The effects of temperature on the kinetics and stability of mesophilic and thermophilic 3-phosphoglycerate kinases

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The effects of temperature on the kinetic parameters k_{cat} and K_m , for three isolates of the highly conserved monomeric enzyme 3-phosphoglycerate kinase (PGK), were investigated in detail using a rapid automated kinetics apparatus. PGK was purified from the thermophilic bacterium *Thermoanaerobacter* sp. Rt8.G4 (optimum growth temperature 68 °C), the mesophile *Zymomonas mobilis* (optimum growth temperature 32 °C) and a second, unidentified, soil mesophile designated *unid A* (optimum growth temperature 27 °C). The kinetic behaviour with temperature of each PGK preparation was distinct, despite the conserved nature of the enzyme. The k_{cat} values increased with temperature, but not as rapidly exponentially, as might be expected from the Arrhenius equation. Maximum k_{cat} values were at much higher temperatures than the optimum growth temperatures for the mesophiles, but for the thermophile the temperature of maximum k_{cat} was close to its optimum growth temperature. K_m values were

in general nearly constant through the lower temperature ranges, but increased substantially as the optimum temperature (highest k_{cat}) was passed. Thermal irreversible denaturation of the PGK proteins was also investigated by measuring loss of activity over time. In a dilute buffer, Arrhenius plots for denaturation were linear, and the calculated apparent energy of activation (E_{act}) for denaturation for the thermophilic PGK was 600 kJ·mol⁻¹, whereas for the mesophilic enzymes the values were 200–250 kJ·mol⁻¹. In the presence of substrates, a considerable stabilization occurred, and in the case of the *Z. mobilis* enzyme, the apparent E_{act} was increased to 480 kJ·mol⁻¹. A theoretical explanation for these observations is presented. Comparing the kinetics data with irreversible denaturation rates determined at relevant temperatures, it was clear that k_{cat} values reached a maximum, and then decreased with higher temperature before irreversible denaturation had any significant influence.

INTRODUCTION

Temperature is one of the most important environmental variables that dictate the viability limits of organisms, because it affects all organisms, and the chance of exposure to extremes that affect the integrity of cells and their components is relatively high. Organisms can tolerate only a defined range of temperatures, above and below which they are adversely affected.

Increasing temperature has two major effects on enzymes; the catalytic rate increases, and ultimately denaturation occurs. Whereas the rate variation (V_{max} or k_{cat}) is frequently presented in studies of temperature effects on enzymes, there are relatively few detailed reports of the changes in other kinetic parameters such as the K_m .

The calculation of k_{cat} requires saturating concentrations of substrates throughout the temperature range. Without a parallel estimation of K_m at each temperature, the best option would be to use substrate concentrations some 100-fold higher than the K_m at room temperature. Usually this is not done for practical reasons, and since K_m can alter quite markedly with temperature, usually increasing as temperature increases, it is likely that reported k_{cat} values have often been underestimated at the higher temperatures. Breaks in Arrhenius plots suggesting structural transitions at high temperatures might in some cases be due to a high K_m causing underestimation of k_{cat} .

The thermal denaturation process of any protein is complex,

but a useful simplification is to consider it to be a two step process. The first step is a reversible conformational change to a partially folded state. This is followed by further modifications to produce an irreversibly denatured state. This process is represented by:

where N represents the native, active enzyme; I represents intermediate forms that are partially denatured, but which are rapidly reconvertible to the native form; and D represents denatured polypeptide which can no longer refold, but will aggregate irreversibly.

The majority of kinetic studies on thermal protein denaturation have been conducted in artificial conditions where aggregation of the unfolded proteins (D) is prevented by the presence of solutes such as urea or guanidine. In such conditions, reversibility of the denaturation process is usually observed ($D \leftrightarrow N$). This allows determination of detailed thermodynamic equilibrium parameters, although most of these are only relevant for the artificial solvent conditions. The more 'natural' thermal denaturation in the absence of such solutes is usually irreversible due to aggregation of the unfolded D polypeptide chain. The only thermodynamic information to be derived from investigations on irreversible denaturation is kinetic parameters associated with

Abbreviations used: PGK, 3-phosphoglycerate kinase; PGA, 3-phosphoglyceric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; 2-ME, 2-mercaptoethanol; k_{cat} , catalytic rate constant; E_{act} , energy of activation; E'_{act} , apparent energy of activation; k_{den} , first-order rate constant for the irreversible denaturation step; k'_{den} , apparent first-order rate constant for denaturation.

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the denaturation rates, such as the energy of activation, E_{act} , and related thermal transition (Eyring) constants.

Of significance to temperature investigations on enzymes is the relationship between catalytic activity and the onset of denaturation. Irreversible denaturation is often assumed to be responsible for the decline in enzyme-reaction velocity above the temperature optimum, which is the temperature at which, under a given set of conditions, maximum product is formed. If this is the only reason for loss of activity at high temperatures, then theoretically the shorter the time taken for measuring activity, the higher the apparent optimum temperature should be. In addition, up to the temperature where significant denaturation occurs, the rate (k_{cat}) should increase exponentially according to the Arrhenius equation. Neither of these features are invariably observed.

The enzyme 3-phosphoglycerate kinase (PGK) is an appropriate model for detailed temperature investigations. The major advantage is that it is a monomer, which avoids complications due to possible subunit dissociation. Being intracellular, it contains no disulphide bonds which could also cause difficulties in thermal-denaturation studies. PGK has been purified from a wide variety of sources [1–3], and its conserved three-dimensional structure is known [4–8]. Detailed kinetic and mechanistic investigations have been conducted with the yeast enzyme [9–15], and most aspects of its kinetic behaviour are understood. However, one complicating factor in using PGK is the occurrence of non-linear kinetics (apparent negative co-operativity) exhibited when PGK is assayed in the reverse direction to glycolysis (the 3-phosphoglyceric acid [PGA] to 1,3-bisPGA direction). One suggested explanation is multiple binding sites for each substrate [16–18], although the crystallographic structure does not directly support this. Another explanation put forward is slow product dissociation at low salt/substrate concentrations [12]. However, linear kinetics are restored by the presence of sulphate or other anions. Although the addition of sulphate simplifies analysis, results are probably not indicative of true enzyme parameters due to interference of the sulphate ion. Alternatively, linear kinetics can be obtained if the concentration of fixed substrate is very high.

Presented in this paper are results of studies on the effects of temperature on three PGK preparations, two from mesophiles, and one from a thermophile. Kinetic experiments to determine k_{cat} and K_m values over a range of temperatures were conducted, and compared with results obtained from irreversible thermal-denaturation experiments under various buffer conditions. We are able to show that, with increasing temperature, k_{cat} reaches a maximum and begins to decrease before significant irreversible denaturation occurs. In all cases K_m values also rose substantially at the extremes of temperature. Despite the conserved nature of PGK, there were substantial differences in behaviour between all three of the enzymes studied.

MATERIALS AND METHODS

Organisms

Zymomonas mobilis strain ATCC 29191 is an aerotolerant anaerobe that has a very rapid fermentation rate on glucose. The cells were grown as described previously [19]. *Thermoanaerobacter* sp. Rt8.G4 (previously described as *Thermoanaerobium brockii* strain Rt8.G4) was a gift from Professor H. Morgan, Waikato University, New Zealand, and was grown as described previously [20]. It is an obligate anaerobe which ferments moderately rapidly on a variety of sugars. *unid A* is an unidentified facultative anaerobe isolated by us from soil, which

has properties relating it to the genus *Aeromonas* (L. Sly, University of Queensland, Australia).

Enzyme sources

PGK was purified from *unid A* (optimum growth temperature 25–28 °C), *Z. mobilis* (optimum growth temperature 30–35 °C) and the *Thermoanaerobacter* sp. (optimum growth temperature 68–70 °C). Plasmid pLOI323, containing *Z. mobilis* PGK and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes [21], was a gift from Professor L. O. Ingram, University of Florida, U.S.A. It was transformed into *Escherichia coli* DH5 α .

Extraction

Cells were extracted at room temperature with 6 volumes of a buffer consisting of 20 mM K-Mes, pH 7.0, containing 5 mM MgCl₂, 0.1 % Triton X-100, 0.2 mg/ml lysozyme, 5 μ g/ml DNase and 10 mM 2-ME (2-mercaptoethanol). *Thermoanaerobacter* extraction was for 1 h, *Z. mobilis* and soil bacteria were extracted overnight. For *E. coli* cells a 30 mM K₂HPO₄/30 mM NaCl buffer, pH 7.0, was used, with Triton X-100, DNase and lysozyme as above, and the addition of 1 % toluene to the lysis mixture. The pH of each of the extracts was adjusted to 6.0 with 1 M Mes.

Molecular-mass estimation

Gel filtration using a calibrated Superdex 75 column (Pharmacia) was employed to determine molecular masses of the isolated enzymes. The applied standards consisted of BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

Enzyme purification

Dye adsorbents were prepared by reaction of the dyes with amine-substituted Sepharose CL-4B [22]. *Z. mobilis* PGK was purified using Procion Navy HE-R as an adsorbent. Following affinity elution with PGK substrates [23], the sample was subjected to Sephacryl 200 gel filtration. The sample was then dialysed and applied to a Q-Sepharose ion-exchange column. The enzyme was eluted with an increasing NaCl gradient. Specific activity at 25 °C was 415 units/mg. SDS/PAGE analysis indicated that the preparation was 90 % pure using densitometry scanning of the stained gel. The same method was used to purify recombinant *Z. mobilis* PGK from *E. coli* cells after a heat treatment (55 °C, 20 min) of the cell extract. No differences could be detected between *Z. mobilis* enzymes isolated from the two sources. *Thermoanaerobacter* PGK was purified using a Procion Brown MX-5BR dye-ligand column as the first step. Subsequent steps were identical to the *Z. mobilis* PGK purification procedure. Specific activity at 25 °C was 240 units/mg. The SDS/PAGE analysis indicated that the main band of PGK represented 70 % of the total protein; most of the other bands were assumed to be peptides from PGK due to nicking proteolysis during purification, as the amounts of these bands increased on storage. Purification of PGK from *unid A* utilized a Procion Blue HE-RD dye-ligand column initially, followed by (NH₄)₂SO₄ fractionation. The salt was added to the dye-column eluate to 60 % saturation. The supernatant was collected and subjected to gel filtration and ion exchange as described for the *Z. mobilis* PGK purification. Specific activity at 25 °C was 215 units/mg. This preparation was estimated by densitometry scanning of SDS/PAGE to be only 50 % pure. As with the *Thermoanaerobacter* enzyme, some of the impurity bands were due to nicking proteolysis.

Routine enzyme assays

PGK was assayed in the direction of 1,3-bisphosphoglycerate production and coupled to GAPDH. A 10 mM K-Mes buffer, pH 6.5, including 30 mM NaCl and 5 mM MgCl_2 , contained 5 mM PGA, 0.15 mM NADH, 1 mM ATP, 10 mM 2-ME and approximately 10 units/ml GAPDH (measured at 25 °C). Stock solutions of ATP, NADH and PGA were buffered to pH 7.0. The mesophilic GAPDH-coupling enzyme was sourced from rabbit muscle, available in the laboratory as an $(\text{NH}_4)_2\text{SO}_4$ suspension. This was passed through an S-200 gel-filtration column before use to remove traces of PGK and all the $(\text{NH}_4)_2\text{SO}_4$. For a high-temperature coupling enzyme, GAPDH was purified from *Thermoanaerobacter* using Cibacron Yellow F-3R dye-ligand chromatography followed by Q-Sepharose ion-exchange and S-200 gel filtration. Enzyme activities are expressed in units of μmol per min.

Kinetic experiments

These utilized a thermostatted version of the automated stopped-flow apparatus described previously [24,25]. The design was such that enzyme sample and substrates (about 0.15 ml each) were separately pre-heated in an aluminium block while the previous mixture was being analysed (10–15 s). They were then mixed and the rate measured after 5 s. For all investigations up to 40 °C, rabbit GAPDH was used as the coupling enzyme. At higher temperatures the *Thermoanaerobacter* GAPDH was used. Temperature was recorded at a point 2 mm from the flow cell, and was accurate to ± 0.5 °C. The variable substrate under investigation was mixed with a known quantity of Rhodamine dye (5–10 μM), which acted as an indicator of substrate concentration; this indicator dye had no effect on the enzymes' activity. The dye concentration was recorded at 552 nm, and the NADH A_{340} was measured simultaneously on a Hewlett-Packard diode-array spectrophotometer. This was programmed to average the dye concentration readings, and to calculate the rate (v) from successive 1 s readings at 340 nm. A warning was given if the rate deviated by more than 10 % between the first and second halves of the time course. A kinetic-analysis programme [24] then determined K_m and $V_{\max}$ from the data by minimizing the root mean squares on the reaction rate (v) versus substrate concentration (s) plot. The values for k_{cat} at 25 °C for each enzyme were calculated from the specific activities at 25 °C, increased by a factor to allow for the impurities present (see above), and using the molecular masses as estimated from gel filtration.

Denaturation experiments

A solution of the enzyme at approximately 10 $\mu\text{g}/\text{ml}$ was heated in a thin-walled glass tube at the specified temperature and continuously sampled at a rate of 0.05 ml/min. The sample cooled (for about 30 s) and was mixed with assay reagents (K-Mes assay buffer, pH 6.5, containing 1 mM PGA, 0.5 mM ATP, 30 mM NADH, 10 units/ml rabbit GAPDH and 10 mM 2-ME) in a flow system, so that the amount of enzyme remaining active was continuously monitored by fluorescence of NADH in a flow cell in a Hitachi 204 fluorimeter. Enzyme preparations were diluted either into a 1 mM K-Mes, pH 6.0, 0.25 mM MgCl_2 buffer, or into the assay buffer as used in the kinetics experiments. From the exponential decrease of activity recorded, the first-order rate constant k'_{den} for denaturation could be determined at each temperature. This value is the 'apparent denaturation rate constant' for $\text{N} \rightarrow \text{D}$, as opposed to the presumed actual rate constant for $\text{I} \rightarrow \text{D}$ as shown above. The apparent energy of

activation for denaturation, E'_{act} , was calculated from the slope of the Arrhenius plot of $\log k'_{\text{den}}$ against $1/T$.

RESULTS

Conditions for kinetic experiments

At low concentrations of fixed substrate (< 1 mM), the isolated bacterial PGK enzymes exhibited the non-linear kinetics (using an Eadie–Hoffstee plot) common for this enzyme [12]. To minimize this phenomenon, the concentration of the fixed substrate was increased. Plots became linear within experimental accuracy when fixed substrate concentrations were at least 5 mM PGA or 5 mM ATP (3 mM ATP was sufficient for *Z. mobilis* PGK). Consequently, all kinetic experiments were carried out with fixed substrate concentrations at these levels or higher.

Molecular mass estimation

The apparent molecular mass of *Z. mobilis* PGK, as determined by gel filtration, was 44 000 Da (41 384 Da from the gene sequence [21]). The estimated masses of *Thermoanaerobacter* PGK and *uid A* PGK by gel filtration were 42 000 and 43 000 Da, respectively.

Effects of temperature on the rate of reaction

An excess of GAPDH was required at all times in the experimental mixture to ensure complete reaction of the 1,3-bisPGA product. At the upper temperature limits of the *Thermoanaerobacter* PGK experiments, tests were conducted to ensure the observed effects were due to decreasing PGK activity, and not because of inactivation of the coupling enzyme. Tests were also carried out to ensure that there was no significant auto-oxidation of NADH at the high temperatures. At the upper temperature range for each enzyme, excess fixed substrate was added to minimize the effects due to increasing K_m values for this substrate. For *Thermoanaerobacter* and *uid A* enzymes, the fixed substrate concentrations were raised to 6 mM; for *Z. mobilis* PGK above 62 °C fixed ATP concentration was increased to 12 mM, and fixed PGA to 8 mM.

The k_{cat} could be calculated at each temperature both from variable PGA and variable ATP experiments. The values were corrected to saturating levels of fixed substrate using the K_m determined for that substrate at that temperature. The data shown in Figure 1 illustrate that the agreement between the two sets of data (each of which was averaged from three estimations at each temperature) was satisfactory. The k_{cat} values increased with temperature, but not as rapidly as would be expected if the reaction rate increased exponentially as defined by the Arrhenius equation. Consequently, Arrhenius plots ($\log k_{\text{cat}}$ against $1/T$) of these data are all non-linear (not shown). In the case of *Z. mobilis*, a sharp optimum-temperature peak was observed at 63 °C, with a drop to near-zero activity by 70 °C. With *uid A*, the peak was reached earlier (53 °C), but the drop-off was more gradual. With each of these two enzymes, the optimum is 25–30 °C above the optimum growth temperature for these species. On the other hand, with the *Thermoanaerobacter* enzyme the optimum (70 °C) is only 2–3 °C above the optimum growth temperature of this species. A fairly rapid drop-off occurred so that by the highest temperature we were able to investigate, 77 °C, the activity was down to about one-third of the value at 70 °C.

Figure 1 Effect of temperature on reaction velocity

The effect of temperature on kinetic parameters were determined in a K-Mes buffer, pH 6.5, for (top panel) *unid A* PGK, (middle panel) *Z. mobilis* PGK and (lower panel) *Thermoanaerobacter* PGK. ● Show experiments varying PGA, whereas data indicated by ○ were obtained when ATP was varied. The k_{cat} data were corrected for the K_m of the fixed buffer substrate (see Figures 2 and 3). The ▲ symbols represent the amount of activity loss that would be expected as a result of irreversible denaturation during the assay time, based on results in Tables 1–3.

The absolute values of k_{cat} were highest for *Thermoanaerobacter* and lowest for *unid A*. At the optimum growth temperatures, the values for k_{cat} were: *unid A*, 300 s^{-1} ; *Z. mobilis*, 450 s^{-1} ; and *Thermoanaerobacter*, 1500 s^{-1} . Expressed as units (at the optimum growth temperature) per g of cells, these values are approximately: *unid A*, 60 $\mu mol \cdot min^{-1} \cdot g^{-1}$; *Z. mobilis*, 1000 $\mu mol \cdot min^{-1} \cdot g^{-1}$; and *Thermoanaerobacter*, 2000 $\mu mol \cdot min^{-1} \cdot g^{-1}$. The low value for *unid A* reflects the fact that it is an aerobe

Figure 2 Effects of temperature on the K_m for PGA

unid A PGK is denoted by ○, *Z. mobilis* PGK by ■ and *Thermoanaerobacter* PGK by △.

Figure 3 Effects of temperature on the K_m for ATP

unid A PGK is denoted by ○, *Z. mobilis* PGK by ■ and *Thermoanaerobacter* PGK by △.

(growing only slowly anaerobically) and so it does not rely solely on glycolysis to produce its energy needs.

Effects of temperature on the K_m values

The K_m values for PGA and ATP for each of the enzymes over the temperature ranges studied are shown in Figure 2 and Figure 3. The values changed little (PGA) or decreased slightly (ATP) as the temperature increased in the lower temperature range, but increased very substantially as the temperature reached and passed the optimum k_{cat} temperature. This increase was very

Table 1 Denaturation rates of *unid A* PGK

Denaturation rates were determined in a 1 mM K-Mes, 0.25 mM MgCl₂ buffer (dilute), pH 6.0, or the assay buffer consisting of 10 mM K-Mes, pH 6.5, including 30 mM NaCl, 5 mM MgCl₂, 0.15 mM NADH, 5 mM PGA, 5 mM ATP and 10 mM 2-ME.

Temperature (°C)	k'_{den} (min ⁻¹)	
	pH 6 Buffer	Assay buffer
43	0.07	
45	0.12	
48	0.33	
50	0.5	
52	0.75	
55	1.17	
57		< 0.01
60	1.86	0.03
63		0.15
65		0.36
68		0.72
70		1.06

Figure 4 Arrhenius plots for the denaturation of *unid A* PGK

○ Represent denaturation in dilute (1 mM) K-Mes buffer, pH 6.0. ● Represent denaturation in the presence of assay buffer plus substrates. Error bars indicate estimated error in measurement of k'_{den} .

marked for ATP. The K_m values for the *unid A* enzyme were significantly lower than those for the other two, except at its highest active temperatures.

The effect of temperature on the specificity constants k_{cat}/K_m can be deduced from Figures 1–3. These values increased in all cases through the lower temperature range due to increasing k_{cat} , but dropped sharply at higher temperatures due both to decreases in k_{cat} and to increases in K_m .

pH Effects

When the kinetic experiments were repeated using Mops in place of Mes in the pH 7.5 buffer described, the overall variations of k_{cat} and K_m with temperature were not significantly altered (data not shown). A reduction in k_{cat} values was observed for *Thermoaerobacter* PGK; this enzyme has an optimum pH of 5.5 [25a].

Table 2 Denaturation rates of *Z. mobilis* PGK

Denaturation rates were determined in a 1 mM K-Mes, 0.25 mM MgCl₂ buffer (dilute), pH 6.0, or the assay buffer consisting of 10 mM K-Mes, pH 6.5, including 30 mM NaCl, 5 mM MgCl₂, 0.15 mM NADH, 5 mM PGA, 5 mM ATP and 10 mM 2-ME.

Temperature (°C)	k'_{den} (min ⁻¹)	
	pH 6 Buffer	Assay buffer
58	0.04	
60	0.07	
62	0.1	
65	0.23	
68	0.61	
70	1.22	0.15
72		0.36
73		0.5
74		1.4
76		2.3

Figure 5 Arrhenius plots for the denaturation of *Z. mobilis* PGK

Details as for Figure 4.

Irreversible denaturation

Denaturation rates were calculated from the experimentally obtained plots of loss of enzyme activity, observed over 1–15 min. *unid A* PGK denaturation rates are presented in Table 1. An Arrhenius plot for the experiments carried out in dilute pH 6.0 buffer was linear, and from the slope the E'_{act} for denaturation was calculated to be 210 kJ·mol⁻¹. Only limited data were obtained for denaturation in the presence of substrates, but a line giving the same slope as in the absence of substrates can be drawn through the higher-temperature values (Figure 4).

For *Z. mobilis* PGK the denaturation rates are given in Table 2. As with the *unid A* PGK results, the Arrhenius plot of the dilute buffer values was linear, and the E'_{act} for denaturation of this enzyme was determined to be 250 kJ·mol⁻¹. However, the Arrhenius plot of the experiments carried out in the presence of substrates (assay conditions) gave a value of 480 kJ·mol⁻¹ (Figure 5).

Table 3 Denaturation rates of *Thermoanaerobacter* PGK

Denaturation rates were determined in a 1 mM K-Mes, 0.25 mM MgCl₂ buffer (dilute), pH 6.0, or the assay buffer consisting of 10 mM K-Mes, pH 6.5, including 30 mM NaCl, 5 mM MgCl₂, 0.15 mM NADH, 5 mM PGA, 5 mM ATP and 10 mM 2-ME.

Temperature (°C)	k'_{den} (min ⁻¹)	
	pH 6 Buffer	Assay buffer
70	< 0.01	
73	0.03	
75	0.05	
77	0.15	
78	0.32	
80	0.92	0.11
82		0.41
83		0.77
84		1.3
86		2.1

Figure 6 Arrhenius plots for the denaturation of *Thermoanaerobacter* PGK

Details as for Figure 4.

Table 3 gives the denaturation rates determined for *Thermoanaerobacter* PGK. An E'_{act} for denaturation of approximately 600 kJ · mol⁻¹ was calculated from the linear Arrhenius plot of the dilute buffer results. In the presence of substrates, there was a 3–4 °C stabilization, with approximately the same value for E_{act} (Figure 6).

Denaturation of PGK in the dilute buffer solution should reflect the true intrinsic thermostability of the proteins. It can be seen that a considerable stabilization occurs when the substrates are added. This stabilization was about 7 °C for *Z. mobilis*, as much as 17 °C for *unid* A, but only 3–4 °C for *Thermoanaerobacter*, compared with the dilute buffer. This is due mainly to the substrates, but also to the buffer ions present and to a lesser extent the small pH difference (T. M. Thomas and R. K.

Scopes, unpublished work). The experiments with substrates present were in conditions identical to those used for the enzyme-activity measurements, except that GAPDH was not included. Consequently, we can presume that catalysis was occurring in these experiments, and an equilibrium was established with all four substrates/products being present.

Activity measurements were recorded in the first 15 s of catalysis. Assuming that a 10 % loss in PGK is required over 15 s to show an effect on the enzyme kinetics, denaturation would not make a significant contribution to the decline in activity until a denaturation rate constant of around 0.4 min⁻¹ was reached. Lines indicating the amount of loss of product in 15 s due to irreversible denaturation are shown in Figure 1. This indicates that irreversible denaturation would not make a significant contribution to the decline in enzyme activity as measured until about 66–67 °C for *unid* A PGK, 73 °C for *Z. mobilis* PGK and 82–83 °C for *Thermoanaerobacter* PGK. In all cases, these temperatures are higher than the highest shown in Figure 1, where little or no activity remains.

DISCUSSION

Examples in the literature of the variation of enzyme activity (specific activity or k_{cat}) with temperature appear, at first glance, to be very similar. Catalysis increases as temperature rises until a maximum velocity is achieved, and with further rises in temperature the enzyme activity declines due to some inactivation process. However, close inspection of this basic trend reveals differences from the simple theory that are significant. The way in which the rate of activity increases for various enzymes is one important difference. Some enzymes, for example α -chymotrypsin [26], *Z. mobilis* glucokinase and glucose 6-phosphate dehydrogenase [27], and *B. megaterium* lactate dehydrogenase [28], have reaction velocities that rise exponentially with temperature until the maximum is reached. These enzymes follow closely the predicted rise in chemical reaction velocity with temperature and give linear Arrhenius plots. Other enzymes, such as trout-brain acetylcholinesterase [29], *Thermotoga maritima* enolase [30], *Pyrococcus furiosus* β -glucosidase [31] and the phosphoglycerate kinases studied in this paper, have much slower and non-exponential increases in reaction velocity. Indeed, the increase in k_{cat} with temperature was practically linear for the PGKs. A non-exponential rise in k_{cat} indicates that the enzyme is unable to continue to achieve maximum catalytic potential as the temperature increases. This will be discussed further below.

The k_{cat} value for *Thermoanaerobacter* PGK was unusually high for a thermophilic enzyme; indeed at intermediate temperatures, e.g. 40–50 °C, it was the highest of all three enzymes studied. This is contrary to the normal observation that thermophilic enzymes are less active than their mesophilic counterparts at a given temperature. Some other enzymes that we have purified from this source have had k_{cat} values less than their mesophilic counterpart; e.g. the GAPDH value was 50 % that of the rabbit enzyme at 40 °C. However, PGK does seem to be an exception, as similarly high values for k_{cat} have been reported for other thermophilic PGKs [2,30,32].

Overall, an increase in K_m at the highest temperatures was observed for each PGK, but each individual enzyme exhibited a distinct K_m variation pattern for each substrate. Increases in K_m with temperature have previously been demonstrated for bacterial and eukaryotic enzymes. Trends similar to those observed for the ATP K_m have previously been reported for various enzymes including the phosphoenolpyruvate K_m s of several fish-muscle pyruvate kinases [33], the glucose 6-phosphate K_m for *Z. mobilis* glucose 6-phosphate dehydrogenase and the pyruvate K_m

of rabbit-muscle lactate dehydrogenase [27]. Examples of more gradual rises in Michaelis constants with temperature, similar to the PGA K_m results described here, include the acetyl-CoA K_m of trout-liver citrate synthases [34], pyruvate K_m of *Bacillus* lactate dehydrogenase [28], the fructose K_m of *Thermotoga neapolitana* xylose isomerase [35] and the ATP K_m of glucokinase from *Z. mobilis* [27].

Enzyme efficiency (k_{cat}/K_m) is relative to the substrate under investigation. Maximum efficiency was reached near the physiological temperature when PGA was the variable substrate. On the other hand, maximum efficiencies when varying ATP for the mesophilic enzymes occurred closer to the temperatures for optimum activity. Since reaction velocity at low substrate concentrations is dependent on the k_{cat}/K_m value, it could be significant that the value is maximum near the physiological temperature for PGA. However, it should be noted that these assays were carried out in the non-physiological direction, so such arguments may have little physiological relevance.

A distinct behaviour of each of the three investigated enzymes was observed for the changes of each kinetic parameter with temperature. It appears that adaptation to optimize performance at different environmental temperatures involves more than a simple shift of all parameters along a temperature scale. In particular, whereas the mesophilic enzymes are active and stable at temperatures well above the survival temperature of the organisms, this is not so for the thermophile, which can survive up to the maximum temperature that its PGK is active, i.e. 75–80 °C.

The denaturation rates calculated in this paper are apparent rates (k'_{den}) for the overall denaturation process. If there are intermediate protein states, I, in equilibrium with the native, active protein, N, the residual activity measured following the heat treatment includes both the N and the I forms, as it may be assumed that the I form(s) all immediately return to N on cooling. This means that the apparent k_{den} is related to the true rate constant for the conversion $I \rightarrow D$ by a parameter that includes the value of the equilibrium constant, K , for the $N \leftrightarrow I$ interconversion. The value of K will be small at low temperatures as there is not enough energy in the system for many protein molecules to unfold. As temperature increases the proportion of the unfolded intermediate states rises. At denaturing temperatures, the value of K can become quite large. If the E_{act} values are calculated on the assumption of a simple $N \rightarrow D$ process, they may be quite in error if it is the I form that actually denatures.

The apparent rate constant k'_{den} is given by the formula:

$$k'_{den} = k_{den} \cdot K/(1 + K) \quad (1)$$

From this equation an expression for the apparent E_{act} (E'_{act}) can be derived:

$$\ln k'_{den} = \ln k_{den} + \ln K - \ln(1 + K)$$

$$\frac{d(\ln k'_{den})}{dT} = \frac{d \ln k_{den}}{dT} + \frac{d \ln K}{dT} - \frac{d \ln(1 + K)}{dT}$$

Since $\frac{\Delta H^0}{RT^2} = \frac{d \ln K}{dT}$ the above expression equates to:

$$\frac{E'_{act}}{RT^2} = \frac{E_{act}}{RT^2} + \frac{\Delta H^0}{RT^2} - \frac{d \ln(1 + K)}{dT}$$

$$E'_{act} = E_{act} + \Delta H^0 - \{RT^2 \cdot d \ln(1 + K)/dT\} \quad (2)$$

When K is very small, the final term in Equation 2 approximates to 0; when K is large, it approaches the value of ΔH^0 . Therefore,

the experimentally determined E_{act} can be as much as the sum of E_{act} for the irreversible $I \rightarrow D$ step and the expected large ΔH^0 value for the reversible partial unfolding of the native protein. Since ΔH^0 is large positive, the value of K must increase rapidly with temperature. Thus E'_{act} should be close to $E_{act} + \Delta H^0$ at lower temperatures when K is much less than 1, and approximate to E_{act} at high temperatures when K is much greater than 1.

Values for E'_{act} of the mesophilic enzymes in dilute pH 6 buffer were at the low end of the scale of E'_{act} values usually obtained for denaturation of proteins [36]. This suggests that in the dilute buffer E'_{act} is close to the true E_{act} , and the ΔH^0 term is not involved. Thus in the denaturation temperature range studied, 58–70 °C, most of the enzyme existed in the I form. But when the denaturation experiments were carried out in assay conditions in the presence of substrates, the E'_{act} for *Z. mobilis* PGK increased from 250 to 480 kJ mol⁻¹. This suggests that in the presence of substrates the enzyme was mainly in the N form, and the E'_{act} value includes the ΔH^0 for the $N \rightarrow I$ conversion. This probably reflects the trapping of the enzyme in the N form by binding to the substrates, although in the temperature range studied (70–76 °C) the enzyme had little if any activity (Figure 1), and very weak affinity for ATP (Figure 3). On the other hand, the experiments with substrates with *uidA* did not have such an effect on the E'_{act} value. In this case with substrates it was necessary to go to much higher temperatures (15–17 °C higher) to get denaturation rates in the measurable range. It is possible that in a lower temperature range (with substrate) the E'_{act} might have been larger, as it was with *Z. mobilis*. Some suggestion that this was so is indicated in Figure 4.

E'_{act} for *Thermoanaerobacter* in dilute pH 6 buffer was much higher than for the mesophilic enzymes, being close to 600 kJ mol⁻¹. In the presence of substrates, it was about the same. In this case it could be that the N form of the thermophilic enzyme is much more stable, and it only converts to the I form (which is very unstable) at very high temperatures. In this case the value of 600 kJ mol⁻¹ would represent the sum of both E_{act} for $I \rightarrow D$, and ΔH^0 for the $N \rightarrow I$ transition.

A comparison of denaturation rates in the presence of assay buffer and the temperatures at which PGK activities began to decline indicated that irreversible denaturation did not account for this reduction in activity. This phenomenon may be a common property of PGK, as well as other enzymes. Another process must be occurring before irreversible denaturation becomes the dominant detrimental influence on enzyme activity.

Based on the arguments above, an explanation is that the reversible unfolding of the protein molecules to the I form, the first step of denaturation, is significant at relatively low temperatures. Such partially unfolded intermediates of PGK are unlikely to be active. Thus as the population of these intermediates rises, the overall catalytic rate will be less than if all species had remained in the N form. However, if the enzyme molecule is saturated with substrates (which is the basis for determining the k_{cat} values in Figure 1), these would tend to keep the enzyme in the N configuration (NS, where S represents substrates). Theoretically, in saturating-substrate conditions no I form could occur unless the enzyme, while still bound with substrates, could assume a partially denatured and inactive I configuration. Thus the process of denaturation in the presence of substrate is: $NS \rightarrow IS \rightarrow I + S \rightarrow D$. At around the optimum temperature a considerable proportion of the enzyme would be converting to the inactive IS, from which the substrate quickly dissociates. The proportion of enzyme in the active NS form, even in the presence of theoretically saturating substrate(s), will depend on the relative rates of dissociation and interconversion between the various

conformations. The data indicate that this proportion declines rapidly with increasing temperature around the optimum value.

An alternative possible explanation for the loss of activity at high temperatures relates to the hinge-bending mechanism of PGK catalysis [5]. As temperature imparts thermal kinetic energy to the protein structure, the conformational interconversions may become too rapid for catalysis to occur efficiently, causing a reduction in reaction velocity before any inactivation process has commenced. Cioni et al. [37] reported that a large number of protein sub-states exist in solution, and the equilibrium between the different forms was affected by the surrounding environment. At high temperatures the interconversions between sub-states, which in PGK can include the 'open' and 'closed' domain forms, became too rapid to distinguish using this experimental method.

A third possible reason for a reduction in catalytic rate before there are any effects of irreversible denaturation is localized unfolding of the protein active site. Shoichet et al. [38], examining the structure and function of T4 lysozyme, concluded that catalytic residues within the enzyme were not optimized for protein stability and that this was a general property of enzyme active sites. Enzyme active sites, therefore, are more susceptible to unfolding than the rest of the protein molecule due to the increased flexibility required for catalytic function. This has been observed for a number of proteins both in the presence of chemical denaturants [39–41], and during thermal denaturation [42–44]. The conclusion is that enzymes unfold in a sequential process, the first stage of which is the localized, reversible unfolding of the active site, followed by the unfolding of the whole protein. Due to its hinge-bending mechanism the PGK active site may be more susceptible to limited and localized unfolding before the more gross global unfolding processes become significant. Some evidence for this has been reported during chemical denaturation of yeast [45,46] and horse-muscle PGK [45]. This is an attractive possible reason for the decline in reaction velocity before there is any significant irreversible denaturation, and is not inconsistent with the concept of intermediate I forms. However, the very large apparent energy of activation for denaturation for *Thermoanaerobacter* PGK suggests that this includes a large contribution from ΔH° for the N $\leftrightarrow$ I interconversion. If this is so, then the reversible change between N and I would also have to involve a substantial conformational change, signifying global rather than local unfolding.

It is also possible that the methods we have used either for measuring denaturation or for determining k_{cat} values are giving false information. If, in the denaturation experiments, on cooling, any significant renaturation (D $\rightarrow$ N) occurs, then the estimate of denaturation rates is incorrect. Testing this by allowing partially denatured samples to cool slowly before sampling did not show any evidence of renaturing; the denatured state D appears to be irreversible in the circumstances described. The hypotheses presented also depend upon the assumption that the partially denatured species I refold totally to N on cooling during the 30 s or so that lapsed before the sample was mixed with assay reagents.

Finally, it is possible that the values of k_{cat} have been underestimated at the higher temperatures because of non-linear kinetics again becoming significant under these conditions despite fixed-substrate concentrations many times the apparent K_m . The substrate-concentration ranges used might have missed a 'very high K_m ' portion of the plots, in which the true k_{cat} was much higher than the extrapolated values. One major problem with investigating this possibility is that using very high substrate concentrations (i.e. > 10 mM) causes major increases in the ionic strength, which has previously been shown to be a complicating

determinant of activity with PGK. All kinetic experiments used a variable substrate-concentration range of 10–15-fold, and were linear within $\pm 5\%$, even at the highest temperatures.

PGK k_{cat} values did not increase exponentially with temperature. This can be explained by an increase in the population of inactive or partially denatured forms as the temperature increased. The K_m s for ATP tended to remain constant or decrease slightly with temperature over most of the investigated temperature range, but rose sharply as the optimum temperatures were reached. The K_m s for PGA were fairly constant in the lower temperature ranges, but increased steadily at higher temperatures. Most notably, the reduction in PGK k_{cat} above the optimum temperatures occurred well before irreversible denaturation became the dominant effect. Whereas there were gross similarities in the variation of kinetic parameters, temperature induced distinctly different kinetic behaviours for the three isolated bacterial PGK enzymes, despite the conserved nature of this enzyme.

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