RESEARCH COMMUNICATION

The effect of low temperatures on enzyme activity

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The stability of two enzymes from extreme thermophiles (glutamate dehydrogenase from *Thermococcales* strain AN1 and β-glucosidase from *Caldocellum saccharolyticum* expressed in *Escherichia coli*) has been exploited to allow measurement of activity over a 175 °C temperature range, from +90 °C to −85 °C for the glutamate dehydrogenase and from +90 °C to −70 °C for the β-glucosidase. The Arrhenius plots of these enzymes, and those for two mesophilic enzymes (glutamate dehydrogenase from bovine liver and β-galactosidase from *Escherichia coli*), exhibit no downward deflection corresponding to the glass transition, found by biophysical measurements of several non-enzymic mesophilic proteins at about −65 °C and reflecting a sharp decrease in protein flexibility as the overall motion of groups of atoms ceases.

INTRODUCTION

It is now accepted that, in general terms, enzyme activity is dependent on protein dynamics [1–5]: in other words, an enzyme must be flexible to function.

A number of biophysical studies [6–12] have shown that the flexibility of mesophilic proteins undergoes a transition at about −65 °C. Below this transition temperature the overall motion of groups of atoms within the protein ceases, and all that is left is the harmonic vibration of individual atoms. These results suggest that protein function will also cease at this temperature. The experiments which come closest to demonstrating this are those of Petsko's group [13], which show that, above the transition temperature, crystals of RNAase A rapidly bind the inhibitor cytidine 2′-monophosphate, but at −61 °C do not. The transition is sharp and is manifested by distinct changes in slope.

Furthermore, if the transition, as expected, reflects a sharp decrease in the flexibility of the protein, then less-flexible proteins may undergo the transition at a significantly higher temperature. We know that the degree of flexibility of both mesophilic and thermophilic enzymes is similar at their respective growth temperatures [14–17], but at any given temperature the thermophilic enzyme is less flexible. It may be that, for an enzyme from an organism growing optimally at 75 °C, the transition will occur near −25 °C instead of −65 °C. This would be useful, because it is harder to measure enzyme activities accurately at lower temperatures. Given the exactly parallel behaviour of mesophilic and thermophilic proteins in terms of activity, flexibility and structure, thermophilic proteins are assumed to have a glass transition similar to that described for mesophilic proteins.

We report here experiments designed to test whether enzyme activity shows the disproportionate decrease at low temperatures which we might expect from biophysical data, and whether such a decrease is seen at a higher temperature in an enzyme from an extreme thermophile. The effect of temperature on the activity of four enzymes has been determined: a β-galactosidase from *Escherichia coli* growing at 37 °C, a β-glucosidase from *Caldocellum saccharolyticum* (70 °C), and glutamate dehydrogenases from bovine liver (37 °C) and *Thermococcus* strain AN1 (75 °C). The thermophilic enzymes in this study are hyperthermophilic proteins because of their stability at temperatures far above the growth temperatures of the organisms.

EXPERIMENTAL

Glucosidases

The thermophilic β-glucosidase was purified from an *E. coli* clone containing the β-glucosidase gene from *C. saccharolyticum* strain Tp8 [21]. The mesophilic β-galactosidase from *E. coli* (catalogue no. G6008) was obtained from Sigma. The enzymes were assayed in 50% (v/v) dimethyl sulphoxide (DMSO) by nitrophenol release from an appropriate substrate (50 mM *p*-nitrophenyl β-D-glucopyranoside for the glucosidase and 60 mM *o*-nitrophenyl β-D-galactopyranoside for the galactosidase). Sodium acetate/acetic acid buffer solutions giving a final pH of 6.2 at the assay temperature [22] and a concentration of 6 mM were used. Enzyme was dissolved in water and then added to the buffer at 0 °C, followed by addition of DMSO to give a final solvent concentration of 50% (v/v). Control measurements were made in the absence of enzyme.

Reactions were started by addition of the substrate, which was dissolved in buffer with 50% (v/v) DMSO. Below −45 °C, substrates were injected by syringe and mixing was achieved by stirring with a precooled glass rod. Between 0 °C and −45 °C the substrate was mixed by using a syringe. Assays times varied according to temperature. Above 0 °C assays were for 5–60 min, while at −70 °C they were for 3–4 months. The reaction was stopped with ethanolamine/DMSO/water (3:5:2, by vol.) equilibrated below the reaction temperature.

Above 20 °C the assays were carried out in sodium citrate/citric acid buffers (6 mM, pH 6.2) without cryosolvent. K_m values for both the glucosidases were determined by using the assay method described above.

Glutamate dehydrogenase (GDH) enzymes

The thermophilic GDH from the *Thermococcales* strain AN1 was prepared as described by Hudson [20]. The mesophilic GDH

Abbreviations used: DMSO, dimethyl sulphoxide; GDH, glutamate dehydrogenase; αOG, α-oxoglutarate ('ketoglutarate').

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from bovine liver (catalogue no. G7882) was obtained from Sigma. Assays for the GDH enzymes were by glutamate detection using a Waters amino acid analyser by using a modified Pico-Tag (Waters Associates) method.

Phosphate buffers (NaH_2PO_4/Na_2HPO_4) adjusted to give a final pH of 8 in 70% methanol at the required temperature [22] were used throughout. Reactions were carried out in 10 mM buffer/0.2 mM NADPH/10 mM α-oxoglutarate (αOG; disodium salt)/100 mM NH_4Cl/70% methanol. Reactions were started by addition of the enzyme. Control measurements were carried out by replacing the enzyme with an aliquot of water equivalent to the volume of enzyme. Reactions were stopped using 60% (v/v) trifluoroacetic acid in dimethylformamide equilibrated below the reaction temperature. Assays below −70 °C required up to 1 month.

Assays without the cryosolvent were carried out using the continuous assay system used by Hudson [20]. Comparison of activities in the presence and absence of the cryosolvent was carried out at 0 °C using the non-continuous method described above.

K_m values for the AN1 GDH in both the aqueous system and the cryosolvent were determined using the continuous assay method used by Hudson [20]. Owing to the instability of the bovine liver GDH at 15 °C in the cryosolvent, the K_m values were determined using a non-continuous assay system at 0 °C. The same procedure as the subzero assays (Figure 2 below) was used, but the production of NADP was monitored (by recording the absorbance at 340 nm) instead of the production of glutamate. The aqueous K_m values for the bovine GDH were also determined using this method.

All measurements are shown in the results for activity measurements in the cryosolvent. Non-cryosolvent assays were checked to be in agreement with previous determinations [20,21]. The activity at each temperature was measured over a single time interval.

RESULTS AND DISCUSSION

No downwards deflection corresponding to a glass transition was observed in any of the Arrhenius plots. The Arrhenius plots for the two thermophilic enzymes and the bovine GDH (Figures 1 and 2) are essentially linear over the whole temperature range, with the activities being inhibited in the cryosolvent. The mesophilic β-galactosidase is not stable at 40 °C, and at that temperature the Arrhenius plot is no longer linear. The Arrhenius plot for mesophilic β-galactosidase (Figure 1) also shows a significant change in slope in the cryosolvent. This may be an indication of a difference in rate-determining step or mechanism in the cryosolvent. Since the Michaelis complex, ES, is proposed to degrade in two steps [23]:

$$ES \xrightarrow{k_2} EG + P_1 \xrightarrow{k_3} E + P_2$$

the change in slope may be a result of the rate constant changing from k_2 to k_3. The enzyme activities in the Arrhenius plots are proportional to $K_{cat.}$, since the substrate concentration is maintained at saturating levels. The cryosolvents used here are the same as those used in biophysical studies of the glass transition.

The dielectric constant, ionic strength and pH may all be affected by the cryosolvent and temperature [18]. Such changes usually limit activity and could be expected to enhance any downward deflection in the Arrhenius plots caused by a glass transition rather than conceal a transition. The absence of such a downward deflection suggests that the precautions taken to restrict or prevent such limitations on activity have been successful. The K_m is also affected by temperature and cryosolvent, but the trends demonstrated in Tables 1 and 2 indicate that substrates did not become rate-limiting at the lower temperatures.

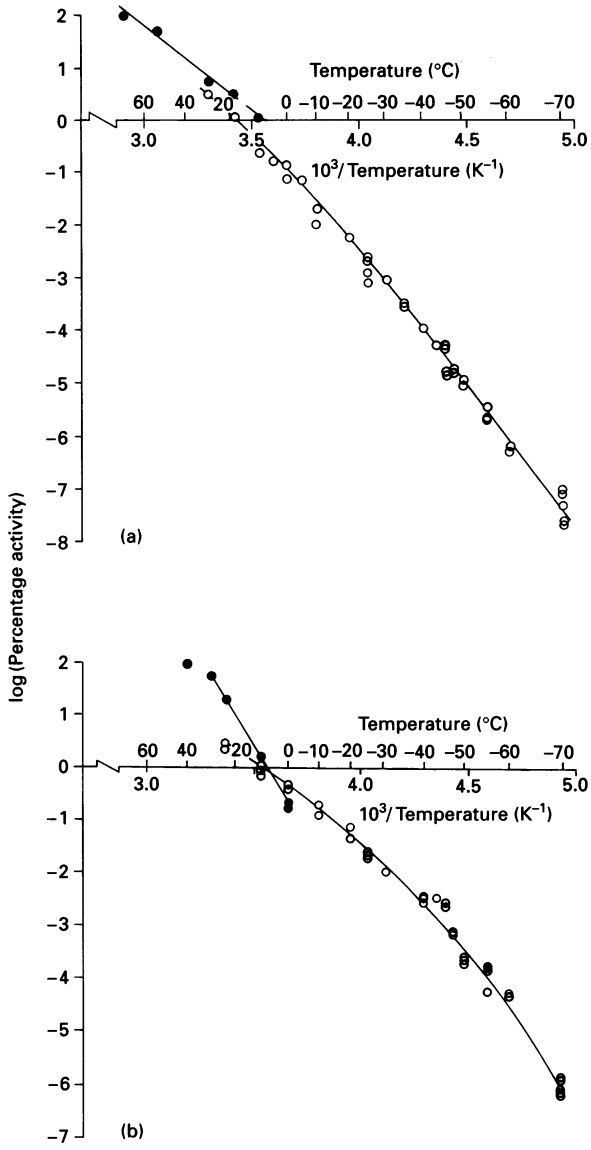

Figure 1 Effect of temperature on the activity (expressed as the percentage residual activity corresponding to 100% activity) of two glucosidases

(**a**) Cloned β-glucosidase from *C. saccharolyticum* Tp8; 100% activity corresponds to 160 μmol of PNP/min per mg of protein. (**b**) β-Galactosidase from *E. coli*; 100% activity corresponds to 95 μmol of PNP/min per mg of protein. ●, Aqueous assays in citrate buffer at pH 6.2. ○, Cryosolvent assays in DMSO/acetate buffer (1:1, v/v), final pH 6.2.

It is possible to argue that the failure to observe a transition at −25 °C for the thermophilic enzymes, on the basis of their lesser flexibility, is due to a tendency for their dynamic behaviour to converge with that of the mesophilic enzymes as both approach the 'freezing point' represented by the transition in flexibility. However, the failure to observe any activity transition with either the mesophilic or thermophilic enzymes down to −85 °C is difficult to explain on the basis of our current assumptions concerning the relationship between enzyme dynamics and activity. The biophysical measurements showing a sharp decrease

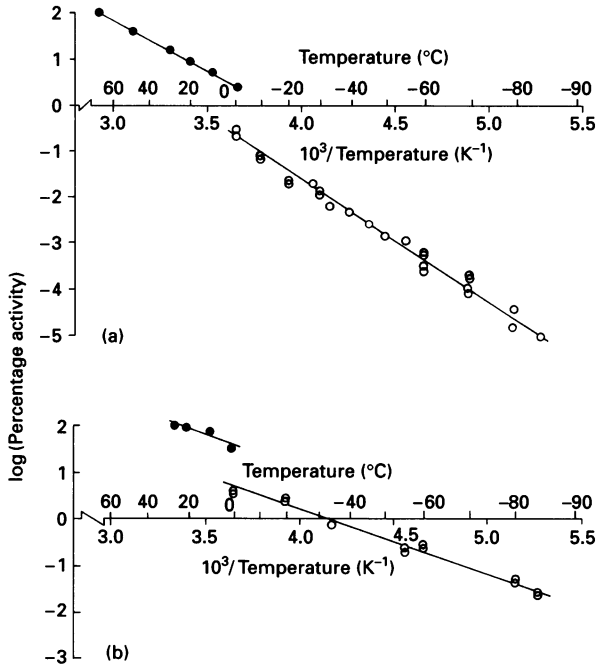

Figure 2 Effect of temperature on the activity (expressed as the percentage residual activity corresponding to 100% activity) of two GDHs

(**a**) GDH from *Thermococcus* strain AN1; 100% activity corresponds to 1212 μmol of glutamate/min per mg of protein. (**b**) GDH from bovine liver; 100% activity correponds to 36 μmol of glutamate/min per mg of protein. ●, Aqueous assays with 10 mM phosphate buffer, final pH 8. ○, Cryosolvent assays in methanol/10 mM phosphate buffer (7:1, v/v), pH 8.

Table 1 Effect of temperature and 50% DMSO on the K_m of the glucosidases

	K_m (mM)			
	β-Glucosidase		β-Galactosidase	
Temperature (°C)	No DMSO	DMSO	No DMSO	DMSO
20	0.78	13.3	0.19	–
0	0.63	8.3	0.22	1.8
−20	–	5.9	–	2.5
−50	–	–	–	3.7

Table 2 Effect of temperature and 70% methanol on the K_m values for the GDH enzymes

			Apparent K_m (mM)		
Enzyme	Solvent	Temperature (°C)	NADPH	NH_4Cl	αOG
AN1	Water*	80	0.066	15.5	1.7
	Water	14	0.019	2.0	0.2
	70% Methanol	14	0.037	6.0	–
Bovine liver	Water	15	1.5	–	0.5
	Water	0	1.1	0.005	0.1
	70% Methanol	0	6	0.03	–

* From Hudson et al. (1993) [20].

in enzyme flexibility centred around −65 °C, which are supported by the results of an inhibitor-binding experiment performed by Rasmussen [13], taken with the expected dependence of activity on flexibility, lead us to expect a similar decrease in activity at this temperature.

There are a number of possible explanations for the absence of an activity transition. It could be that the motion which is still occurring below the transition temperature is sufficient for low enzyme activity. The catalytic-centre activities ('turnover numbers') of the enzymes are extremely low at these low temperatures; for example, the thermophilic β-glucosidase turns over once every 17 days at −70 °C and the thermophilic GDH turns over once every 10 s at −80 °C. Such low activity may not require rapid motion, even taking into account the multiple motions required for one turnover. A very low rate of conformational change below the transition temperature may be sufficient to maintain low levels of activity. And it could be that the motions required for such activity may not be detectable using the flexibility techniques reported.

On the basis of RNAase A failing to bind an inhibitor below the transition temperature, Rasmussen et al. [13] concluded that RNAase A loses activity below the transition temperature. Inhibitor binding and catalytic transformation involve different processes which may be affected differently by temperature. However, even if this were the case, the flexibility required for catalysis will be more complex than for inhibitor or substrate binding, so catalysis should not be expected to operate when inhibitor binding is no longer possible. Parak et al. [12] have demonstrated a transition in activity of the membrane proteins of the chromophores in *Rhodospirillum rubrum* as measured by electron transfer between two of the acceptors. However, the environment in which this experiment was carried out is unclear, and a phase transition in the membrane could have been the cause of such a transition. Also, the flexibility required for electron transfer will be less than that required for substrate binding, catalytic transformation and product release.

Another possibility is that enzymes may not necessarily need to change conformation to be slightly active. A single substate or conformation may be responsible for the low levels of activity reported here at low temperatures. At ambient temperatures the substrate induces the correct conformation and the reaction can proceed. Below the transition temperature enzymes become locked within a distribution of substates, favouring the most stable conformations. If the catalytic conformation is present below the transition, activity will be observed, but if the catalytic substate is not favoured in the absence of the substrate, then it will not be present below the transition temperature and activity will be expected to be absent. The presence or absence of activity would be enzyme-dependent. However, this hypothesis assumes that a single substate can carry out binding of the substrate as well as the catalytic transformation. Results from transition-state inhibitor studies [19] do not support this hypothesis.

This work has used the stability of enzymes from extreme thermophiles to enable enzyme activity to be measured over a greater temperature range than has previously been attempted. The activation energies for both the thermophilic enzymes were constant over the whole temperature range, implying that enzymes from extreme thermophiles are not specifically adapted for activity at high temperatures, except insofar as this is conferred by their higher thermostability. This is consistent with some currently held views on the interrelationship between enzyme stability, activity and dynamics [24,25].

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