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Thermodynamic Study of Yeast Phosphoglycerate Kinase

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ABSTRACT: Enthalpies of binding of MgADP, MgATP, and 3-phosphoglycerate to yeast phosphoglycerate kinase have been determined by flow calorimetry at 9.95–32.00 °C. Combination of these data with published dissociation constants [Scopes, R. K. (1978) *Eur. J. Biochem. 91*, 119–129] yielded the following thermodynamic parameters for the binding of 3-phosphoglycerate at 25 °C: $\Delta G^\circ = -6.76 \pm 0.11$ kcal mol^{-1}, $\Delta H = 3.74 \pm 0.08$ kcal mol^{-1}, $\Delta S^\circ = 35.2 \pm 0.6$ cal K^{-1} mol^{-1}, and $\Delta C_p = 0.12 \pm 0.32$ kcal K^{-1} mol^{-1}. The thermal unfolding of phosphoglycerate kinase in the absence and presence of the ligands listed above was studied by differential scanning calorimetry. The temperature of half-completion, $t_{1/2}$, of the denaturation and the denaturational enthalpy are increased by the binding of the ligands, the increase in $t_{1/2}$ being a manifestation of Le Chatelier's principle and that in enthalpy reflecting the enthalpy of dissociation of the ligand. Only one denaturational peak was observed under all conditions, and in contrast with the case of yeast hexokinase [Takahashi, K., Casey, J. L., & Sturtevant, J. M. (1981) *Biochemistry 20*, 4693–4697], no definitive evidence for the unfolding of more than one domain was obtained.

Yeast phosphoglycerate kinase (EC 2.7.2.3) (PGK)[1] consists of a single polypeptide chain of some 420 amino acid residues and has a molecular mass of 45 000–50 000 daltons (Scopes et al., 1973). High-resolution X-ray studies (Banks et al., 1979) show that in the crystalline state the molecule is composed of two globular lobes of approximately equal size separated by a cleft. As with other kinases (Anderson et al., 1979), substrate binding induces a hinge-bending conformational change which closes the cleft.

In previous work from this laboratory (Takahashi et al., 1981) the thermodynamics of the binding of glucose to yeast hexokinase and of the thermal unfolding of the enzyme in the absence and presence of glucose were investigated. In this paper we report similar work on yeast phosphoglycerate kinase in the absence and presence of the ligands 3-phosphoglycerate, MgATP, and MgADP.

[1] Abbreviations: PGK, yeast phosphoglycerate kinase; 3-PG, 3-phosphoglycerate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DTE, dithioerythritol; DSC, differential scanning calorimetry.

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Table I: Enthalpy of Binding of MgATP, MgADP, and 3-PG to PGK

temp (°C)	ΔH_b (kcal mol^{-1})		
	MgADP	MgATP	3-PG
9.95	−7.86 ± 0.19[a] (8)[b]	−7.70 ± 0.04[a] (8)[b]	−0.77 ± 0.01[a] (8)[b]
17.50	−8.59 ± 0.62 (9)	−6.80 ± 0.21 (7)	2.01 ± 0.08 (7)
25.00	−9.36 ± 0.77 (9)	−7.56 ± 0.01 (8)	3.74 ± 0.08 (8)
32.00	−12.03 ± 0.33 (8)	−7.43 ± 0.33 (8)	2.07 ± 0.02 (8)

[a] Uncertainties are expressed as the standard error of the mean. [b] The numbers in parentheses indicate the number of measurements.

Materials and Methods

Yeast phosphoglycerate kinase was purchased from Sigma Chemical Co. (lot nos. 100F-8170 and 64F-8130) as an ammonium sulfate precipitate. Enzyme activity was assayed in the back-reaction, leading from 3-phosphoglycerate to 1,3-diphosphoglycerate, as described by Bücher (1947). The values of specific activity obtained were close to those stated by Sigma. Prior to use, the ammonium sulfate precipitate was spun down, dissolved in a buffer containing 50 mM PIPES, pH 7.0, and 0.1 mM DTE, and then exhaustively dialyzed against the same buffer. For studying the effect of various buffers and varying pH, the enzyme was dialyzed against the appropriate buffer as indicated in the text. All dialyses were performed at 4 °C. After dialysis, the enzyme solution was centrifuged at about 10000*g* for 1 h in order to remove any residual solid material. Protein concentrations were calculated from spectrophotometric measurements by using an E_{280nm} for a 1 mg mL^{-1} solution of 5.7 (Blake et al., 1972). All protein solutions were used within 2 days of preparation.

The magnesium salt of ATP, the potassium salt of ADP, and the disodium salt of D-(−)-3-phosphoglyceric acid (3-PG) were purchased from Sigma Chemical Co. All other chemicals were of reagent grade. Doubly deionized water was used for preparing all solutions.

Calorimetry. Determinations of the enthalpy of binding of the ligands to PGK were made in a flow microcalorimeter of the heat-conduction type designed by Takahashi and Fukada (1985). A stopped-flow method was used for all measurements. The solutions of ligand and protein were each flowed at a rate of 9.2×10^{-4} cm^3 s^{-1} for 60–100 s. The concentrations after mixing of protein and substrate were about 35 μM and 2 mM, respectively. A buffer solution containing 50 mM PIPES, pH 7.0, 4 mM $MgCl_2$, and 10^{-4} M DTE was used. The calorimeter was calibrated by measuring both the heat of neutralization of NaOH with HCl and the heat of dilution of sucrose.

All differential scanning calorimetric (DSC) experiments were performed with a DASM-4 microcalorimeter (Privalov, 1980) at a scan rate of 1 K min^{-1}. The thermodynamic parameters were obtained from the DSC curves according to the procedures outlined by Privalov and Khechinashvili (1974), with base lines drawn as described by Takahashi and Sturtevant (1981). The effective cell volumes were determined over the appropriate temperature range by measuring the total heat capacities of the cells filled with water and with N_2, using the known specific heats and densities.

Results and Discussion

Isothermal Calorimetry. The enthalpies of binding of MgADP, MgATP, and 3-PG to PGK obtained at four different temperatures are summarized in Table I. Only in the case of MgADP binding does there appear to be a significant dependence of the enthalpy on temperature. The thermodynamic parameters at 298.15 K calculated from these enthalpies and the dissociation constants published by Scopes (1978) are listed in Table II. The binding of the two nucleoside phosphates results from a favorable enthalpy change while that of 3-PG results from a favorable entropy change. The thermodynamics of the binding of 3-PG to PGK is similar to that observed by Takahashi et al. (1981) for the binding of D-glucose to yeast hexokinase in that the favorable free energy change (−5.12 ± 0.04 kcal mol^{-1} at 25 °C) is primarily due to a favorable entropy change (14.7 ± 3.1 cal K^{-1} mol^{-1}).

Thermal Unfolding of PGK at pH 7.0. The thermal unfolding of PGK was studied at pH 7.0 over a concentration range, in various buffers, and in the presence of 0.3 M KCl and 4 and 100 mM $MgCl_2$. This process is apparently irreversible under all conditions employed, as indicated by the fact that no endotherm is seen on rescanning any of the solutions. The results of these measurements are summarized in Table III. Several interesting points may be noted. The calorimetric enthalpy (column 5) is essentially constant over the range of experimental conditions employed, including three buffers with different heats of ionization. This shows that little or no change in protonation of the enzyme takes place on denaturation. The overall mean enthalpy of 204.2 ± 2.6 kcal mol^{-1} corresponds to 4.34 ± 0.06 cal g^{-1}, which is a usual value for a globular protein. The temperature of half-denaturation $t_{1/2}$ (column 4) is constant except in the presence of $MgCl_2$ when

Table II: Thermodynamic Parameters for Binding of MgADP, MgATP, and 3-PG to PGK at 25 °C, pH 7.0

substrate	K_b[a] (M^{-1})	$\Delta G°$ (kcal mol^{-1})	ΔH (kcal mol^{-1})	$\Delta S°$ (cal K^{-1} mol^{-1})	ΔC_p (cal K^{-1} mol^{-1})
MgADP	(1.00 ± 0.20) × 10^5	−6.82 ± 0.12	−9.36 ± 0.77	−8.52 ± 3.0	−180 ± 50
MgATP	(1.11 ± 0.12) × 10^5	−6.89 ± 0.07	−7.56 ± 0.01	−2.28 ± 0.25	0
3-PG	(9.09 ± 1.65) × 10^4	−6.76 ± 0.11	3.74 ± 0.08	35.2 ± 0.6	120 ± 320

[a] Scopes (1978).

Table III: Thermal Denaturation of D-Phosphoglycerate Kinase at pH 7.0

buffer	protein concn (mg mL^{-1})	no. of detn's	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$
50 mM cacodylate (ΔH_i = −0.47 kcal mol^{-1})	0.732–1.829	7	56.31 ± 0.03	203.7 ± 3.5	1.82 ± 0.10	159.7 ± 1.9	0.78 ± 0.01
50 mM PIPES (ΔH_i = 2.75 kcal mol^{-1})	0.413–1.893	7	56.26 ± 0.05	207.3 ± 1.3	1.43 ± 0.05	178.4 ± 2.2	0.87 ± 0.02
50 mM imidazole (ΔH_i = 8.78 kcal mol^{-1})	0.502–2.141	7	56.03 ± 0.04	198.2 ± 2.7	1.43 ± 0.05	159.4 ± 3.9	0.80 ± 0.02
50 mM PIPES, 4 mM $MgCl_2$	0.636–1.388	8	55.22 ± 0.08	207.7 ± 2.3	1.54 ± 0.05	159.1 ± 1.8	0.77 ± 0.01
50 mM PIPES, 100 mM $MgCl_2$	0.475–1.583	6	54.58 ± 0.11	209.1 ± 1.5	1.67 ± 0.24	232.9 ± 3.7	1.12 ± 0.02
50 mM imidazole, 100 mM $MgCl_2$	0.410–1.578	10	53.07 ± 0.05	194.3 ± 4.3	2.21 ± 0.20	223.4 ± 4.3	1.12 ± 0.03
50 mM imidazole, 300 mM KCl	0.395–1.538	6	56.37 ± 0.08	209.3 ± 1.9 204.2 ± 2.6[a]	1.24 ± 0.13 1.62 ± 0.14[a]	234.2 ± 1.9	1.12 ± 0.02

[a] Mean of values ± SE.

Table IV: Effect of pH on Thermal Unfolding of PGK

buffer	pH	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$
acetate	5.40	53.5	165.0	1.69	165.3	1.00
PIPES	5.88	53.9	171.2	1.55	166.1	0.97
PIPES	6.50	55.8	223.0	1.85	159.5	0.72
PIPES	7.04	55.7	214.6	1.27	158.0	0.74
PIPES	7.46	55.2	213.7	1.32	152.5	0.71
PIPES	7.96	54.7	210.4	0.93	152.7	0.73
borate	8.50	54.5	199.7	0.77	205.8	1.03
borate	9.00	54.4	174.8	0.95	184.1	1.05

it is lowered by as much as 3 °C. The change in heat capacity (column 6) is also approximately constant. The values of the van't Hoff enthalpy listed in column 7 were calculated according to

$$\Delta H_{vH} = ART_{1/2}^2 C_{ex,1/2}/\Delta H_{cal} \quad (1)$$

where the constant A has the value 4.00 for a simple two-state process not involving association or dissociation, $T_{1/2} = t_{1/2} + 273.15$, $C_{ex,1/2}$ is the excess apparent heat capacity at $t_{1/2}$, and ΔH_{cal} is the calorimetric enthalpy. Strictly speaking, eq 1 can only be applied to a reversible process, but we have given empirical evidence in earlier papers (Edge et al., 1985; Manly et al., 1985) that at least some apparently irreversible protein denaturations accurately follow predictions based on equilibrium thermodynamics. It is seen that the ratio $\Delta H_{vH}/\Delta H_{cal}$ (column 8) is somewhat less than unity except in the presence of 100 mM $MgCl_2$ or 300 mM KCl. A value of this ratio of less than unity indicates a significant population of one or more intermediate states between the native and denatured states, whereas a ratio greater than unity indicates intermolecular cooperation, which may require some degree of molecular association. A possible interpretation of these data is that a low ionic strength or low $MgCl_2$ concentration the denaturation involves the unfolding of two more or less independent domains (however, see the discussion below based on curve fitting), while at high ionic strength or high $MgCl_2$ concentration, interaction between the domains is increased sufficiently to cause a more nearly two-state denaturation, apparently with increased intermolecular association.

The thermal denaturation of yeast hexokinase at low ionic strength (Takahashi et al., 1981) gave a much clearer indication of the unfolding of two lobes than did that of PGK. Presumably the two domains of PGK interact more strongly than do those of hexokinase.

Figure 1 shows a trace of a typical DSC curve for which $\Delta H_{vH}/\Delta H_{cal} = 0.87$. This curve shows a distinct asymmetry, being somewhat more gradual on the low-temperature side than on the high-temperature side. Since such asymmetry could be due to intermediate steps in the unfolding and since the protein appears to be composed of two lobes, an attempt was made to resolve the curve into the sum of either two sequential two-state steps or two independent two-state steps by a procedure previously outlined (Edge et al., 1985). Neither model led to a standard deviation of calculated points from the observed points which was significantly lower than that obtained for a simple single two-state process. Another possible source for the asymmetry seen in Figure 1 is dissociation of an oligomer (Takahashi & Sturtevant, 1981), and indeed, fitting of the data to a single two-state step involving dissociation of a dimer at unfolding gave a substantially lower standard deviation. However, as seen in Table III, t for the denaturation is independent of protein concentration so that this model is not satisfactory. Despite the good reproducibility of the data and the high signal-to-noise ratio, we have been unable to develop a reasonable model that accounts for the data with a satisfactorily low standard deviation. The remaining possibility is that the high-temperature side of the curve is sharpened by an exothermic, and necessarily rate-limited, aggregation of the denatured protein. This possibility has not been explored since it would involve at least four additional parameters and could not lead to anything approaching a unique solution.

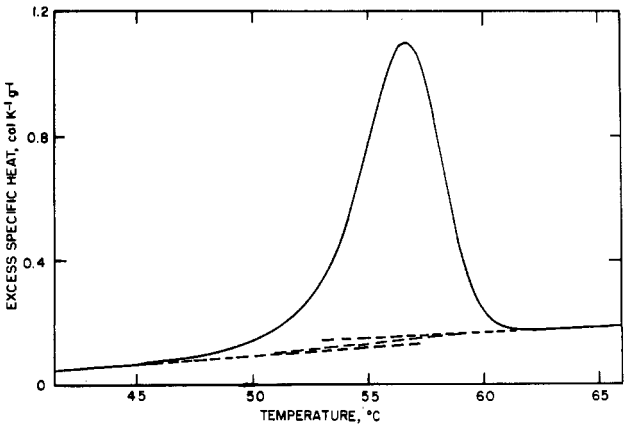

FIGURE 1: Tracing of DSC curve observed with phosphoglycerate kinase in 50 mM PIPES buffer at pH 7.0: protein concentration, 1.239 mg mL^{-1}; scan rate, 1 K min^{-1}. The electrical noise level at the sensitivity employed corresponded to less than 0.01 cal K^{-1} g^{-1}. The initial, final, and calculated base lines are shown as dashed lines.

Effect of pH on the Thermal Unfolding of PGK. The effect of pH was studied over the range pH 5.40–9.00, with the results given in Table IV. Two experiments were performed at each pH. The lack of significant change in $t_{1/2}$ with pH at neutral pH supports the conclusion reached above that the protein undergoes no change in protonation during denaturation at pH 7.0. The increase of ΔH_{cal} with increasing pH at low pH and the decrease at high pH are both too large to be purely ΔC_p^d effects, in contrast to what has been observed with several other proteins (Privalov & Khechinashvili, 1974). These changes must be ascribed to unknown changes in the structure of either the native or the denatured protein, or both, as must also the large decrease in ΔC_p^d with increasing pH. The variation of $\Delta H_{vH}/\Delta H_{cal}$ indicates that at both low and high pH the denaturation is quite closely two-state in character.

Krietsch and Bücher (1970) examined the effect of pH on the activity of muscle PGK and found a dependence similar to that found here for $t_{1/2}$ and ΔH_{cal}.

Thermal Unfolding of PGK in the Presence of Nucleoside Phosphates. The effects of MgADP and MgATP on the thermal denaturation of PGK were studied at protein concentrations of 0.71–1.12 mg mL^{-1} in 50 mM PIPES buffer containing 0.1 M $MgCl_2$ at pH 7.0, with the results summarized in Table V. It is evident that $t_{1/2}$, ΔH_{cal}, and ΔC_p^d all suddenly decrease at nucleotide concentrations of 18 mM and above, presumably because of effects arising from occupancy of additional binding sites on the native and/or the denatured protein(s). At lower ligand concentrations the value of $t_{1/2}$ increases with increasing total ligand concentration $(L)_0$ in the manner predicted by the van't Hoff equation, as expected if

Table V: Thermodynamics of Thermal Unfolding of PGK in the Presence of MgADP and MgATP at pH 7.0

concn of ligand (mM)	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$
			MgADP[a]		
1.0	56.30	228.6	1.78	265.3	1.16
2.0	57.00	219.6	1.83	267.1	1.22
3.0	57.35	230.0	1.87	278.9	1.21
5.0	57.75	226.6	2.02	264.9	1.17
7.5	58.00	231.5	1.87	276.5	1.19
10.0	58.20	227.7	1.72	268.6	1.18
		227.3 ± 1.7[b]	1.85 ± 0.04[b]	270.4 ± 2.2[b]	1.19 ± 0.01[b]
18.0	54.45	185.8	0.81	180.6	0.97
22.0	47.93	117.8	0.00	116.1	1.00
25.0	44.33	46.0	−1.17	128.7	2.80
			MgATP		
1.0	54.90	215.1	1.33	244.1	1.13
2.0	54.98	210.1	1.25	225.5	1.07
3.0	55.35	213.6	1.11	250.1	1.17
5.0	55.65	206.3	1.34	235.8	1.14
7.5	56.05	215.8	1.25	252.2	1.17
10.0	56.28	208.3	1.14	243.9	1.17
14.0	56.50	215.3	1.10	246.3	1.14
		212.1 ± 1.4[b]	1.22 ± 0.04[b]	242.6 ± 3.5[b]	1.14 ± 0.02[b]
18.0	56.23	204.8	1.21	235.2	1.15
22.0	55.20	196.2	0.88	214.3	1.09
25.0	52.73	166.5	0.44	174.8	1.05

[a] Additional $MgCl_2$ at the same concentration as K-ADP was added to the solutions of K-ADP. [b] Mean of values ± SE.

Table VI: Enthalpy of Thermal Unfolding of PGK in the Presence of Various Ligands

ligand	ΔH_{vH}[a] (kcal mol^{-1})	ΔH_{vH}[b] (kcal mol^{-1})	ΔH_{cal}[c] (kcal mol^{-1})
MgADP	270.4 ± 2.2	263.6	227.3 ± 1.7
MgATP	242.6 ± 3.5	313.5	212.1 ± 1.4
3-PG	247.6 ± 5.1	236.4	227.9 ± 2.3

[a] Mean of values calculated by eq 1. [b] Values obtained from the slopes of the van't Hoff plots in Figure 2. [c] Mean of the values obtained by integration of the observed DSC curves.

Table VII: Enthalpies of Dissociation of Ligands from PGK at 56.26 °C in PIPES Buffer at pH 7.0

ligand	ΔH_{dissoc} (kcal mol^{-1})	$\Delta C_{p,dissoc}$[a] (cal K^{-1} mol^{-1})
MgADP	17.8	270
MgATP	5.5	−66
3-PG	21.2	800

[a] Calculated from values for ΔH_b at 25 °C (Table I).

the ligand is dissociated on denaturation (Fukada et al., 1983). The appropriate van't Hoff plots are A (MgADP) and B (MgATP) in Figure 2. The van't Hoff enthalpies derived from these plots ($\Delta H_{vH} = -RS$ where S is the slope of the plot) are listed in column 3 of Table VI, where they are compared with the means of the values calculated by eq 1 and the mean calorimetric enthalpies. There is rough agreement between the two estimates of ΔH_{vH}, both of which are considerably larger than ΔH_{cal}. The data indicate that one ligand molecule is bound per protein molecule in the concentration range up to 10–14 mM and that the protein is somewhat associated in the presence of the nucleotides. Vas and Batke (1984) concluded on the basis of kinetic measurements that only one molecule of MgATP or of 3-PG binds to a molecule of PGK.

As indicated in previous papers (Fukada et al., 1983; Manly et al., 1985), the enthalpy of dissociation of a ligand that dissociates completely on denaturation is equal to the difference between the enthalpies of denaturation of the protein in the presence and absence of the ligand, provided the protein is essentially saturated by the ligand before denaturation. The enthalpies being compared must, of course, be calculated to the same temperature by use of the appropriate values of ΔC_P^d; this requirement poses some difficulties in cases where the temperature of denaturation depends on either protein or ligand concentration. Using mean values for $t_{1/2}$, ΔH_{cal}, and ΔC_P^d in the presence of ligands leads to the values for the enthalpies of ligand dissociation given in Table VII. Included in the table are values for $\Delta C_{P,dissoc}^d$ based on the values for ΔH_b at 25 °C given in Table I. These values differ markedly from those listed in Table II and are probably more reliable because of the wider temperature range involved.

Thermal Unfolding of PGK in the Presence of 3-PG. The results of measurements made on the denaturation of PGK in the presence of 3-PG in 50 mM PIPES buffer containing 0.1 M $MgCl_2$ at pH 7.0 are listed in Table VIII. As was observed with added MgADP or MgATP, at high 3-PG concentration both $t_{1/2}$ and ΔH_{cal} show decreases. In contrast to the situation with the nucleotides, 3-PG causes a sharp increase in ΔC_P^d at high concentration. At lower concentrations

Table VIII: Thermodynamics of Thermal Unfolding of PGK in the Presence of 3-PG at pH 7.0

concn of 3-PG (mM)	$t_{1/2}$ (°C)	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{cal} (kcal mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$
2.0	54.83	1.72	221.6	232.2	1.05
5.0	55.30	2.00	225.0	239.5	1.07
10.0	56.00	1.95	227.7	250.0	1.10
18.0	56.65	1.87	230.4	258.8	1.12
25.0	57.03	1.95	234.9	257.2	1.10
		1.90 ± 0.05[a]	227.9 ± 2.3[a]	247.6 ± 5.1[a]	1.09 ± 0.01[a]
50.0	56.65	2.16	217.2	242.0	1.16
75.0	55.98	2.45	186.5	219.7	1.18
100.0	55.63	2.75	179.0	226.2	1.27

[a] Mean of values ± SE.

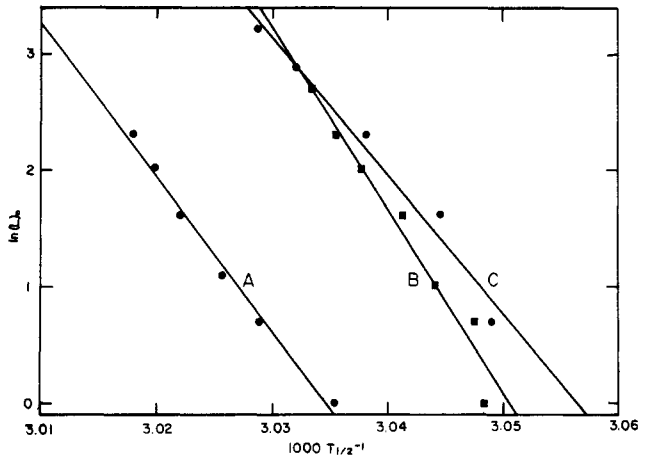

FIGURE 2: van't Hoff plots derived from DSC experiments on phosphoglycerate kinase with added MgADP (plot A), MgATP (plot B), and 3-phosphoglycerate (plot C). The logarithm of the total ligand concentration, $(L)_0$, is plotted against $1000/T_{1/2}$, where $T_{1/2}$ is the absolute temperature at which the denaturation is half completed. The lines were determined by least squaring the data.

the binding of 3-PG follows the van't Hoff equation, as shown by plot C in Figure 2. As seen in Table VI, in the case of 3-PG the two estimates of ΔH_{vH} and that of ΔH_{cal} are all in reasonably good agreement, so we can again conclude that only one ligand molecule is bound per protein molecule up to ligand concentrations of 25 mM. As outlined in the preceding section, it can be estimated that the enthalpy and heat capacity changes in the dissociation of 3-PG from PGK are 21.2 kcal mol^{-1} and 0.80 kcal K^{-1} mol^{-1}, respectively (Table VII).

The results of curve fitting applied to DSC curves obtained in the presence of ligands were as inconclusive as those obtained for PGK alone, with about the same standard deviation, 4–6% of the maximal value of C_{ex}, with a two-step model as with a one-step model.

In conclusion we may cite the following as two important conclusions from the present work:

(1) The thermal denaturation of PGK over the pH range 5.4–9.0 is irreversible as judged by the complete lack of a denaturational endotherm on rescanning solutions that had previously been heated to a high enough temperature to cause complete denaturation. Nevertheless, as we have found previously (Edge et al., 1985; Manly et al., 1985), the apparently irreversible denaturation of the protein in the presence of ligands proceeds according to predictions based on equilibrium thermodynamics.

(2) We have studied by means of DSC three proteins that according to X-ray crystallographic data have bilobate structures, namely, yeast hexokinase (Takhashi et al., 1981), the arabinose binding protein of *Escherichia coli* (Fukada et al., 1983), and phosphoglycerate kinase. Of these three only the first gave definite DSC indication of the more or less independent unfolding of the two lobes, and this only in the absence of substrate. We may conclude that the interactions between the lobes in the other two cases are strong enough to cause approximately simple two-state unfolding of the molecule as a whole.

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