Assaying Enzymes from Hyperthermophiles

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Abstract

The determination of kinetic and thermodynamic data from hyperthermophilic enzymes at physiological temperature (i.e. ≥ 80 °C) raises a number of technical and fundamental problems. Based on studies of purified enzymes from the model organism *Pyrococcus furiosus* several of these problems are identified and explored here. It is proposed that kinetic and thermodynamic data on hyperthermophilic enzymes be reported at the organism's growth temperature or, alternatively, at a lower temperature compatible with practical assay conditions with additional data obtained at yet lower temperatures to allow for extrapolation.

Introduction

The native structure of biomacromolecules is metastable with respect to a number of physico-chemical parameters: e.g., ionic strength, pH, radiation, pressure, temperature. To a limited extent the cell can develop specific biochemical capacities to protect itself from the detrimental effects of the extreme values of these environmental parameters. A well-known example is the capacity of the bacterium *Helicobacter pylori* to raise locally acidity of the human stomach from a nominal pH value of 1.5 to circa 5 – 6, by producing large quantities of the nickel enzyme urease for the production of ammonia from human-made urea [1]. A second example is the capacity of many halotolerant or halophilic micro-organisms to take up or to synthesize organic compatible solutes, such as the quaternary amine betaine, and thus to balance osmotic potential in an environment of high ionic

strength [2]. However, for other environmental boundary conditions, notably extremes in temperature, micro-organisms have apparently been unable to develop machineries to stabilize their interior at mesophilic values. Thus, e. g., the archaeon *Pyrococcus furiosus* that grows optimally at an environmental temperature of 100 °C [3], also has an intracellular temperature of 100 °C, and, therefore, it must have its *entire* biochemistry adapted to this biologically extreme temperature. In this framework the enzymologist is not only faced with a fundamental problem (what is the biochemical nature of high-temperature adaptation) and with a practical problem (how does one measure biological activities at high temperatures), but also with a problem of normalization (under what conditions should 'hot' enzymes be assayed to maximize comparability with 'regular' enzymes).

Some two decades of biochemical research on hyperthermophiles has until now left the notion of unity in biochemistry unshaken. No fundamentally new concepts have been discovered related to the central pillars of life: the bioenergetics of oxidative phosphorylation, the transcription of DNA, translation of RNA, chaperone-assisted protein folding, and so on. Also, the use that hyperthermophiles make of building blocks (ATP), metabolites (glyceraldehyde-3-phosphate), and cofactors (NADPH) appears to be completely conventional, and this is remarkable in view of the limited lifetime versus thermal degradation of these compounds in dilute aqueous solution at 100 °C [4]. How hyperthermophiles succeed in stabilizing thermolabile intermediates is an unsolved problem. A partly solved problem is the thermostability of proteins from hyperthermophiles: comparisons with mesophilic counterparts at different levels ranging from pair wise comparison of 3D structures to predicted proteins from multiple genomes [5,6] suggest that the determinant is a multifaceted one, encompassing an increased number of salt bridges, hydrogen bridges, beta sheets, shortened loops, altered amino acid usage, etc. The implication is that protein thermostability in general is not predictable at this time, therefore, that mutagenesis towards increased stability is not yet possible in a rational way.

Our work on high-temperature enzymology has focused on the hyperthermophilic, anaerobic, marine euryarchaeoton *Pyrococcus furiosus* as a model system for several reasons. The organism is readily grown, e. g., on starch in 100 litre batch cultures at circa 93 °C with a doubling time of circa 40 minutes. Its biochemistry has been under study for nearly two decades. Its complete genome and also those of half a dozen closely related species (*Thermococcales* spp) are freely available. Several of its genes have been found to be readily (over)expressed in *Escherichia coli* as functional proteins. The biochemistry of *P. furiosus* and related species is mildly idiosyncratic, for example, in its strong preference for (and absolute dependence on) the 5 d transition element tungsten. In our ongoing studies we have identified a number of fundamental and practical problems, illustrated below, that may be of general relevance to quantitative 'hot' enzymology.

High Temperature Adapted Assay Instrumentation

The conceptually simple rise in temperature required to measure enzymes from hyperthermophiles at or near their physiological temperatures poses technical problems of variable complexity depending on the type of assay. Our work on *P. furiosus* has focused on redox catalysis and thus on the use of on-line assays based on the detection of redox dyes (spectrometry), electrons (direct electrochemistry) and also of gaseous substrates/products (amperometry).

UV-visible research spectrometers are commonly equipped with variable temperature accessories; however these usually have not been designed to operate in the 80 – 110 °C range. Furthermore, they are made to accept 1 cm square cuvettes, some of which, notably the fused quartz type, do not withstand high temperature for prolonged periods, and so high temperature colour-based assays can prove to be a costly exercise.

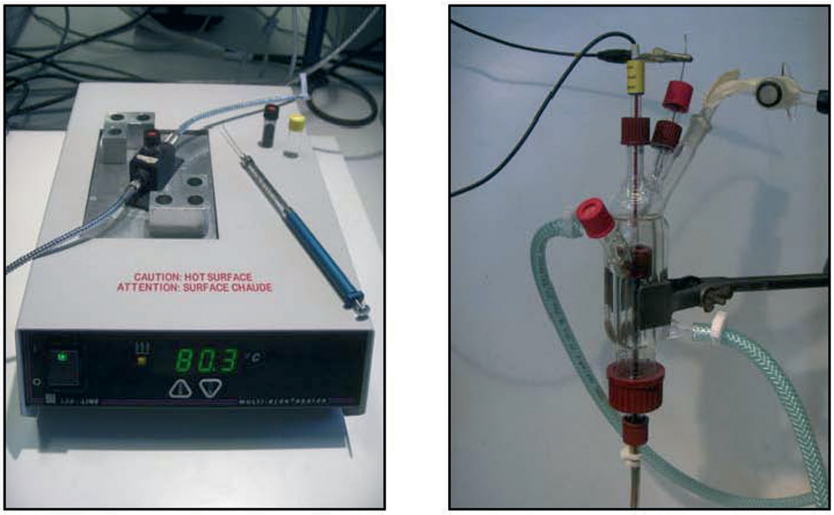

Figure 1. High temperature adaptations of routine assay equipment: (A) UV-vis spectrometry; (B) direct electrochemistry.

Figure 1a is a picture of a relatively simple solution suitable for routine assay of numerous samples, e. g., during protein purification. The cuvette house of a fibre optic spectrometer (Avantes – The Netherlands) has been built into an aluminum block that is part of a heating plate. The cuvette house has been adapted to take round glass bottles (HPLC type; 1 cm diameter). The heating block also contains additional holding positions for the pre-thermostatting of cuvettes. The HPLC bottles are closed with a septum which does not only allow for anaerobicity but also for the temperature to be raised up to a few degrees above 100 °C. The plastic cap allows for rapid cuvette transfer by hand at high temperature.

Direct electrochemistry on solid electrodes allows determining reduction potentials of electron transfer proteins and of some enzymes by cyclic voltammetry; it can also be used to assay redox enzymes in combination with natural or artificial electron transfer partners by measuring the extent of a catalytic wave in cyclic voltammetry. We have previously described a simple three electrode electrochemical cell for direct protein electrochemistry built around a small drop of solution (typically 10 – 50 µl) on top of a flat activated glassy carbon disc as the working electrode [7]. This design can be readily adapted for high temperature studies up to circa 90 °C by surrounding the cell with a thermostatted water jacket connected to a circulating water bath (Fig. 1b). The drop of solution is protected from evaporation by overlaying it with a small amount of immersion oil as used in microscopy or in PCR instruments for DNA multiplication. The reference electrode is of the Ag/AgCl type (saturated KCl) which, contrast to calomel eletrodes, can be operated up to at least 100 °C and provides a – temperature dependent – well defined reference potential [8].

A similar solution of isothermal junctions between electrodes by means of a temperature controlled water jacket is possible for amperometrically assaying gaseous substrates (O_2, H_2, NO, N_2O) with the familiar membrane covered electrochemical Clark cell, or oxygraph, the combined electrode of which is platinum versus Ag/AgCl. Here, solvent evaporation is not an issue in view of the larger cell volume (1 – 2 ml) and smaller evaporating surface (1 mm diameter port for injections/degassing). Temperature specs for commercial versions of the Clark cell will typically be limited to rather low values (40 ° C), but jacketed cells are readily home made of polycarbonate and can be run up to nearly boiling temperature.

The Problem of Choosing a Proper Assay Temperature

Figure 2 is a 'typical' plot of enzyme activity versus temperature [9]. The example is from one of the tungsto-enzymes of *P. furiosus*, Aldehyde OxidoReductase. AOR catalyses the two-electron oxidation of a range of aldehydes to their corresponding acids. The highest catalytic competence (k_{cat}/K_M) is for the substrate crotonaldehyde when assayed with benzyl viologen as electron acceptor (Fig. 2):

$$CH_3CHCHCHO + H_2O \leftrightarrow CH_3CHCHCOOH + 2\,H^+ + 2e^-$$

but the natural substrate(s) has not been unequivocally identified [10]. AOR is thought to function in catabolism of proteinaceous material (amino acid degradation). Figure 2 illustrates two key aspects of 'hot' enzymology.

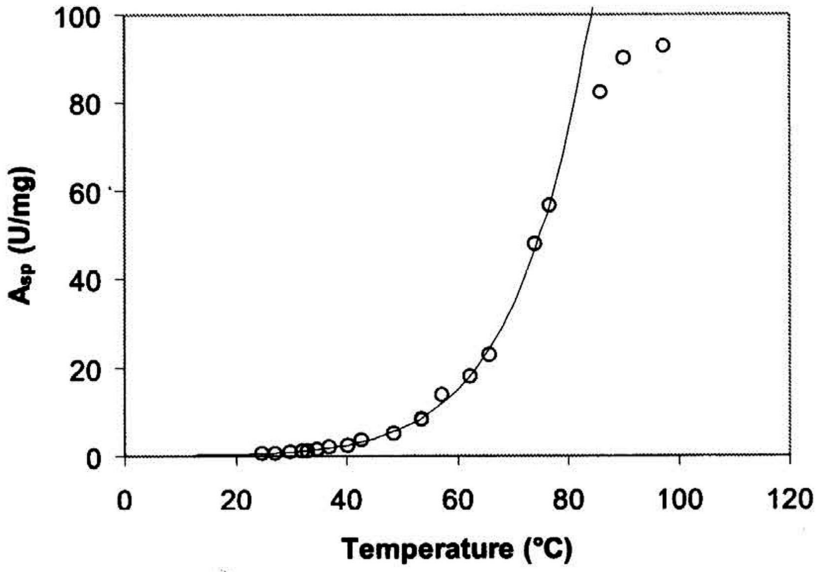

Figure 2. Apparent crotonaldehyde oxidation activity of *P. furiosus* aldehyde oxidoreductase as a function of temperature.

Firstly, as with *any* enzyme activity, the reaction rate is seen to approximately double with every 10 degrees increase in temperature. However, for hyperthermophilic enzymes the *dynamic range* of temperatures over which activity is practically measurable, is much greater than for mesophilic enzymes. This fact implies improved possibilities for, e.g., the study of temperature-dependent protein conformational changes, and the determination of activation energies associated with enzyme catalysis. Furthermore, a poor-man's version of pre-steady-state kinetics presents itself: at laboratory ambient temperatures hyperthermophilic enzymes are slowed down to the extent that trapping of enzyme kinetic intermediates would appear to no longer require advanced (stopped flow; rapid quench) equipment. This fascinating possibility of facilely creating kinetically relevant intermediates on a 'hand-mixing' time scale remains largely unexplored to date.

Secondly, and again as with *any* enzyme, increasing the assay temperature will eventually lead to the thermal degradation of activity. However, when employing the operational definition of physiological temperature as the temperature at which the hyperthermophilic organism exhibits maximal growth rate under optimized laboratory conditions, it is found more often than not that hyperthermophilic enzymes degrade thermally at a faster rate than would be compatible with the time scale of their activity assays. In Fig. 2 this is seen as a deviation of experimental points at high temperature from the fitted exponential. The dilemma for the enzymologist is obvious: the assay temperature has to be lowered to a value such that the enzyme will be stable at least for the time span that it takes to measure its activity. In the example of Fig. 2 this corresponds to circa 80 °C, which can still be

considered a – suboptimal – physiological temperature as *P. furiosus* will still grow at 80 °C (be it at a reduced rate). However, different enzymes from the same species vary drastically in their thermal stability in dilute aqueous solution (*cf.* ferritin, below) and a single standard temperature for all enzymes could only be defined after all enzymes would have been purified and characterized. Clearly, such a normalized temperature would not be equal to the temperature of maximal growth. It is therefore suggested that kinetic data on (hyper)thermophilic enzymes be reported at the highest temperature at which their activity is stable over the time period required for a reliable assay (if lower than the temperature of maximal growth), and that this information be extended with data on activity and stability as a function of temperature.

Interference of Non-Catalytic Reaction at High Temperature

Ferritin is a small (circa 20 kDa) α-helical protein that spontaneously polymerizes into a cage-shaped homo 24-mer, and that ubiquitously occurs in all domains of life. Its main physiological function is thought to be the storage of iron and/or a protection against oxidative stress [11]. Ferritin takes up Fe(II) ions and converts these in the presence of an oxidant, e. g., O_2, into a core of ferrihydrite. Its activity can be assayed by measuring the increase in light scattering at, e. g., 315 nm from the growing Fe(III) core. Non-biological oxidation of Fe(II) by O_2 at ambient temperatures is usually very much slower than ferritin-catalysed oxidation. A structural ferritin gene in *P. furiosus* can be cloned and overexpressed in *E. coli* resulting in an extremely thermostable 24-mer the Fe(II) oxidation activity of which is resistant to 10 h boiling at 100 °C or 30 min autoclaving at 120 °C [12].

When assayed at 25 °C this ferritin exhibits cooperative kinetics (Hill coefficient $n \approx 2$) and a half-maximal activity for $K_{0.5} = 5$ mM Fe(II). At 25 °C the non-catalytic rate of Fe(II) oxidation for [Fe(II)] = 5 mM is negligible compared to the ferritin catalysed rate. When the temperature is raised to 85 °C the Fe(II) oxidation activity increases by circa two orders of magnitude consistent with an approximate two-fold increase in rate for every 10 °C increase in temperature. However, this activity can only be measured at relatively low [Fe(II)] ≤ 0.3 mM. At [Fe(II)] = 5 mM the rate of non-catalytic oxidation is comparable to that of ferritin-catalysed oxidation, and this interference precludes a complete kinetic analysis at high temperature. This example illustrates interference of a background reaction under *in vitro* assay conditions to the extent that kinetic analysis is limited to non-physiological temperature (*P. furiosus* does not grow at 25 °C).

High-temperature studies of *P. furiosus* ferritin have pointed to another technical problem of 'hot' biochemistry: the extreme thermostability of this protein is reflected in the fact that differential scanning calorimetric measurements fail to reveal a 'melting' temperature up to 120 °C [12]. It appears that calorimetry is not a practical option to study unfolding of these types of proteins.

Determination of Reduction Potentials at High Temperture

A key thermodynamic parameter to be determined in the study of redox proteins is the reduction potential, E_m, of the prosthetic group(s). An E_m value reflects the relative stability of the oxidized versus the reduced form of a compound and thus can be dependent on a number of environmental parameters, e. g., pH and ionic strength, but also: temperature. This raises the question at what temperature redox properties of (hyper)thermophilic proteins should be determined and reported. E_m values of proteins are not necessarily linear functions of temperature, e. g., due to temperature-dependent protein conformational changes. Consequently, E_m values should preferably be determined at physiological temperature, which is, however, not a trivial problem as can be illustrated on the example of *P. furiosus* rubredoxin, a small (6 kDa), thermostable electron transfer protein with a single Fe(II/III) redox prosthetic group. Its E_m can be determined as a function of temperature by direct voltammetry on activated glassy carbon as shown in Fig. 3. Apparently, no temperature-dependent conformational changes occur over the studied temperature range because the reduction potential is found to be linear in T with an approximate -1.5 mV change per degree increase [13].

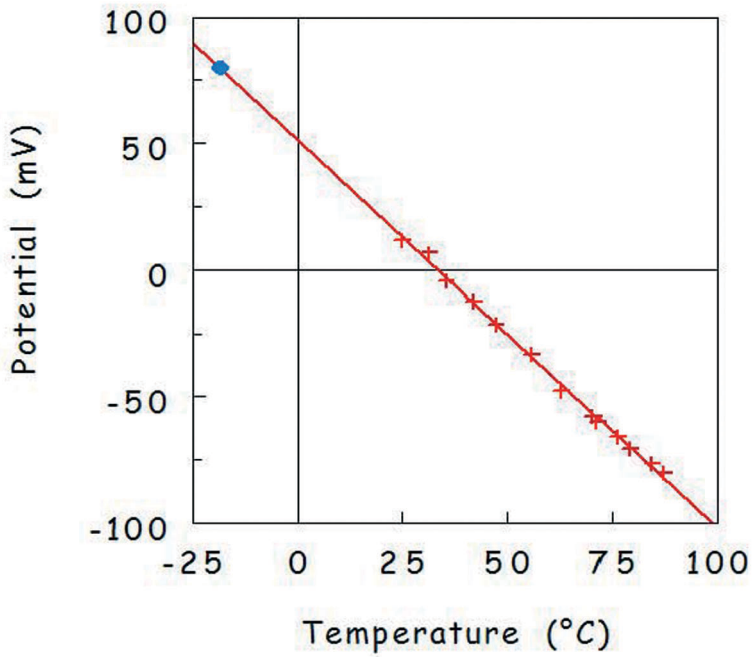

Figure 3. Reduction potential of *P. furiosus* rubredoxin as a function of temperature as determined with direct electrochemistry (((plus))) and with EPR monitored titration (●).

An alternative technique to determine E_m values is bulk titration monitored with EPR spectroscopy. Substoichiometric additions of reductant (dithionite) or oxidant (ferricyanide) are made to the protein solution in the presence of a cocktail of redox mediators to ensure redox equilibrium between the protein and the detection electrode (platinum), and when equilibrium is reached (i.e. a constant voltage reading) then a sample is drawn and rapidly frozen for cryogenic EPR analysis in order to determine the extent of reduction of the prosthetic group. The importance of this method lies in (1) its general applicability to metalloproteins that usually exhibit an EPR signal either in their oxidized or in their reduced state, and (2) the finding that many proteins, notably redox enzymes do not exhibit finite electron transfer rates with bare electrodes, which precludes general application of the direct voltammetry method. Remarkably, when the bulk titration method is applied to *P. furiosus* rubredoxin, the outcome is $E_m \approx +80$ mV *in*dependent of whether the titration is done at 20 °C or at 80 °C [13]. This observation suggests that, during the cooling down of the EPR sample towards its freezing point the rubredoxin protein sufficiently rapidly adapts its structure so that the determined E_m value always corresponds to the sample's freezing temperature whatever the initial sample temperature was (*cf.* Fig. 3). The general implication would be that reduction potentials of (hyper)thermophilic electron transfer proteins at physiological temperatures cannot be determined accurately by EPR monitored redox titrations. Whether this conclusion also holds for larger proteins, notably enzymes, is not yet clear at this time.

Enzyme Activation During Heat-Up

The enzyme hydrogenase catalyses the activation of molecular hydrogen in nature either for its oxidation to protons or for its formation from protons [14]:

$$2\,H^+ + 2e^- \leftrightarrow H_2$$

The most common form of the enzyme has an active centre consisting of a heterodinuclear cluster of nickel and iron bridged by cysteinato sulfurs and with structural CO and CN^- ligands coordinating the Fe ion. In purified NiFe-hydrogenase this unusual centre is frequently found to be in an inactivated form which can be activated by anaerobic incubation under hydrogen. The activation process may involve the removal of a bridging oxo or peroxo group and the concomitant or subsequent reduction of the dinuclear cluster [14]. The rate of activation depends on the source of the enzyme and on its history. *P. furiosus* makes a soluble NiFe-hydrogenase the metal centre of which is in an inactive, oxidized form after purification of the enzyme at ambient temperature. EPR spectroscopic studies have shown that this hydrogenase goes through an auto-activation cycle when heated up anaerobically to, e.g., 80 °C [15]. The mechanism of auto-activation is unknown, but it may involve one or more molecules of H_2 trapped in the protein's H_2 channelling system during

isolation. The general implication of these observations is that (hyper)thermophilic enzymes may exhibit a broad range of extents of activation depending not only on the temperature of assay, but also on the time of pre-incubation at this temperature.

Change of Substrate Solvation with Temperature

In addition to the already mentioned aldehyde oxidoreductase, AOR, *P. furiosus* synthesizes at least four more tungsten-containing oxidoreductases, one of which is named formaldehyde oxidoreductase, FOR, because for all tested aldehyde substrates this enzyme has the highest k_{cat} for formaldehyde [16]. The physiological relevance of this observation has however been questioned because the apparent K_M for formaldehyde is unrealistically high, namely of the order of 10^{-2} M [16]. As with AOR, also FOR is thought to function in a degradation pathway of proteinaceous material.

Formaldehyde is a unique aldehyde in its very strong tendency to be hydrated in aqueous solution. The hydration equilibrium lies well towards the direction of methylene glycol formation such that only a very small fraction is in the free formaldehyde form:

$$HCHO + H_2O \leftrightarrow HOCH_2OH$$

So what is the actual substrate of the FOR-catalysed oxidation reaction: is it methylene glycol or formaldehyde? A reasonable answer is suggested by temperature-dependent Michaelis–Menten analysis. At 20 °C the K_M= 40 mM and at 80 °C the K_M= 6 mM for total formaldehyde (free plus hydrated); however, when these values are re-calculated for free formaldehyde using the temperature dependence of the dissociation constant for methylene glycol [17] the $K_M \approx 0.03$ mM is *in*dependent of temperature [18]. It thus appears that *free* formaldehyde is an excellent substrate for the enzyme FOR. This example illustrates another general rule of 'hot' biochemistry: the non-biological hydration chemistry of the substrate may be very different at mesophilic versus (hyper)thermophilic temperatures.

Conclusions

In comparison to the study of their mesophilic counterparts exploration of the quantitative enzymology of thermophilic enzymes, and, *a fortiori*, of hyperthermophilic enzymes raises a number of additional problems of a practical and also of a fundamental nature.

Established methods of analysis have to be adapted to handle samples at high temperatures. For some methods this can be a relatively simple technical adjustment (colorimetric assays, *cf.* Fig. 1), but for other methods attempts at adaptation can reveal intrinsic limitation (EPR monitored redox titration, *cf.* Fig. 3).

Unfolding of hyperthermophilic proteins may be intrinsically difficult to study as these proteins may not exhibit melting in the temperature range in which commercial calorimetries operate.

The study of redox properties of hyperthermophilic redox enzymes may be intrinsically difficult because common methods to determine reduction potentials do not work at high temperatures.

Activation mechanisms for enzymes purified in an inactivated form can be a complex function of temperature.

Non-biological substrate chemistry (e.g., hydration; oxidation on air) may strongly vary with temperature and may thus complicate temperature-dependent enzymology.

When physiological temperature is defined as the optimal growth temperature of a hyperthermophilic micro-organism, it is frequently difficult to assay activities at this temperature because the enzymes may have limited stability in isolated form in dilute solutions.

In summary, with a view to the standardization of assay conditions for hyperthermophilic enzymes it is advised that data be reported either at optimal growth temperature or, if this is not feasible, at the highest possible temperature at which assays can be reliably run. Particularly in the latter case it is of relevance also to obtain data at lower temperatures to allow for cautious extrapolation.

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