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A Differential Scanning Calorimetric Study of the Binding of Sulfate Ion and of Cibacron Blue F3GA to Yeast Phosphoglycerate Kinase[†]

Cui Qing Hu and Julian M. Sturtevant*

Departments of Chemistry and of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

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ABSTRACT: In continuation of earlier work [Hu, C. Q., & Sturtevant, J. M. (1987) *Biochemistry 26*, 178–182], differential scanning calorimetry has been employed in a study of the effects on the thermal denaturation of yeast phosphoglycerate kinase of two inhibitors of the enzyme, sulfate ion and the dye Cibacron blue F3GA. Sulfate ion, as is usual with ligands that dissociate during unfolding of the host protein, raises $t_{1/2}$, the temperature of half-completion of the denaturation, has only a modest effect, stemming from the enthalpy of dissociation of the ligand, on the enthalpy of denaturation, and has little or no effect on the heat capacity change resulting from denaturation. In sharp contrast, Cibacron blue F3GA lowers $t_{1/2}$ and drastically decreases both the enthalpy and heat capacity changes due to denaturation. The DSC results with sulfate ion are consistent with previous kinetic data [Scopes, R. K. (1978) *Eur. J. Biochem. 91*, 119–129; Khamis, M. H., & Larsson-Raznikiewicz, M. (1981) *Biochim. Biophys. Acta 657*, 190–194], which indicate two binding sites for sulfate ion at one of which the ligand acts as a competitive inhibitor. The results with Cibacron blue F3GA indicate that the dye induces a major destabilizing structural change in the enzyme in addition to rendering it enzymically inactive.

In earlier work (Hu & Sturtevant, 1978) the thermodynamics of the binding of MgADP, MgATP, and 3-phosphoglycerate (3-pG)[1] to yeast phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) (PGK) and of the thermal unfolding of the enzyme in the absence and presence of these ligands was studied. In this paper we extend our differential scanning calorimetric (DSC) study to include the effects on the thermal denaturation of PGK of two inhibitors of the enzyme, sulfate ion and Cibacron blue F3GA (CB3GA), in the absence and the presence of 3-pG and MgATP. Sulfate ion has been reported to play a dual role, as both an activator at low concentration and a competitive inhibitor at high concentration (Scopes, 1978; Khamis & Larsson-Raznikiewicz, 1981; Mas et al., 1988), with two dissimilar binding sites on the protein. CB3GA also appears to behave as a competitive inhibitor with either one (Thompson & Stellwagen, 1976) or two (Beissner & Rudolph, 1979) binding sites on the protein.

MATERIALS AND METHODS

Yeast phosphoglycerate kinase was purchased from Sigma Chemical Co. (lot no. 64F-8130) as an ammonium sulfate precipitate. Enzyme solutions were prepared by exhaustive dialysis against a buffer containing 50 mM PIPES and 0.1 mM DTE at pH 7.00. Protein concentrations were determined spectrophotometrically by taking the absorbance of a 1 mg mL^{-1} solution at 280 nm to be 0.57[2] (Blake et al., 1972). Protein solutions were used within 2 days of preparation.

The magnesium salt of ATP and the disodium salt of D-(−)-3-phosphoglyceric acid were products of Sigma Chemical Co. CB3GA was purchased from Fluka. All other chemicals were of reagent grade. Doubly deionized water was used throughout.

The DSC experiments were performed with a DASM-4 instrument (Privalov, 1980) purchased from Mashpriborintorg, Moscow, USSR. All scans were run at 1 K min^{-1}. Base lines were drawn as described by Takahashi and Sturtevant (1981), and enthalpies were evaluated by planimeter integration. van't Hoff enthalpies were calculated by the equation

$$\Delta H_{\mathrm{vH}} = 4RT^2_{1/2}C_{\mathrm{ex},1/2}/\Delta H_{\mathrm{cal}}$$

where $T_{1/2} = 273.15 + t_{1/2}$, $t_{1/2}$ is the temperature in degrees Celsius of half-completion of the denaturation, $C_{\mathrm{ex},1/2}$ is the

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[1] Abbreviations: CB3GA, Cibacron blue F3GA; DSC, differential scanning calorimetry; DTE, dithioerythritol; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); 3-pG, 3-phosphoglycerate; PGK, yeast phosphoglycerate kinase.

[2] This value is listed by Blake et al. (1972) and by Pickover at al. (1979) and was used by us in our earlier paper (Hu & Sturtevant, 1987), whereas Krietsch and Bucher (1970) and Thompson and Stellwagen (1976) gave the value 0.49. All of our values for ΔH_{cal} and ΔC_p^{d} can be brought into line with any future version of the absorbance by simple multiplication by the appropriate factor. The values for ΔH_{vH} would remain unchanged.

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Table I: Thermodynamics of the Thermal Unfolding of PGK in the Presence of Sulfate Ion[a]

SO_4^{2-} concn (mM)	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$
1.0	56.15	226.3	2.08	184.1	0.81
2.0	56.45	228.7	2.14	203.1	0.89
3.0	56.65	226.3	1.96	217.4	0.96
5.0	57.30	230.0	2.22	230.7	1.00
7.0	57.55	233.4	2.14	242.8	1.04
10.0	58.05	229.7	2.26	250.6	1.09
15.0	58.55	237.6	2.02	264.8	1.11
18.0	58.90	242.1	2.14	262.3	1.08
25.0	59.25	245.2	2.14	280.4	1.14
40.0	60.00	240.3	2.57	284.4	1.18
50.0	60.25	243.3	2.14	275.4	1.13
60.0	60.75	246.2	2.45	291.0	1.18
75.0	61.10	237.5	1.96	291.7	1.23
90.0	61.75	247.1	2.14	302.8	1.23
100.0	62.00	243.0	1.84	296.8	1.22
120.0	62.70	250.2	2.45	301.9	1.21
mean (60 °C) ± SE		239.6 ± 1.2	2.15 ± 0.05		

[a] Protein concentration 1.1–1.8 mg mL^{-1}.

apparent excess heat capacity relative to the base line at $t_{1/2}$, and ΔH_{cal} is the molar enthalpy change at $t_{1/2}$.

RESULTS AND DISCUSSION

A DSC trace for PGK in PIPES buffer at pH 7.0, which is similar to those observed in this work, is shown in our earlier paper (Hu & Sturtevant, 1987). No visible noise was present, and straight base lines were observed before and after the transition. It was ascertained by experiments at concentrations of PGK from 0.6 to 5.4 mg mL^{-1} that there is no significant variation with protein concentration in any of the quantities derived from the DSC data. In particular, the invariance of $t_{1/2}$ indicates that there is no change in the degree of oligomerization of PGK due to denaturation. Presumably both the native and denatured forms of the protein are essentially monomeric.

Thermal Unfolding of PGK in the Presence of Sulfate Ions. The concentration of sulfate ion was varied from 1 to 120 mM. The denaturation was operationally irreversible as was the case with PGK alone (Hu & Sturtevant, 1987) but was nevertheless found to follow quantitatively predictions based on equilibrium thermodynamics. A similar situation has previously been observed with the core protein of *lac* repressor (Manly et al., 1985) and with the subunits of aspartate transcarbamoylase (Edge et al., 1985).

The results are summarized in Table I. The temperature, $t_{1/2}$, increases with increasing sulfate concentration, as is to be expected for a reversible reaction accompanied by dissociation of the ligand. The increase in ΔH_{cal} with increasing sulfate concentration is in part due to the increase in $t_{1/2}$ coupled with the large value of ΔC_p^d, as is shown by the fact that conversion of the values to 60 °C gives the mean value 239.6 kcal mol^{-1} with a standard error of 1.2 kcal mol^{-1}. The fact that this value is significantly higher than the value 204.2 ± 2.6 kcal mol^{-1} reported earlier (Hu & Sturtevant, 1987) is due to the enthalpy of dissociation of sulfate from the native protein (Edge et al., 1988). The permanent changes in apparent heat capacity listed in column 4 of Table I are independent of sulfate concentration within experimental uncertainty and have the mean value 2.15 ± 0.05 kcal K^{-1} mol^{-1}. This value is not unusually large for a protein of molecular weight 47 000. The ratio $\Delta H_{vH}/\Delta H_{cal}$ listed in column 6 is seen to increase with increasing sulfate concentration. Judging by our earlier results (Hu & Sturtevant, 1987), this increase may be due to increasing ionic strength as the concentration of sulfate is increased. Interpreted literally (Sturtevant, 1987), the values for the ratio indicate a small population of intermediate states at low ionic strength and a small extent of oligomerization of either the native or denatured protein, or both, at high ionic strength.

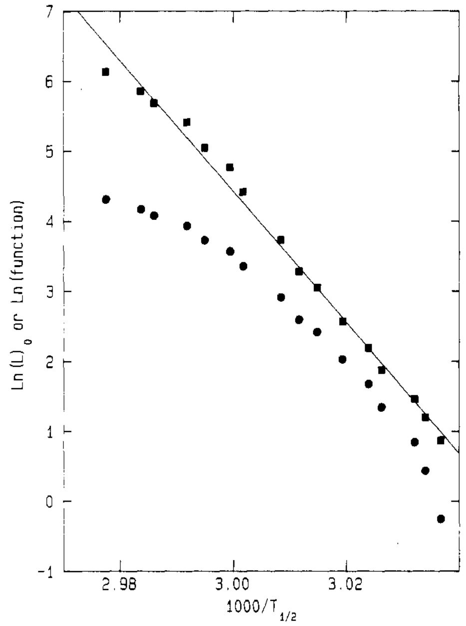

FIGURE 1: van't Hoff plots of the DSC data obtained in the presence of SO_4^{2-}. Filled circles: plot of ln $[L]_o$ vs $1000/T_{1/2}$, where $[L]_o$ is the total sulfate concentration and $T_{1/2}$ is the absolute temperature of half-completion of the denaturation. Filled squares: plot of ln (function) vs $1000/T_{1/2}$, where the function is given in eq A5 in the Appendix. The line is the least-squared line through the squares.

A van't Hoff plot of the logarithm of SO_4^{2-} concentration vs $1/T_{1/2}$ shows distinct curvature (filled circles in Figure 1), indicating the existence of a more complex equilibrium than simple single-site binding. This is consistent with the conclusion based on kinetic data (Khamis & Larsson-Raznikiewicz, 1981) that PGK has two unequal binding sites for anions. The data in Table I can be fitted to the simplest two-site model:

$$A + L \underset{\Delta h_1}{\overset{k_1}{\rightleftharpoons}} Al \qquad AL \underset{\Delta H_1}{\overset{K_1}{\rightleftharpoons}} B + L \tag{1}$$

$$AL + L \underset{\Delta h_2}{\overset{k_2}{\rightleftharpoons}} AL_2 \qquad AL_2 \underset{\Delta H_2}{\overset{K_2}{\rightleftharpoons}} B + 2L \tag{2}$$

where A and B are respectively the native and denatured forms of the protein and L is the ligand. The fitting procedure, which includes the effect of ΔC_p, the denaturational change in heat

Table II: Values at 56 °C for the Parameters for Fitting the Data of Table I to the Model of Equations 1 and 2

no.	k_1 (mM^{-1})	Δh_1 (kcal mol^{-1})	k_2 (mM^{-1})	Δh_2 (kcal mol^{-1})	ΔH_{vH}[a] (kcal mol^{-1})	r^2[b]	ΔH_0[c] (±SE) (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_0$
1	0	0	0	0	147	0.948	231.0 ± 1.2	0.64
2	0.50	-20	0.10	-5	208	0.990	214.0 ± 1.0	0.97
3	1.00	-20	0.10	-5	217	0.987	213.0 ± 1.0	1.02
4	0.50	-10	0.10	-5	208	0.990	222.8 ± 1.0	0.93
5	0.50	-20	0.05	-5	186	0.992	214.8 ± 1.0	0.87
6	0.50	-20	0.10	-10	203	0.989	211.1 ± 1.1	0.96

[a] The product of −1.987 times the slope of a modified (eq A5) van't Hoff plot. [b] r^2 is the coefficient of determination showing the adequacy of fit in least squaring of the van't Hoff plots according to eq A5. [c] The enthalpy of denaturation after deducting the calculated enthalpies of dissociation of sulfate ions.

Table III: Thermodynamics of the Thermal Denaturation of PGK in the Presence of SO_4^{2-} and of MgATP or 3-pG[a]

SO_4^{2-} concn (mM)	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$
10 mM MgATP					
2.0	60.20	238.7	2.18	215.7	0.90
4.0	60.35	236.9	2.18	220.4	0.93
8.0	60.55	238.5	1.94	230.7	0.97
10.0	60.70	245.0	2.18	236.1	0.96
15.0	60.90	242.1	2.33	247.7	1.02
20.0	61.00	240.7	1.94	245.0	1.02
40.0	61.40	248.5	2.33	264.4	1.06
60.0	61.90	241.1	2.33	279.1	1.16
80.0	62.30	241.1	2.33	288.4	1.20
mean (60 °C) ± SE		239.1 ± 1.2	2.19 ± 0.06		
10 mM 3-pG					
2.0	60.35	246.0	1.70	285.3	1.16
4.0	60.35	240.1	1.90	291.4	1.21
8.0	60.45	240.1	2.04	295.3	1.22
10.0	60.50	244.4	2.38	292.7	1.20
15.0	60.50	244.3	1.90	296.5	1.21
20.0	60.60	251.9	2.38	286.5	1.14
40.0	60.80	250.3	2.38	300.8	1.20
60.0	61.15	250.0	2.38	295.8	1.18
80.0	61.55	250.7	2.72	301.7	1.20
mean (60 °C) ± SE		244.9 ± 1.5	2.20 ± 0.12	292.5 ± 1.9	1.19 ± 0.01

[a] Protein concentration 1.40–1.60 mg mL^{-1}.

capacity, is outlined in the Appendix. As is illustrated in Table II, essentially equally good fits can be obtained with a range of values for the parameters involved in the model. The choice of values depends largely on whether one wishes to obtain near equivalence of ΔH_{vH} and ΔH_0, the value of ΔH_{cal} corrected for ligand dissociation (rows 2 and 3 in the table), or a value for the ratio $\Delta H_{vH}/\Delta H_{cal}$ equal to that observed with PGK alone (row 5). The latter selection gives the van't Hoff plot represented by the filled squares and least-squared line in Figure 1. The parameters in rows 2, 3, 5, and 6 all give values for ΔH_0 in good agreement with the value of ΔH_{cal} in the absence of ligands (Hu & Sturtevant, 1987). The values in row 5 extrapolated to 25 °C by means of the Gibbs–Helmholtz equation, assuming $\Delta C_p = 0$ for the first equilibrium in eq 1 and 2, give 0.083 mM and 9.03 mM for k_1 and k_2, respectively, in sharp disagreement with the values 1.6 mM and 150 mM reported by Scopes (1978), though with roughly the same ratio. This discrepancy can be largely removed by assuming a value of approximately −1.1 kcal K^{-1} mol^{-1} for ΔC_p in the first two steps in eq 1 and 2. Although this is a very large value, values of this magnitude have been observed in other protein–ligand systems (Sturtevant, 1977). In view of the fact that this value for ΔC_p is not at all well established, we have not included it in the fitting procedure outlined in the Appendix.

Effect of Sulfate Ion on the Denaturation of PGK in the Presence of a Fixed Concentration of MgATP or 3-pG. The data obtained for SO_4^{2-} in the presence of MgATP or 3-pG are listed in Table III. When SO_4^{2-} is added to PGK containing either 10 mM MgATP or 10 mM 3-pG, it has very little effect on $t_{1/2}$ until the concentration of SO_4^{2-} reaches 20 mM or so. As seen in the van't Hoff plots in Figure 2, eventually, as the SO_4^{2-} displaces the other ligand, it starts to exert the effect on $t_{1/2}$ seen in the absence of a second ligand. The final slope reached at 80 mM SO_4^{2-} concentration is approximately the same as that found at 10 mM SO_4^{2-} (solid line in the figure) in the absence of a second ligand, and it is still decreasing as shown in Figure 2. This behavior is consistent with SO_4^{2-} competing with MgATP or 3-pG for the weaker of its two binding sites (Khamis & Larsson-Raznikiewicz, 1981).

The values for ΔH_{cal} listed in Table III, after correction to fixed temperature using the observed values for ΔC_p^d, are constant within experimental uncertainty, as are also the values for ΔC_p^d. The ratio $\Delta H_{vH}/\Delta H_{cal}$ increases in the case of added MgATP in much the same way as it does with SO_4^{2-} alone but is quite constant when the extra ligand is 3-pG.

Effect of MgATP or 3-pG on the Denaturation of PGK in the Presence of a Fixed Concentration of Sulfate Ion. Tables IV and V list the data with varying MgATP or 3-pG concentrations, respectively, in the presence of 10 or 60 mM SO_4^{2-}. van't Hoff plots of ln $[L]_0$ vs $1000/T_{1/2}$ are quite accurately linear in all four cases. This indicates that the ligand binding in each case is governed by a single equilibrium constant, as was also the case in the absence of SO_4^{2-} (Hu & Sturtevant, 1987). Quantitative interpretation of the apparent van't Hoff enthalpies listed in Tables IV and V, obtained by multiplying the slopes of the plots by −1.987, is difficult since the substrates undoubtedly displace increasing fractions of the bound SO_4^{2-}

Table IV: Thermodynamics of the Thermal Denaturation of PGK in the Presence of MgATP and SO_4^{2-} [a]

MgATP concn (mM)	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$
		10.0 mM SO_4^{2-}			
1.0	58.30	231.5	2.10	236.9	1.02
2.0	58.80	244.1	2.55	230.0	0.94
3.0	59.15	240.9	2.42	227.7	0.95
5.0	59.65	245.6	2.04	230.9	0.94
7.5	60.15	245.5	2.55	235.2	0.96
10.0	60.60	249.8	2.55	240.8	0.96
14.0	60.95	253.0	2.31	243.9	0.96
18.0	61.10	247.7	2.42	247.1	1.00
mean (60 °C) ± SE		245.1 ± 1.9	2.37 ± 0.08	236.9 ± 2.2	0.97 ± 0.01
app ΔH_{vH} = 212 kcal mol^{-1}					
		60.0 mM SO_4^{2-}			
1.0	60.60	239.0	1.84	288.4	1.21
2.0	60.85	241.8	2.20	289.5	1.20
3.0	61.00	246.5	1.84	281.6	1.14
5.0	61.40	246.2	2.20	286.6	1.16
7.5	61.70	248.0	2.20	287.7	1.16
10.0	61.90	255.1	2.20	281.3	1.10
14.0	61.90	246.1	2.20	281.0	1.14
18.0	61.40	240.0	2.20	269.5	1.12
mean (60 °C) ± SE		242.5 ± 1.8	2.11 ± 0.06	280.4 ± 2.8	1.15 ± 0.02
app ΔH_{vH} = 400 kcal mol^{-1}					

[a] Protein concentration 1.43–1.71 mg mL^{-1}.

Table V: Thermodynamics of the Thermal Unfolding of PGK in the Presence of 3-pG and SO_4^{2-} [a]

3-pG concn (mM)	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p^d (kcal K^{-1} mol^{-1})	ΔH_{vH} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$
		10 mM SO_4^{2-}			
2.0	59.30	243.4	2.28	273.4	1.12
4.0	59.80	249.2	2.28	274.6	1.10
6.0	60.10	249.9	2.28	282.4	1.13
10.0	60.60	252.9	2.13	286.6	1.13
14.0	60.90	249.7	2.28	297.5	1.19
18.0	61.10	258.2	2.28	290.7	1.13
20.0	61.20	249.9	2.44	300.5	1.20
24.0	61.35	258.0	2.28	299.3	1.16
mean (60 °C) ± SE		250.2 ± 1.5	2.28 ± 0.03	287.0 ± 3.8	1.15 ± 0.01
app ΔH_{vH} = 262 kcal mol^{-1}					
		60 mM SO_4^{2-}			
2.0	60.60	248.3	1.97	280.3	1.13
4.0	60.85	250.9	1.97	287.2	1.14
6.0	61.05	249.4	1.97	291.6	1.17
10.0	61.35	251.0	2.10	292.6	1.17
14.0	61.50	252.5	2.10	293.4	1.16
18.0	61.65	251.6	1.64	299.4	1.19
20.0	61.75	251.6	1.97	299.6	1.19
24.0	61.90	255.8	1.97	294.9	1.15
mean (60 °C) ± SE		248.8 ± 0.6	1.96 ± 0.06	289.8 ± 2.3	1.16 ± 0.01
app ΔH_{vH} = 421 kcal mol^{-1}					

[a] Protein concentration 1.43–1.66 mg mL^{-1}.

with increasing substrate concentration. Qualitatively, it may be noted that at higher SO_4^{2-} concentration the added substrate has less effect on $t_{1/2}$ than it has at lower SO_4^{2-} concentration because of the increased competition by SO_4^{2-}.

Thermal Unfolding of PGK in the Presence of CB3GA. The binding of CB3GA produces very unusual effects on the thermal denaturation of PGK. (a) The temperature of denaturation is lowered. The easiest interpretation of this phenomenon is that the dye is more tightly bound to the denatured than to the native form of the protein. (b) The enthalpy change on unfolding is markedly lowered from about 210 kcal mol^{-1} in the presence of 0.01 μM CB3GA to 103 kcal mol^{-1} in the presence of 300 μM CB3GA. If the interpretation of the effect on $t_{1/2}$ given above is correct, then it would appear that the binding of CB3GA to denatured PGK is accompanied by a large decrease in enthalpy. On the other hand, if CB3GA causes a structural change, it may be to a form with very much lower enthalpy of unfolding. Since the free energy of unfolding is not much changed ($t_{1/2}$ not greatly affected), there would have to be a large compensating decrease in the entropy of unfolding. (c) The denaturational change in heat capacity goes from 1.00 kcal K^{-1} mol^{-1} in the presence of 0.01 μM CB3GA to −3.3 kcal K^{-1} mol^{-1} at 300 μM CB3GA. This dramatic change is suggestive of an important structural change that results in a decrease instead of an increase in the exposure of hydrophobic groups during denaturation, to an increase in the exposure of charged groups, or to a decrease in the number of soft internal vibrational modes (Sturtevant, 1977). It should be noted that the large negative values for ΔC_p^d observed at high CB3GA concentration mean that the observed enthalpy

Table VI: Constants in the Equation $Y = A + Bc^a$

property	system	no. of expts	A	B	SD	r^2
$t_{1/2}$	PGK alone	9	55.51	−13.76	±0.12	0.99
	PGK + 10 mM MgATP	8	60.19	−33.79	±0.47	0.98
	PGK + 10 mM 3-pG	17	60.63	−31.30	±0.31	0.99
	PGK + 5 mM SO_4^{2-}	5	57.28	−25.30	±0.06	1.00
	PGK + 50 mM SO_4^{2-}	5	60.49	−35.60	±0.24	0.99
ΔH_{cal}	PGK alone	9	209.5	−381.2	±7.5	0.97
	PGK + 10 mM MgATP	8	223.5	−359.6	±7.7	0.96
	PGK + 10 mM 3-pG	17	240.6	−458.1	+6.9	0.98
	PGK + 5 mM SO_4^{2-}	5	227.1	−587.0	±10.0	0.96
	PGK + 50 mM SO_4^{2-}	5	238.4	−611.8	±11.7	0.94
ΔC_p^{d}	PGK alone	9	0.74	−16.57	±0.75	0.86
	PGK + 10 mM MgATP	8	0.21	−10.87	±0.94	0.61
	PGK + 10 mM 3-pG	17	1.13	−13.83	±0.71	0.79
	PGK + 5 mM SO_4^{2-}	5	0.24	−21.32	±1.56	0.54
	PGK + 50 mM SO_4^{2-}	5	0.79	−17.74	±1.07	0.63
$\Delta H_{\mathrm{vH}}/\Delta H_{\mathrm{cal}}{}^b$	PGK alone	9	0.69	2.329	±0.11	0.86

[a] Y is $t_{1/2}$, ΔH_{cal}, or ΔC_p^{d}, and c is the concentration of CB3GA in millimolar. [b] The variation of this ratio was not linear in the presence of the other ligands. Protein concentration 1.2–1.8 mg $\mathrm{mL^{-1}}$.

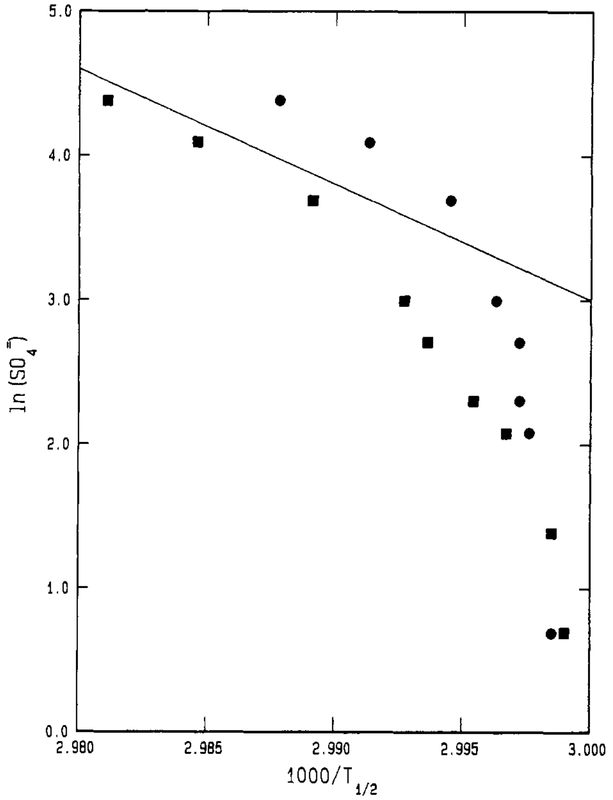

FIGURE 2: van't Hoff plots of ln [SO_4^{2-}] against $1000/T_{1/2}$. Filled circles: data obtained in the presence of 10.0 mM 3-pG. Filled squares: data obtained in the presence of 10.0 mM MgATP. The line shows the slope of the plot of ln $[\mathrm{L}]_0$ vs $1000/T_{1/2}$ in Figure 1 at [SO_4^{2-}] = 10 mM.

decreases would be somewhat larger if calculated to a fixed temperature. (d) The ratio $\Delta H_{\mathrm{vH}}/\Delta H_{\mathrm{cal}}$ changes from 0.78 observed in the presence of 0.01 μM CB3GA to 1.42 in the presence of 300 μM CB3GA.

All these effects are linearly dependent on the concentration of CB3GA. Table VI lists the constants in the equation $Y = A + Bc$, where Y is $t_{1/2}$, ΔH_{cal}, ΔC_p^{d}, or $\Delta H_{\mathrm{vH}}/\Delta H_{\mathrm{cal}}$ and c is the millimolar concentration of CB3GA. The linearity is quite precise for $t_{1/2}$ and ΔH_{cal}, less so for ΔC_p^{d} and $\Delta H_{\mathrm{vH}}/\Delta H_{\mathrm{cal}}$.

The binding of CB3GA to PGK has been studied by spectrophotometry, equilibrium dialysis, kinetic measurements, and column chromatography (Thompson & Stellwagen, 1976; Beissner & Rudolph, 1979). While there has been some question as to whether the protein has one or two binding sites for this inhibitor, the dissociation constants reported have been of the order of 10 μM. The experiments summarized above were run at a protein concentration of 34.7 μM. If we assume a single binding site with a dissociation constant of 10 μM, it follows that at 300 μM CB3GA the protein should have been 95% saturated. It thus appears that, even allowing for a substantial change in the dissociation constant due to the relatively high temperatures at which the DSC experiments were necessarily conducted, if the DSC effects were due to binding at the site(s) involved in the earlier measurements on this system, then marked deviations from the linearity shown in Table VI should have been observed within the concentration range employed in our work. We therefore conclude that relatively weak binding at a site or sites in addition to the inhibitory site(s) is responsible for the large effects we have observed.

Effects of CB3GA on the Thermal Denaturation of PGK in the Presence of MgATP, 3-pG, or SO_4^{2-}. The effects of CB3GA observed in the presence of other ligands are similar to those discussed above, including an approximately linear dependence on dye concentration (Table VI). The slope B for each property is approximately unaffected by the presence of another ligand, indicating, as concluded above, that the effects observed in this study result from binding at site(s) other than the more tightly binding inhibitory site(s).

APPENDIX

Interpretation of the DSC data observed in the presence of SO_4^{2-} in terms of a van't Hoff plot of ln $[\mathrm{L}]_0$, the logarithm of the total ligand concentration, as a function of $1/T_{1/2}$ involves including both the effect of ΔC_p^{d} on ΔH_{vH}, assumed to be the same as its effect on ΔH_{cal}, and the results of binding SO_4^{2-} at two sites with unequal binding constants. If ΔC_p^{d} is independent of temperature, its effect is expressed by replacing ln $[\mathrm{L}]_0$ in the van't Hoff plot by

$$\ln [\mathrm{L}]_0 - \frac{\Delta C_p^{\mathrm{d}}}{R}\left(\frac{T_0}{T_{1/2}} + \ln T_{1/2}\right)$$

where T_0 is an arbitrarily selected reference temperature. The value of ΔH_{vH} at $T = T_0$ is then $-RS$, where S is the slope of the modified van't Hoff plot. In cases where ΔC_p^{d} is large, it is convenient to add to ln $[\mathrm{L}]_0$ the integer value of the mean value of

$$\frac{\Delta C_p^{\mathrm{d}}}{R}\left(\frac{T_{\mathrm{o}}}{T_{1/2}} + \ln T_{1/2}\right)$$

The equilibria involved in ligand binding are given by eq 1 and 2 in the text. All the enthalpies are expressed in calories per mole, and Δh_1 and Δh_2 are assumed to be independent of temperature. If $[\mathrm{L}]_{\mathrm{o}}$ is much greater than the total protein concentration $[\mathrm{A}]_{\mathrm{o}}$, it follows that

$$[\mathrm{A}] = \frac{[\mathrm{A}]_{\mathrm{o}}}{D} \qquad [\mathrm{AL}] = \frac{k_1[\mathrm{A}]_{\mathrm{o}}[\mathrm{L}]_{\mathrm{o}}}{D}$$
$$[\mathrm{AL}_2] = \frac{k_1k_2[\mathrm{A}]_{\mathrm{o}}[\mathrm{L}]_{\mathrm{o}}^2}{D} \qquad [\mathrm{B}] = \frac{k_1K_1[\mathrm{A}]_{\mathrm{o}}}{D} \tag{A1}$$

where

$$D \equiv 1 + k_1[\mathrm{L}]_{\mathrm{o}} + k_1k_2[\mathrm{L}]_{\mathrm{o}}^2 + k_1K_1 \quad (K_1 = k_2K_2) \tag{A2}$$

At $t = t_{1/2}$

$$[\mathrm{A}] + [\mathrm{AL}] + [\mathrm{AL}_2] = [\mathrm{B}] \tag{A3}$$

so that K_1 at $t_{1/2}$ has the value

$$K_{1,1/2} = \frac{1 + k_{1,1/2}[\mathrm{L}]_{\mathrm{o}} + k_{1,1/2}k_{2,1/2}[\mathrm{L}]_{\mathrm{o}}^2}{k_{1,1/2}} \tag{A4}$$

Therefore, including ΔC_p^{d}, the appropriate van't Hoff plot is

$$\ln\left(\frac{1 + k_{1,1/2}[\mathrm{L}]_{\mathrm{o}} + k_{1,1/2}k_{2,1/2}[\mathrm{L}]_{\mathrm{o}}^2}{k_{1,1/2}}\right) - \frac{\Delta C_p^{\mathrm{d}}}{R}\left(\frac{T_{\mathrm{o}}}{T_{1/2}} + \ln T_{1/2}\right) \text{ vs } \frac{1}{T_{1/2}} \tag{A5}$$

and again ΔH_{vH} at T_{o} is given by $-RS$.

Values for t_{o}, k_1, and k_2 at t_{o} and Δh_1 and Δh_2 are selected subject to the limitations outlined below. Then

$$k_{1,1/2} = k_1 \exp\left[\frac{\Delta h_1}{R}\left(\frac{1}{T_{\mathrm{o}}} - \frac{1}{T_{1/2}}\right)\right] \tag{A6}$$

$$k_{2,1/2} = k_2 \exp\left[\frac{\Delta h_2}{R}\left(\frac{1}{T_{\mathrm{o}}} - \frac{1}{T_{1/2}}\right)\right] \tag{A7}$$

The selection of k_1, k_2, Δh, and Δh_2 is limited in two ways: (1) The slope of the modified van't Hoff plot depends strongly on k_1 and k_2 and to a lesser extent on Δh_1 and Δh_2. Thus the possible values for k_1 and k_2, since we have assumed two-state behavior, should lead to a van't Hoff slope that makes ΔH_{vH} equal to ΔH_{cal} at $t = t_{\mathrm{o}}$. We also wish to maximize the value of r^2, the coefficient of determination, for the van't Hoff plot when least squared. (2) We want the values of $\Delta H_{\mathrm{obsd}} = \Delta H_{\mathrm{cal}} + \Delta H_{\mathrm{dissoc}}$, where $\Delta H_{\mathrm{dissoc}}$ is the actual heat of dissociation of the ligands, to become as nearly constant as possible when corrected to t_{o} with the mean value of ΔC_p^{d} and corrected for the heats of ligand dissociation. We also want the corrected mean value, ΔH_{o}, to be reasonable for PGK observed in the absence of ligands.

Since $[\mathrm{B}] = 0$ at the start of a DSC experiment, D in eq A2 has the value

$$D_{\mathrm{o}} = 1 + k_1[\mathrm{L}]_{\mathrm{o}} + k_1k_2[\mathrm{L}]_{\mathrm{o}}^2 \tag{A8}$$

Since the dissociation of AL involves Δh_1 and that of AL_2 involves $\Delta h_1 + \Delta h_2$, it follows that

$$\Delta H_{\mathrm{dissoc}} = -\frac{k_1\Delta h_1}{D_{\mathrm{o}}}[[\mathrm{L}]_{\mathrm{o}}(1 + k_2[\mathrm{L}]_{\mathrm{o}})] - \frac{k_1k_2}{D_{\mathrm{o}}}\Delta h_2[\mathrm{L}]_{\mathrm{o}}^2 \tag{A9}$$

In this expression k_1 and k_2 should have values appropriate for the temperature at which denaturation starts. An adequate estimate of that temperature is

$$t_{\mathrm{start}} = t_{1/2} - 2\Delta t \tag{A10}$$

where

$$\Delta t = 0.69T^2_{1/2}/\Delta H_{\mathrm{cal}} \tag{A11}$$

so that

$$k_{1,\mathrm{start}} = k_1 \exp\left[\frac{\Delta h_1}{R}\left(\frac{1}{T_{\mathrm{o}}} - \frac{1}{T_{\mathrm{start}}}\right)\right]$$
$$k_{2,\mathrm{start}} = k_2 \exp\left[\frac{\Delta h_2}{R}\left(\frac{1}{T_{\mathrm{o}}} - \frac{1}{T_{\mathrm{start}}}\right)\right] \tag{A12}$$

Registry No. PGK, 9001-83-6; 3-PG, 820-11-1; CB3GA, 12236-82-7; MgATP, 1476-84-2; $\mathrm{SO_4^{2-}}$, 14808-79-8.

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