delta G = -nFE \quad (1)$$

$$\Delta S = nF\left(\frac{\delta E}{\delta T}\right) \quad (2)$$

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$$\Delta H = nF\left[T\left(\frac{\delta E}{\delta T}\right) - E\right] \quad (3)$$

As mentioned before, cytochrome c is probably the most commonly investigated biomolecule and this is also reflected in the temperature dependent studies. In several publications thermodynamical parameters of cytochrome c of various organisms were determined using temperature dependent electrochemistry in a variety of experimental set-ups. A selection of these will be discussed in the following paragraphs.

Benini *et al.* have observed a quasi reversible diffusion controlled redox process using cyclic voltammetry on cytochrome c_{553} from *Bacillus pasteurii*.[8] *B. pasteurii* is a gram positive bacterium of which only very few soluble cytochrome c proteins have been isolated and investigated. In the temperature dependent experiment with non-isothermal cells the authors observed a discontinuity at 35 °C. Here, the slope of linear E/T changed and therefore so did the thermodynamics of the redox process (Fig. 4). In the low temperature range (2–35 °C), the entropy change for the reduction was determined to be −162.7 J mol^{-1} K^{-1} and the enthalpy to be −53.0 kJ mol^{-1}. Both parameters increased in the high temperature range (35–50 °C) to −294.1 J mol^{-1} K^{-1} and −93.4 kJ mol^{-1}, respectively. This clearly indicates a conformational change of the protein. Both thermodynamical values together lead to a slight increase of the "theoretical" standard reduction potential as well (+47.0 mV → +60.3 mV) showing that the high temperature species is easier to be reduced in comparison. In both conformers entropy favours the oxidized form while enthalpy favours the reduced form. NMR experiments indicate that all ligands stay attached to the iron in both oxidation states and at different temperatures, especially the axial ligand methionine is not removed, although a re-orientation of the axial ligands was found to accompany the redox process. This means that the protein is not denatured by heating but indeed only changed with respect to its conformation. It is assumed that the ligand sphere of iron changes upon oxidation of ferricytochrome c to ferrocytochrome c. In comparison to the data available for the most often used horse heart cytochrome c, the difference in redox potential shows that the major factor for the *B. pasteurii* cytochrome c is indeed the entropy. In all cytochrome c proteins

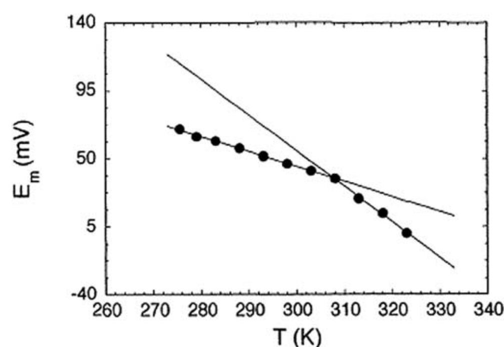

Fig. 4 The different slopes of E/T in the low T (275–308 K) and high T (308–323 K) ranges. The continuous lines represent linear fits of the experimental data. Reproduced with kind permission from Springer Science + Business Media. Copyright SBIC 1998. [Benini *et al.*, Modulation of *Bacillus pasteurii* cytochrome c_{553} reduction potential by structural and solution parameters, *J. Biol. Inorg. Chem.*, **3**, 1998, 371.]

the entropy was found to be negative for the reduction which has been interpreted as an increase in protein rigidity in the reduced state. Benini *et al.* now suggest that it could also be attributed to a change in the hydrogen bonding arrangement of water molecules and therefore to hydration entropy changes. Hydrogen bonding responsible for entropy at a cytochrome c includes heme propionates, neighbouring amino acid residues and solvent water in the heme crevice. If the orientation of the axial ligands changes upon a redox process, this may very well affect hydrogen bonding, and therefore induce a rather high entropy change. The authors conclude, combining all their observations, that the number of water molecules extruded upon reduction of the iron from the active site should be larger in the high temperature range conformer explaining the increased figure for the entropy change compared to the low temperature conformer. They were therefore able to develop a picture of the active site with respect to the role and relative number of water molecules that would not otherwise be easily obtainable.

A conformational change of the cytochrome c proteins is not only caused by a temperature change but also by modifying the pH value. Battistuzzi *et al.* investigated the thermodynamics of the alkaline transition (AT) of beef heart cytochrome c.[9] This transition can easily be detected since a second redox process evolves in the voltammograms due to the formation of the alkaline conformer. They combined pH titrations with temperature dependent cyclic voltammetry experiments in a range of 5–65 °C. Here, the slope of the plot of pK_{app} (apparent pK value) *vs.* $1/T$ gave the value for ΔH_{AT}. ΔS_{AT} was determined from the intercepts ($T = 0$) of these van't Hoff plots. They observed two distinctive sets of thermodynamic data for the alkaline transition below and above *ca.* 40 °C. In the low temperature region this process is endothermic with a small positive entropy. At high temperature this is dramatically changed resulting in a slightly smaller negative enthalpy but a considerable loss of entropy in contrast with the low T conformer. The alkaline transition related conformational changes are interpreted as changes in the hydration sphere or the binding of different lysins to the iron centre upon pH increase. In all cases the reduced is the thermodynamically more stable form based mainly on the enthalpy (higher affinity of methionine's thioether ligand to iron(II) than to its oxidized form). Remarkably, the pH value has no influence on the temperature at which the temperature dependent conformational change occurs. The transition of the two native conformers (low and high T conformer) to the alkaline forms are therefore clearly thermodynamically distinct processes.

Not only alkaline transitions of proteins can be observed but also acid transitions. Battistuzzi *et al.* investigated the thermodynamics of the acid transition in blue copper proteins (Fig. 5).[10] In the mononuclear type 1 copper proteins, which are responsible for electron transfer (one e$^-$ at a time), the active site geometry plays an important role. Its entatic state lowers the activation energy of the redox process and the more tetrahedral than square planar geometry causes a slightly raised redox potential. The authors studied the acid transition, which involves protonation and subsequent detachment of one of the histidine ligands from the central copper ion, of a number of blue copper proteins: plastocyanins from spinach and cucumber, stellacyanin from *Rhus vernicifera*, the cucumber basic protein (CBP) and amicyanin from *Paracoccus versutus.* The experiments involved direct protein electrochemistry at varying pH and temperature. For almost all

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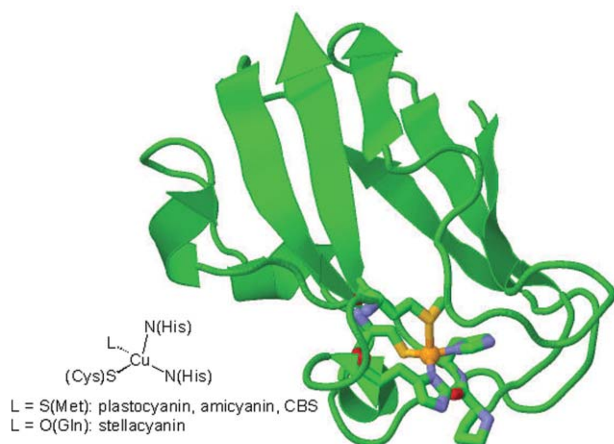

Fig. 5 The active site structures of blue copper proteins investigated by Battistuzzi *et al.* (CBS = cucumber basic protein) and the crystal structure of spinach plastocyanin downloaded from the Protein Data Bank (1AG6).

investigated proteins (except CBP) the acid transition is exothermic. The entropy, however, was surprisingly strongly dependent on the protein species. The thermodynamic driving force was found to be of enthalpic nature for plastocyanins but of entropic nature for phytocyanins, indicating a general difference between the respective domains of life. The Cu–His bond is in all systems analogous except for amicyanin, which is exceptional in various other aspects as well. By using temperature dependent electrochemistry, the acid transitions' entropies could be determined, which depend strongly on structural changes and solvent reorganization effects upon protonation and the subsequent detachment of one histidine. In a similar manner as that described for the alkaline transition of cytochrome c, using van't Hoff plots the enthalpies were determined as well and consequently also the free energies $\Delta G_{AcT}°$. The transition thermodynamics are related to the enthalpic and entropic balance of bond breaking, bond making and protein and solvent reorganization processes. Changes in the hydrogen bonding network in the hydration sphere of a chemically active biomolecule generally include often self compensating enthalpic and entropic effects, which was indeed the case here. The authors conclude that $\Delta G_{AcT}°$ values for the acid transition in blue copper proteins are almost exclusively determined by the strength of the copper-histidine bond and its immediate environment.

Horseradish peroxidase on the other hand was shown by Battistuzzi *et al.* to be a protein whose redox entropy is most likely determined by solvation effects only.[11] The active site of horseradish peroxidase (Fig. 6) consists of iron equatorially bound to heme b (protoporphyrin IX) with one histidine as axial ligand. The authors studied this enzyme in its native form (fife-coordinate, high-spin) and as a cyanide adduct (six-coordinate, low-spin). The entropies for the redox process obtained from the gradients of the redox potentials *vs.* temperature were shown to be significantly different, resulting in a substantial decrease of the redox potential of 124 mV for the cyanide adduct. The enthalpy in this respect was less important for the overall change in thermodynamics compared to the entropy. Native horseradish peroxidase exhibits a complicated array of hydrogen bonds in the heme pocket, which is easily accessible by solvent through the substrate channel. It was therefore concluded that solvent reorganization contributes significantly to the entropy changes upon reduction. This was

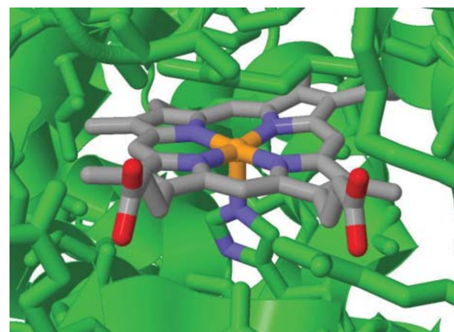

Fig. 6 Crystallographic active site structure of horseradish peroxidase downloaded from the Protein Data Bank (1ATJ).[12,13]

supported by the fact that the entropy of the cyanide adduct on the other hand was remarkably lowered owing to the fact that the sixth ligand prevents solvent molecules from accessing the active site. Other effects, like a change in protein flexibility upon reduction, could not however be excluded to contribute to the entropies of both studied forms.

Yet another protein was the focus of a study by Arendsen *et al.* who investigated a 4Fe-4S-ferredoxin (Fig. 7) from *Desulfovibrio vulgaris* (Hildenborough).[14] Though containing four iron atoms, ferredoxins are in general only able to transfer one electron, since only two oxidation states are accessible. Interestingly, in this case the protein can be isolated in two different forms: a major form that is tightly bound to RNA, and a minor form free of nucleic acid. The RNA bound protein could be stripped of RNA by extraction with phenol but not by employing nucleases or chromatography. Both forms show deviating spectroscopic properties and the RNA bound form does not show a signal when investigated electrochemically. Spectroscopic data furthermore suggests that an intact iron sulfur cluster is a prerequisite for RNA binding. For the nucleic acid free protein, a reversible diffusion controlled redox process could be detected using cyclic voltammetry, which was investigated temperature dependently. The entropy of the redox process (from gradient E *vs.* T) was found to be huge with –230 J K^{-1} mol^{-1}. This indicates a large conformational

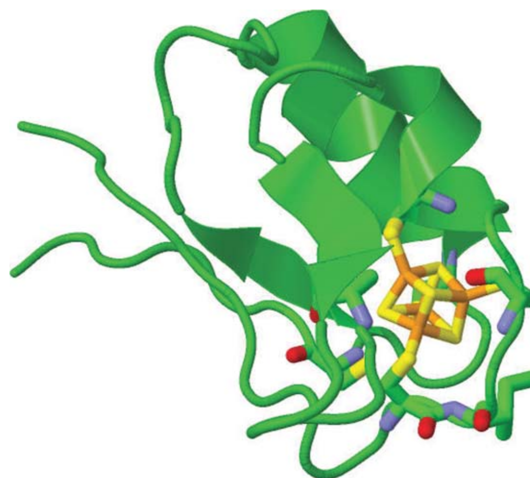

Fig. 7 Crystallographic structure of a 4Fe4S-ferredoxin with its geometrically quite rigid iron sulfur cluster downloaded from the Protein Data Bank (1DAX).

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change of the peptide (the iron sulfur clusters geometry is rather rigid) and an unusual flexibility for a protein with an electron transfer function since a redox-state-independent structure would be beneficial here. A large flexibility on the other hand would allow protein substrate (for instance RNA) interactions. All this indicates that the investigated ferredoxin may be associated with a regulatory function rather than with electron transfer which is quite unusual for this type of protein and a striking observation.

Even the roles of distinct amino acids for the active sites of redox active proteins can be investigated using temperature dependent electrochemistry and the thermodynamic values obtained by it.

Catalase-peroxidases (KatGs with the same active site as horseradish peroxidase,[15] Fig. 6) from different organisms contain an unusual specific distal methionine-tyrosine-tryptophane cross-link, which appears to be important for the catalase ($2H_2O_2 \rightarrow O_2 + 2H_2O$; disproportionation of peroxide) but not the peroxidase ($H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O$; reduction of peroxide) activity of the KatGs which can catalyse both reactions. Bellei *et al.* undertook a spectroscopic and electrochemical investigation of the wild-type KatG from *Synechocystis* PCC 6803 and in comparison of its mutated form, in which the tyrosine of the cross link (Y249) is replaced by a phenylalanine (KatG(Y249F)).[16] By determining the thermodynamics of the ferric reduction of the two KatGs, they were able to examine the impact of the cross-link on the enzyme's redox properties and therefore on its function. Entropy as well as enthalpy for the reduction of the central iron was determined by temperature dependent electrochemistry using a heated working electrode, while keeping the rest of the set-up at ambient temperature. In both proteins, wild-type and mutant, the oxidised form is enthalpically stabilised but entropy is lost upon reduction. The cross-link appears to have no influence on the value of the redox potential, but the thermodynamics (ΔS and ΔH) of the redox process are changed in the mutant. Furthermore, the catalase activity is completely lost when the tyrosine in question is exchanged for phenylalanine while the mutated enzyme still catalyses the reduction of peroxide. Obviously the redox potential is not responsible for the loss of peroxidase activity and most probably the binding of the proximal histidine to the iron is also not altered. The enthalpy of the iron reduction in the wild-type KatG is higher and the entropy less negative. The latter is of course not unexpected considering that without the cross-link the flexibility of the protein in the mutant should be greater and therefore the conformational change upon reduction more pronounced. The enthalpy is mainly linked to metal ligand binding interactions and the electrostatics at the interface metal/protein/solvent molecules. Entropy determination, on the other hand, is based on oxidation state dependent changes in conformational degrees of freedom and reorganisation effects. Conformational changes and solvent reorganisation do deviate in both enzymes resulting in different values for ΔS and ΔH. In conclusion it was shown that the oxidation of peroxide does not depend directly on the metal's properties, but instead on the substrate channel and the protein integrity, which are both changed in the mutant KatG, whereas peroxide reduction is not compromised by the mutation.

Thermophiles and hyperthermophiles. Especially interesting is temperature dependent electrochemistry with respect to organisms that live at unusual temperatures like the thermophilic bacteria and archae and the hyperthermophilic archae.

The temperature dependent conformational behaviour of *Pyrococcus furiosus* ferredoxines and rubredoxines (mononuclear iron sulfur proteins) in earlier work was determined by EPR spectroscopy of shock frozen samples taken at different temperatures. In these studies, break points indicating conformational changes were found. Hagedoorn *et al.* were suspicious about these results and re-evaluated the redox chemistry of both enzymes using electrochemistry.[17] They could clearly demonstrate that the temperature dependence of both investigated redox proteins was strictly regular without any break points up to 90 °C, and that EPR investigations in this respect lead to incorrect results. They suggest that this is due to the fact that even when carried out rapidly, shock freezing itself leads to conformational changes of the enzymes. Thermodynamic parameters were determined with negative entropies and enthalpies for the reduction, resulting in a negative ΔG^0 for rubredoxine, favouring the reduced form and a positive ΔG^0 for ferredoxine, favouring the oxidised form.

Gillès de Pélichy *et al.* investigated the rubredoxine with the Fe(S-Cys$_4$) active site of a mesophilic organism (*Clostridium pasteurianum*) in comparison with that of a hyperthermophillic organism (*Pyrococcus furiosus*).[18] The redox potential of *P. furiosus* at 25 °C is substantially higher (31 mV) than at 95 °C (−93 mV) indicating that the properties of this thermophile's enzymes are quite different at ambient temperature compared to its usual working environment. The comparative redox potentials of the enzymes were similarly and linearly dependent on the temperature. Using the slopes of E/T, enthalpies and entropies were determined for the redox process at the central iron of the rubredoxines. Entropies for the reduction are negative for both enzymes. This means that the oxidized form is the more stable one at higher temperatures. The authors explain this with reference to the decreasing dielectricity constant of water with increasing temperature that accordingly prefers the lesser charged species. Most importantly, however, is the fact, that ΔH and ΔS for the reduction are very similar for the two organisms showing that the structural differences of both rubredoxines which are responsible for the thermostability in *P. furiosus* do actually not influence the electron transfer activity.

In my opinion, a very interesting aspect of thermophiles and hyperthermophiles is the analogous use of tungsten instead of molybdenum at the active sites of their oxidoreductases. Since the thermophiles and hyperthermophiles belong to the earliest organisms on earth, it is assumed that there was an evolutionary change from tungsten to molybdenum, and the reasons for this change are discussed regarding different aspects. One reason is most probably the very low abundance of molybdenum at thermophillic conditions resembling those on the early earth and its higher abundance compared to tungsten at today's ambient conditions. Furthermore, the different redox potentials of both metals and their different temperature dependencies may play a role. We have comparatively investigated the different temperature dependencies of analogous molybdenum and tungsten complexes using models for the active sites of the oxidoreductases, and also strictly inorganic compounds.[19] We discovered that the redox potential (at least for the transition $M^{IV} \leftrightarrow M^{V}$) of tungsten is in general more sensitive towards temperature changes than that of molybdenum (Fig. 8). This could indeed be an evolutionary advantage for molybdenum, which is able to provide more stable redox potentials for the enzymatic turnover, making this change

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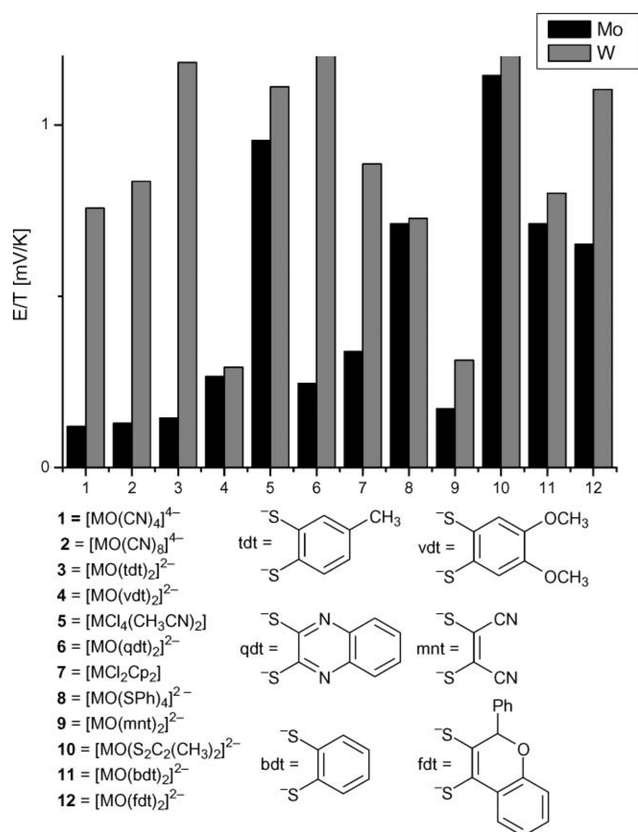

Fig. 8 Different absolute values for the temperature dependencies of the redox potentials of molybdenum and tungsten complexes.

not only based on abundance but most probably also on function and further explaining today's overwhelming use of molybdenum.

Kinetics

The denaturation temperature and the rate of the electron transfer over a temperature range of 10–45 °C of not only one protein, but of a complex system involving two proteins and the substrate were investigated by Long *et al.*[20] The study was aimed at understanding the temperature dependence of the electron transfer reactions that occur between cytochrome c (Cyt c), cytochrome c oxidase (Cox) and oxygen, thus the processes at the end of the respiratory chain in vertebrates where oxygen is converted into water and energy stored in form of ATP (adenosine triphosphate). Cytochrome c was attached to an indium oxide electrode surface heterogeneously and consequently able to reduce the cytochrome c oxidase using electrons provided by the electrode. The presence of excess oxygen ensured that the dissolved cytochrome c oxidase was oxidised at all times and therefore permanently able to oxidise cytochrome c. Interestingly, the thermal denaturation of cytochrome c oxidase at about 52–56 °C destabilized cytochrome c (decrease of denaturation temperature by 15°) whereas the presence of cytochrome c did not affect the denaturation temperature of cytochrome c oxidase. The authors propose two different denaturation mechanisms for cytochrome c based on this observation: (a) a catalytic denaturation of cytochrome c upon interaction with thermally denatured cytochrome c oxidase and (b) a denaturation of cytochrome c by unknown extraneous material equally accumulated at higher temperatures as denatured cytochrome c oxidase. An irrevocable experimental distinction between both was not possible but suggestion (a) was clearly favoured.

The detailed interpretation of the electrochemical studies involved the heterogeneous electron transfer kinetics between the indium oxide electrode and cytochrome c, the rates of homogeneous electron transfer between reduced cytochrome c and cytochrome c oxidase, the mechanism of the denaturation (see above) and fitting and modelling of all these data. It was found that the catalytic regeneration step of cytochrome c oxidation by cytochrome c oxidase is influenced by several parameters that could be controlled by the experimental conditions. This intermediate redox process (in between initial cytochrome c and final oxygen reduction) becomes more important at high temperature, with a lower scan rate and higher concentration of cytochrome c. In all experiments, the amount of cytochrome c bound to the electrode exceeded that of the dissolved cytochrome c oxidase while oxygen was present in large excess. At higher cyclic voltammetric scan rates, the oxidation of cytochrome c occurred at pseudo-first-order conditions. At lower scan rates on the other hand the redox process became second order with respect to both enzymes (reactant and substrate). The authors assume that with lower scan rates, a mixture of cytochrome c oxidase in two states is present in the diffusion layer around the electrode, which means that the observed rate values are actually mixtures of two different values. They therefore calculated redox rates only from those experiments at higher scan rates. As expected, the reaction rates for both redox processes (Cyt c and Cox reduction) increased with higher temperatures until, at elevated temperatures, the reaction between cytochrome c and cytochrome c oxidase became decoupled owing to the denaturation of the latter. They also calculated the activation energies using the Arrhenius equation from the observed reaction rates at different temperatures and found that they decreased with reduced cytochrome c concentrations. One could interpret this by interactions between Cox and reduced and oxidised cytochrome c proteins as well, where the oxidised Cyt c blocks the ready Cox or by a decelerated diffusion of Cox. In conclusion they have shown that the redox process between cytochrome c and cytochrome c oxidase in vitro is susceptible to various parameters and that they all have to be considered when these experiments are to be evaluated.

In order to ascertain enzyme turnover kinetics of arsenite oxidase (Fig, 9) from *Alcaligenes faecalis* Hoke *et al.* used temperature dependent catalytic (this means that substrate conversion is monitored at a high over potential) protein film voltammetry where the protein is directly attached to the electrode.[22] Arsenite oxidase belongs to the group of molybdopterin dependent enzymes that contain molybdenum or tungsten at their active sites and catalyse two electron redox processes. The redox processes of these enzymes are of course mainly determined by the metal, but the molybdopterin being a non-innocent ligand is also assumed to have an influence. A peculiarity of this study was that the usually used limiting current at high over potential could not be determined easily since the curve shapes depended on substrate concentration and temperature, and were therefore altered throughout the experiments. At high concentrations and high temperatures, a limiting plateau could not be ascertained (Fig. 10) owing to a residual slope that was probably caused by enzyme molecules attached to the electrode surface in a less

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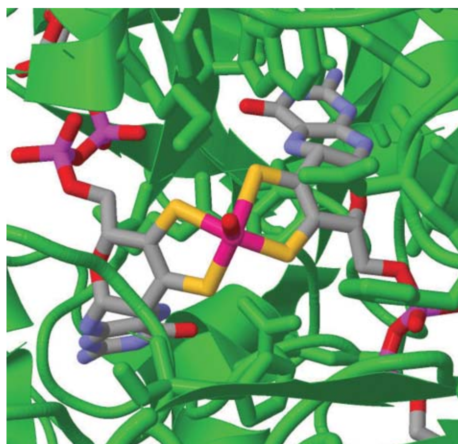

Fig. 9 Active site structure of arsenite oxidase (PDB: 1G8J) as determined by Ellis *et al.*[21] The molybdenum coordination sphere consists of two molybdopterin ligands and one apical oxo ligand.

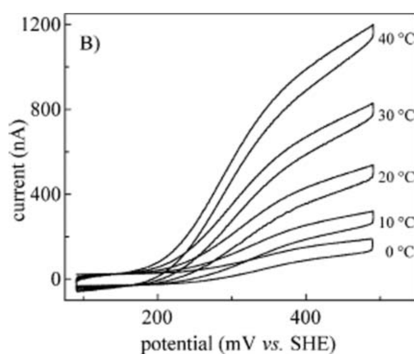

Fig. 10 Influence of the temperature on the catalytic current at high over potential for arsenite oxidase. Reprinted with permission from [Hoke *et al.*, *Biochemistry*, 2004, **43**, 1672]. Copyright 2004 American Chemical Society.

favourable orientation. Since this residual slope depends on the turnover rate of the enzyme and the electrochemical electron exchange constant (which is however dependent on the enzymes' orientations and therefore includes a dispersion), it should be proportional to the unattained limiting current and conditioned by the same factors as the electron transfer turnover rate. The authors consequently used this slope to extract catalytic parameters. They obtained a lower limit for the turnover number k_{cat} of considerable 50 s^{-} showing that the enzyme was fully active and a value for the Michaelis constant K_M of 6 µM being consistent with a value found in solution studies. More importantly, they discovered that the residual slope increased with increased temperature. This clearly indicates that the activation energy for the enzyme's turnover is higher than that for long-range electron transfer, and that the former is the rate-limiting step.

Voltage clamp experiments. A human protein was the focus of voltage clamp experiments undertaken by Mackenzie *et al.*[23] Voltage clamp means that a potential is applied across for instance a membrane and the resulting current is recorded. A simplified schematic for this kind of electrochemical set-up, which is considerably different from those that are aimed at measuring redox potentials is shown in Fig. 11. The current in this experiment depended on the number of ions crossing the membranes either way. The essential micronutrient vitamin C (L-ascorbic acid)

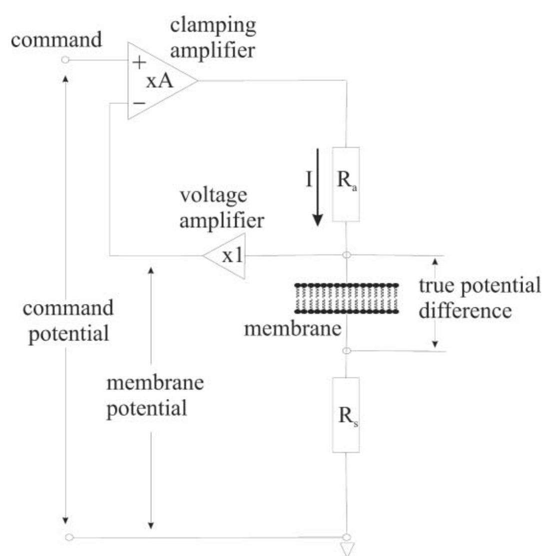

Fig. 11 Simplified schematic of a voltage clamp experiment that applies a potential across a membrane and measures the current.[24] R_a = resistance consisting partly of the cytosolic part of the cell; R_s = resistance outside the cell.

cannot be sythesised by humans and must be taken with food and intestinally absorbed. The protein responsible for vitamin C absorption is the epithelial sodium dependent L-ascorbic acid co-transporter SVCT1. The cells with which the experiments were conducted were *Xenopus* oocytes expressing the human SVCT1. The studies revealed that in order to transport one vitamin C through the membrane, two sodium molecules need to be transported as well. Therefore the sodium dependency of SVCT1 has now been quantified. Binding to the transporter was found to be in the order Na^+–vitamin C–Na^+. The transport is driven by an electrochemical gradient for Na^+. Transporter expressing cells produced currents of about −250 nA, whereas controls only gave currents of about −3 nA. In the absence of vitamin C, the current decreased at steadily applied potential in a Boltzmann curve. The recorded currents were found to be strongly temperature dependent and were measured in a temperature range from 18–30 °C. These data were used to calculate the activation energy for the vitamin C transport *via* an integrated Arrhenius function: $\ln(-I) = \ln A - (E_a/RT)$ with E_a being approximately 23 kcal mol^{-1}. The strong temperature dependence furthermore confirms that it is an active kind of transporter and not a simple channel, because it strongly suggests that conformational changes of the peptide are involved.

Biotechnology

In combination with thermophiles but also mesophiles electrochemistry has proved very useful with respect to biotechnological applications. In the selected examples, the higher temperature is the key to an enhanced performance of the different applications and set-ups.

Chlorinated organics are widely used in research and applications. Their disposal, however, constitutes a severe environmental problem. With the use of thermophilic cytochrome P450 (CYPP119) from extremophilic *Sulfolobus solfataricus*, Blair *et al.* achieved the exceptional electrocatalytic 8 electron reduction of

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specifically toxic CCl_4 to methane.[25] This reaction is commonly known to be quite difficult. The archaeon *S. solfataricus* does not only show rather high denaturation temperatures (90 °C), but it is also tolerant to extreme pH values and pressures in solution. The authors isolated P450 CYP119 and immobilised it in a dimethyldidodecylammonium bromide (DDAB) film on the surface of a pyrolitic graphite electrode. They investigated the effect of temperature on the electrochemical activity of this enzyme, and found that if used as a sol-gel film (even although the film was stable until 60 °C) the observed current decreased (from 30 °C to 60 °C by 55%). By contrast in combination with a polymer (poly-*p*-styrene sulfonate), even at 80 °C 93% of the current at 30 °C had been retained. Further, the typical heme Soret band did not change in accordance with temperature, underlining the integrity of the protein. The redox potential decreases linearly with increasing T. The presence of chlorinated C_1 species caused an increase of the cathodic current, showing the catalytic reduction of the substrate formally according to: $Fe^{I} + RCl + H^{+} \rightarrow Fe^{III} + RH + Cl^{-}$. The maximal catalytic current grew steadily from 25 °C to 55 °C indicating an enhanced performance based on better kinetics, better diffusion and increased solubility of the substrate. In total, the methane production at 55 °C was a remarkable 35 times higher than at 25 °C. C_2 species as side products were never observed. The authors have therefore developed a very efficient biotechnological device using higher temperature for dramatically increasing the activity of the destruction of environmentally problematic chlorinated C_1 species.

On electrode surfaces immobilised proteins can either be used to catalyse chemical redox reactions that would be otherwise very difficult to accomplish or they can exploit the fact that the presence of substrate and its subsequent catalytic turnover increases the observed current at applied potential. In the latter case, a signal increase would indicate the presence of a certain substrate and therefore could be used for the sensing of this molecule. Casalini *et al.* covalently attached a Met80Ala cytochrome c mutant to gold electrodes.[26] By removing the axial methionine ligand, one coordination position becomes available for substrates. This modified cytochrome c could catalytically reduce elemental oxygen as well as nitrite, and the observed currents were directly linked to the concentration of substrate. The thermodynamics and kinetics of the redox process at the iron (without substrate turnover) were investigated by the previously described methods using temperature dependent electrochemistry. The catalytic performance was also studied at different temperatures and over a broad pH range, showing that the protein–gold surface system was remarkably sensitive (especially at high temperatures), stable and efficient at varied conditions. Based on this, the authors envision the utilisation of an improved version of their system as an efficient, low-cost nitrite biosensor.

The compound luminol has become quite famous for its criminological use as detector of blood in even tiny amounts, and it is also widely used for visualisation in biochemical standard procedures like ELISA (Enzyme-Linked Immuno Sorbent Assay). Based on luminol Lin *et al.* have developed an electrochemiluminescent biosensor for hypoxanthine by using a heated carbon paste electrode modified with xanthine oxidase (Fig. 12).[27] Hypoxanthine is associated with the degradation of adenine nucleotide and its detection and quantification is very important for the quality control of sea food. The electrode in the

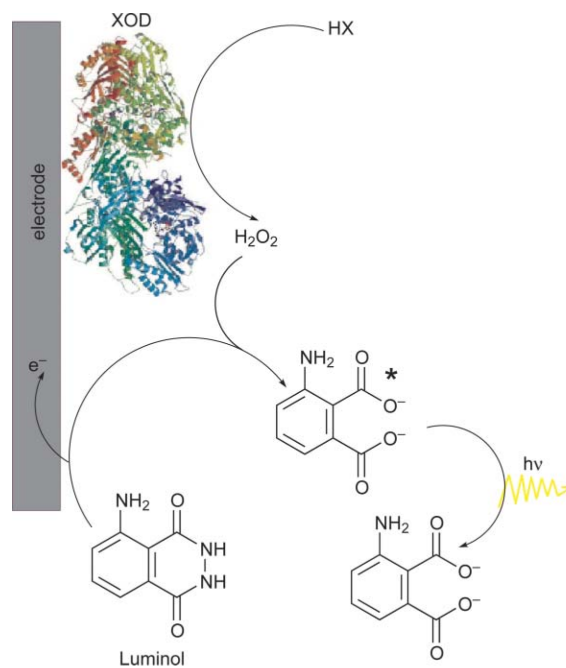

Fig. 12 The electrochemical detection device for hypoxanthine (HX) developed by Lin *et al.* using xanthine oxidase (XOD) attached to the electrode and luminol with an optimum working temperature of 35 °C.

presented system is able to mediate the redox processes that are needed to let luminol luminesce, and its luminescence is greatly enhanced in presence of hydrogen peroxide. The latter is actually the reason why the new system can be used as hypoxanthine sensor. The xanthine oxidase on the electrodes surface is able to catalyze the oxidation of hypoxanthine to uric acid by molecular oxygen. In this process hydrogen peroxide is evolved, consequently increasing luminol's luminescence. The activity of xanthine oxidase is of course kinetically increased with higher temperatures as long as it is not denatured. And also diffusion and convection are affected by the temperature. Hydrogen peroxide on the other hand is not very stable and its disproportionation to oxygen and water would increase at higher temperatures as well. Increasing the temperature of the whole system would therefore have advantages and disadvantages. By using the heated electrodes, however, the authors achieved an increase in the xanthine oxidase activity without fuelling hydrogen peroxide decomposition. They tested several different temperatures and found that the optimum was about 35 °C with a striking 30-fold lower detection limit for hypoxanthine compared to 25 °C. Above 35 °C, however, the signal to noise ratio degraded. The authors point out that the development of devices like this would be useful for those enzymes that naturally work at higher temperatures (thermophyllic/hyperthermophyllic) in particular. It is going to be rather interesting to follow the ongoing research in this respect.

In summary, it can be concluded that temperature dependent electrochemistry is conveniently used in applications and analysis, and is able to deliver a wide range and variety of information about biologically relevant redox systems within a single analytical experiment that are otherwise not as easily obtainable. It therefore seems surprising that it has not been used more often and by more groups until now. It has been neglected especially with respect to bioinorganic model chemistry. There are a number

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of studies published that compare kinetics of models with those of very different enzymes using other methods but they are mostly concerned with reactions that involve substrates being transformed into products or substrate binding because the data is obtained by measuring the respective concentrations.[28–34] Equally interesting results can be obtained from comparative studies of models and enzymes addressing the thermodynamics.[35] Such studies using temperature dependent electrochemistry promise to yield valuable and very detailed information without much effort. A comparison of entropies between models and enzymes, for instance, could help to further understand the peptide's (general environment and direct coordination sphere) and active site's thermodynamical role in proteins. This is especially true for electron transfer proteins. An advantage of temperature dependent electrochemistry is certainly the possibility to determine kinetic as well as thermodynamic data without changing the set-up. The method's high potential, in any case, should be able to prompt an increasing number of researchers to turn to it at least occasionally.

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