Yeast 3-Phosphoglycerate Kinase: Sulfate and Substrate Binding, Their Effect on the Conformational State of the Enzyme†

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ABSTRACT: Anions and particularly sulfate are known to interact with 3-phosphoglycerate kinase and to induce an increase of its catalytic efficiency. The present work affords information on the location of the anionic site and on the conformational change produced by the sulfate binding. We have established that sulfate is able, first, to modify the environment of some critical amino acids (cysteine and arginines) located in the N-terminal half of the protein, second, to induce perturbation of aromatic residues as judged by spectrophotometry, and, third, to slightly decrease the magnitude of the Cotton effect at 233 nm. All these modifications are produced by sulfate concentrations required for the activation of the enzyme. The most striking result consists in a large change in the hydrodynamic properties of the protein upon sulfate interaction as determined by analytical ultracentrifugation studies. Thus, sulfate modifies the shape of the molecule, causing it to become more compact. Furthermore, a study of the binary and ternary complexes between yeast 3-phosphoglycerate kinase and its substrates suggests that such a change of the shape of the molecule only occurs in sulfate–enzyme with or without substrates and in ATP (with or without Mg^{2+})–3-phosphoglycerate–enzyme complexes.

Kinetic studies of yeast 3-phosphoglycerate kinase have shown a complex and anomalous catalytic behavior of this monomeric enzyme where substrates act as activators (Scopes, 1978a). For an explanation of these observations, recent studies suggested that substrates could bind not only at the catalytic site but also at other sites of weaker affinity (Scopes, 1978b; Schierbeck & Larsson-Raznikiewicz, 1979; Rao et al., 1978). Furthermore, it was reported that some anions were able to modify the catalytic activity of the enzyme. Steady-state kinetic studies have shown that sulfate in the range 1–10 mM induces an increase of the catalytic activity of the enzyme (Larsson-Raznikiewicz & Jansson, 1973; Scopes, 1978a). From NMR studies, it was suggested that a specific conformational change was induced by the interaction of sulfate with yeast 3-phosphoglycerate kinase (Tanswell et al., 1976). The evidence for a second site distinct from the active site and possessing a high affinity for a variety of anions and negatively charged substrates was also supported by the experiments of Wrobel & Stinson (1978), who studied the effect of these ligands on the environment of a chromophoric reagent specifically bound to the single cysteine residue. These authors suggest that a cluster of positively charged amino acid residues may participate in the binding of these ligands.

If one considers the crystallographic data which provide the three-dimensional structure of 3-phosphoglycerate kinase either from yeast or from muscle (Bryant et al., 1974; Watson et al., 1977; Banks et al., 1979), two remarkable features appear at the level of the structure of this monomeric enzyme.

First, the polypeptide chain is organized in two large and distinct domains of equal size separated by a thin waist.

Second, the secondary structure within the two domains is characterized by an $\alpha\beta$ structure typical of the nucleotide binding structure found in dehydrogenases and some kinases (Rossman et al., 1974). From this structure, two nucleotide binding sites could be predicted; nevertheless, only one has been localized in the domain which corresponds to the C-terminal half of the polypeptide chain (Blake & Evans, 1974; Watson et al., 1977). The other domain probably contains the binding site of 3-phosphoglycerate (Banks et al., 1979); it corresponds to the N-terminal half of the molecule and is characterized by a high level of positively charged amino acids (Fattoum et al., 1978).

If one considers the distance of at least 10 Å which separates these two sites (Banks et al., 1979; H. C. Watson, personal communication), it appears that the two substrates are too far apart to allow the γ-phosphoryl transfer. A motion of the two domains then would be required for catalytic efficiency (Banks et al., 1979; Anderson et al., 1979). We have previously reported some spectrophotometric and chemical data which were consistent with this hypothesis (Desvages et al., 1980; Roustan et al., 1973).

Using small-angle X-ray scattering from solutions of 3-phosphoglycerate kinase, Pickover et al. (1979) found that the radius of gyration of the enzyme decreases upon binding Mg-ATP and 3-phosphoglycerate. From their results, they suggest that the conformational change observed would result from a hinge motion of the two domains which produces a closing of the cleft between them.

Considering the effect of sulfate on the enzymic activity of 3-phosphoglycerate kinase and the possible effect of this anion on a conformational state of the enzyme, we initiated the present work to study the effect of the interaction of sulfate on the molecular properties of 3-phosphoglycerate kinase. The problem was approached by the use of some physical techniques such as ultracentrifugation, optical rotatory dispersion, and differential spectrophotometry but also by studying the effect of sulfate toward the specific modification of several reactive residues, some of them having been localized in the primary structure.

Then, using the same experimental approach, we studied the formation of the binary and ternary complexes between the enzyme and its substrates, with and without sulfate, and their relation to the conformational states of the enzyme.

This paper presents the results obtained from these studies.

Materials and Methods

3-Phosphoglycerate kinase from baker's yeast was prepared according to Scopes (1971). Its specific activity was about 1300 units/mg at 30 °C and pH 7.5, as determined by a spectrophotometric method (Bücher, 1947). Its purity was checked by polyacrylamide gel electrophoresis (Devis, 1964). Prior to use, 3-phosphoglycerate kinase was extensively dia-

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lyzed against buffer to remove ammonium sulfate completely. Glyceraldehyde-3-phosphate dehydrogenase (80 units/mg at 25 °C) was purchased from Boehringer.

DTNB[1] was obtained from Pierce Chemical Co.; phenylglyoxal monohydrate was from Aldrich Chemical Co. 7-Chloro-4-nitrobenzofurazan was from Merck. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate was purchased from Fluka. Nitrotyrosyl ethyl ester was synthesized according to Dall'Asta & Ferrario (1962).

Chemical Modifications. Chemical modifications were performed with or without sulfate in order to test its protective effect.

(*1*) *Reaction with DTNB.* 3-Phosphoglycerate kinase (0.7 mg/mL) in 1 mL of 0.02 M Tris-HCl buffer, pH 8, was incubated at 30 °C in the spectrophotometric cell. The reaction was started with DTNB (1 mM). The liberation of thionitrophenolate was monitored at 412 nm (Ellman, 1959).

(*2*) *Reaction with Phenylglyoxal.* The rate of inactivation by phenylglyoxal was determined under the following conditions. 3-Phosphoglycerate kinase (2 mg/mL) in 35 mM veronal buffer, pH 7.5, at 30 °C was reacted with 2 mM phenylglyoxal.

(*3*) *Reaction with NBD-Cl.* Before modification, the SH group of the enzyme was masked with *p*-(chloromercuri)-benzoate (molar ratio 1:1). All operations were carried out at 30 °C with 3 mg of 3-phosphoglycerate kinase/mL in 0.05 M Tris-HCl buffer, pH 8. The reaction was initiated by 3 mM NBD-Cl.

(*4*) *Reaction with Carbodiimide.* 3-Phosphoglycerate kinase (2 mg/mL) was incubated with 100 mM CMC and 30 mM NTEE in 10 mM Mes buffer, pH 6.1, at 17 °C.

In the three last cases, at specific time intervals, aliquots of the reaction mixture were removed and assayed for enzymic activity. The data were analyzed according to Scrutton & Utter (1965) by plotting V_a/V_o vs. $[1 - (V_a/V_o)](1/S)$; V_a and V_o were pseudo-first-order rate constants of the reaction with or without the effector S, respectively. To test the effect of substrates on the inactivation by carbodiimide, we simply deduced the protective effect from the ratio of the corresponding pseudo-first-order rate constants.

Physical Measurements. (*1*) *UV Difference Spectra.* UV difference spectra were obtained with either a Cary 15 or a Cary 219 spectrophotometer as previously described (Roustan et al., 1968, 1970). In some experiments, the use of matches of 0.15-cm light path cells instead of 0.437-cm light path cells enabled us to use higher ligand or enzyme concentration. The binding constants and the molar absorption of the ligand–enzyme complex were obtained from the variation of the differential absorption at fixed wavelength vs. ligand concentration. The number of binding sites (n) was determined from difference spectra by plotting either $1/(1 - X)$ vs. $C/(XE)$ (Klotz, 1944; Stockel, 1959; Pantaloni & Dessen, 1969) (X = fraction of spectral effect, C = ligand concentration, and E = enzyme concentration) or log X vs. log (total ligand concentration = C) (Beaulieu & Raynaud, 1970). The former plot gives directly the number n of binding sites which is the value of $C/(XE)$ for $1/(1 - X) = 0$. In the latter, the intersect of the two tangents to the experimental curve gives the value of log nE from which n is calculated.

(*2*) *Optical Rotatory Dispersion.* ORD measurements were performed on a Fica type Spectropol I spectropolarimeter. The dispersion curves in the UV region were obtained with 0.1–0.2 mg/mL enzyme in 1-cm light path cells; the effect of sulfate on the magnitude of the Cotton effect of the protein was detected at 233 nm by differential measurements using a matched cell of 1-cm light path. Sucrose was added in one compartment to quench the intrinsic rotation of the protein at 233 nm.

(*3*) *Ultracentrifuge Experiments. Apparatus.* Ultracentrifugation was conducted in a MSE analytical ultracentrifuge. The absorption optics possessed a monochromator and photoelectric scanner. The absolute temperature of the rotor was measured and was stable to ±0.1 °C. Usually, samples were analyzed in a six-hole rotor by using cells of 1-cm light path. The distance from the inner reference line to the axis of the rotor is 5.7 cm.

Measurements of Sedimentation Velocity. Sedimentation velocity measurements were made at about 50 000 rpm and at 20 °C.

In most cases the derivation of the absorbance of the protein at 278 nm was recorded vs. r. However, in the presence of ATP, a wavelength of 290 nm was taken on account of the absorbance of the adenine ring. The measurements of log r vs. time were fitted by regression analysis. Up to 15 determinations were used per experiment. The sedimentation coefficient was determined from the corresponding slope. The $s_{20,w}$ was obtained after correction for viscosity and density (Schachman, 1957). A partial specific volume of 0.748 cm^3 g^{-1} deduced from the amino acid composition of 3-phosphoglycerate kinase (Desvages et al., 1980) was used in our calculations.

To study the effect of substrates on the state of the enzyme, we conducted the s determinations at the same enzyme concentration (2 mg/mL) in 0.02 M Tris-HCl buffer, pH 7.5, with or without NaCl to obtain ionic strengths between 0.02 and 0.1. The results obtained are not ionic strength dependent.

(*4*) *Centrifugal Equilibrium Experiments.* Samples (0.8 mg/mL) were run at 12 800 rpm at 17 °C. When the equilibrium was obtained (43 h), the absorbances at 280 nm were recorded along the cell. Molecular weights were obtained from plots of log absorbance vs. r^2. The corresponding data fitted straight lines.

(*5*) *Estimation of Preferential Hydration.* Preferential hydration was determined by means of ultracentrifugation (Katz & Schachman, 1955). Sedimentation velocity was checked by using various sucrose concentrations (0–40 g %) in order to obtain samples of different densities. Overfilling cells with two sectors were used in which one sector was filled with buffer and the other with enzyme solution. The experiments were run at 54 000 rpm at 20 °C.

Results

Characterization of Anionic Site by the Protective Effect. In order to determine the specificity of binding of sulfate at low concentration in relation to its activating effect, we calculated the dissociation constant between sulfate and 3-phosphoglycerate kinase from the decrease of the rate of the reaction between some reactive groups of the enzyme and specific reagents. The data are analyzed according to Scrutton & Utter (1965).

(*1*) *Cysteinyl Group.* Total protection was afforded by sulfate against DTNB incorporation on cysteine. Furthermore, a corresponding dissociation constant for sulfate of 0.7 mM was determined (Figure 1, Table I).

(*2*) *Arginyl Residue.* The rate of inactivation of 3-phosphoglycerate kinase by phenylglyoxal was decreased when

[1] Abbreviations used: PGK, 3-phosphoglycerate kinase (EC 2.7.2.3); PGA, 3-phosphoglycerate; PG, phenylglyoxal; DTNB, 5,5′-dithiobis(2-nitrobenzoate); NBD-Cl, 7-chloro-4-nitrobenzofurazan; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; NTEE, nitrotyrosyl ethyl ester.

Table I: Effect of Sulfate on the Chemical Modification of 3-Phosphoglycerate Kinase[a]

amino acid modified	reagent	buffer	protection[b] (%)	$K_s(SO_4^{2-})$[b] (mM)
Cys	DTNB	0.02 M Tris-HCl, pH 8	100	0.7
Arg	PG	0.035 M Veronal, pH 7.5	80	2.8
Tyr	NBD-Cl	0.02 M Tris-HCl, pH 8	100	25
Glu	CMC + NTEE	0.01 M Mes, pH 6.1	5	

[a] For experimental conditions, see Materials and Methods. [b] Obtained by plotting $V_a/V_o = f[1 - (V_a/V_o)]\,(1/[SO_4^{2-}])$ according to Scrutton & Utter (1965), where V_a and V_o were pseudo-first-order rate constants of the reaction with and without sulfate, respectively.

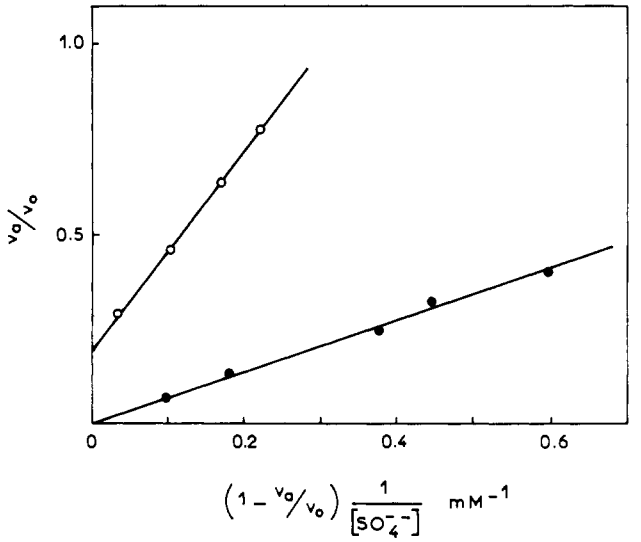

FIGURE 1: Protective effect of sulfate against modification of the cysteine by DTNB (●) and the critical arginine by phenylglyoxal (○). V_a/V_o is plotted vs. $[1 - (V_a/V_o)](1/[SO_4^{2-}])$ (mM^{-1}). For experimental conditions and calculations, see Materials and Methods.

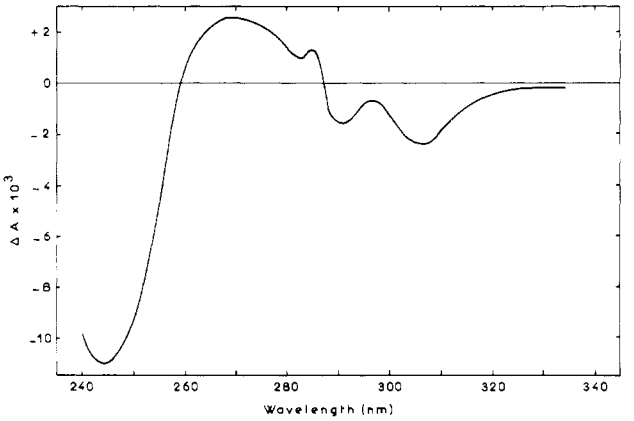

FIGURE 2: Difference spectrum induced by sulfate binding to 3-phosphoglycerate kinase. 33 μM enzyme and 10 mM sulfate in 0.02 M Tris-HCl buffer, pH 7.5; temperature 19 °C, 0.437-cm light path cells.

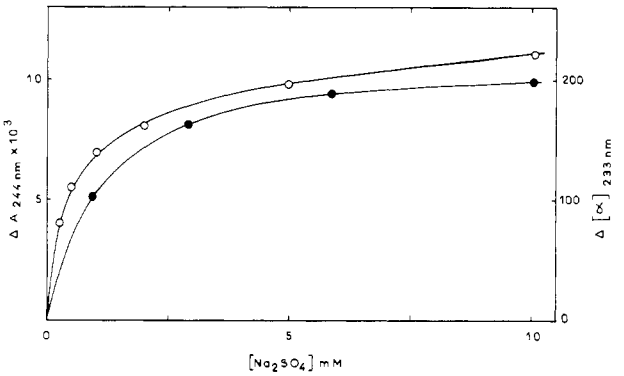

FIGURE 3: Binding of sulfate to 3-phosphoglycerate kinase. Effect of sulfate concentration (1–10 mM) on the difference spectrum followed at 244 nm (○), experimental conditions of Figure 2, and effect of sulfate on the Cotton effect at 233 nm (●), 3 μM enzyme in 0.05 Tris-HCl buffer, pH 7.5.

sulfate was bound to the enzyme. So, a partial protection of 80% occurred. The corresponding dissociation constant for sulfate was about 2.8 mM (Figure 1, Table I).

(*3*) *Tyrosyl Group.* In the case of the modification of the critical tyrosine by NBD-Cl, a total protection by sulfate against inactivation was observed. However, we may note that the dissociation constant is 1 order of magnitude higher than that determined above (Table I).

(*4*) *Glutamyl Residue.* Finally, the sulfate binding does not provide any significant protection against the inactivation by CMC and NTEE (Table I).

Binding of Sulfate. (1) Difference Spectrophotometry. The spectral effect induced by the interaction of sulfate with the enzyme is presented in Figure 2. Its magnitude is related to sulfate concentration (Figure 3). A K_s of 1.0 ± 0.3 mM can be estimated. The maximum magnitude of the spectrum is proportional to the enzyme concentration. Its ϵ_M at 244 nm is about 800.

(*2*) *Optical Rotatory Dispersion.* The binding of sulfate slightly affects the magnitude of the intrinsic Cotton effect of 3-phosphoglycerate kinase at 233 nm. The maximum variation of $\Delta\alpha_{233nm}$ is about 200 °C and is dependent on sulfate concentration (Figure 3). K_s = 1.1 mM can be estimated.

(*3*) *Ultracentrifugation.* The sedimentation velocity was measured at different concentrations of enzyme (0.35–4.0 mg/mL) with or without sulfate (Table II). The concentration of sulfate usually tested was between 5 and 10 mM. The results obtained from native and sulfate-liganded enzyme were not dependent on the ionic strength (0.02–0.12) used in our experimental conditions. For the native enzyme, values of $s^0_{20,w}$ display a low dependence on enzyme concentration (c) which can be expressed as $s = s^0(1 - gc)$ (Figure 4). The value of g was estimated at 0.03 ± 0.01 L/g. The corresponding value for $s^0_{20,w}$ was 3.30 ± 0.04 S. For the sulfate-

Table II: Molecular Properties of Yeast 3-Phosphoglycerate Kinase

parameters	native enzyme	sulfate-liganded enzyme
sedimentation constant = $s^0_{20,w}$	3.30 ± 0.04 S	3.90 ± 0.05 S
concn dependency of $s^0_{20,w}$ = g	0.03 ± 0.01 L/g	undetermined
anhydrous partial sp vol = $\bar{\nu}$[a]	0.748 $cm^3\ g^{-1}$	0.748 $cm^3\ g^{-1}$
mol wt		
equilibrium sedimentation M_r	44 800	42 100
amino acid composition[a] (416 amino acids)	45 000	
diffusion coeff = D_{20}[b]	(7.03 ± 0.06) × 10^{-11} $m^2\ s^{-1}$	(8.32 ± 0.10) × 10^{-11} $m^2\ s^{-1}$
frictional ratio = f/f_o[c]	1.27 ± 0.01	1.07 ± 0.01

[a] Determined from amino acid composition according to Desvages et al. (1980). [b] Estimated from the relation $D = RTs/[M_r(1 - \bar{\nu}\rho)]$. [c] Derived from s and M_r data.

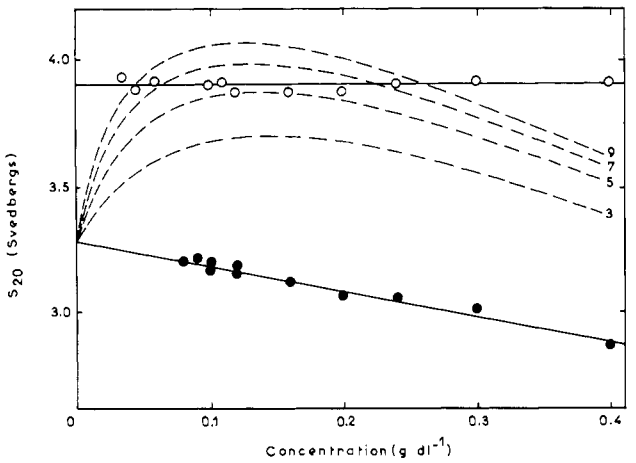

FIGURE 4: Effect of enzyme concentration on the sedimentation constants. (●) Free enzyme; (○) sulfate-liganded enzyme; (---) theoretical curves calculated for a rapid self-dimerization of the enzyme according to Gilbert & Gilbert (1973) for association constants, L, of 3, 5, 7, and 9 dL g^{-1}.

liganded enzyme, an increase of the $s^0_{20,w}$ was observed, $s^0_{20,w}$ = 3.90 ± 0.05 S. No significant concentration dependence was noticed (Figure 4). At high sulfate concentration (i.e., = 40 mM) the sedimentation coefficient takes a value similar to that of the unliganded enzyme.

From these data, the diffusion coefficient and the ratio f/f_0 were estimated (Table II). In all calculations, a molecular weight (M_r) of 45 000 was used. It was obtained from the amino acid composition reported by Desvages et al. (1980) and from the X-ray data at 2.5-Å resolution (H. C. Watson, personal communication) which give a value of 416 amino acids for the whole protein.

In order to explain the results obtained above, we compared the molecular weight of the native and the sulfate-liganded enzymes obtained by equilibrium sedimentation. Similar values were obtained for the two samples (Table II).

The preferential association of water to 3-phosphoglycerate kinase in a third component system was obtained according to Katz & Schachman (1955). For the same concentration of protein (2 mg/mL), the sedimentation velocities were determined for increasing concentrations of sucrose. From these data values of 0.40 ± 0.03 and 0.17 ± 0.03 g of H_2O/g of protein were calculated for native and sulfate-liganded 3-phosphoglycerate kinase, respectively. The value of 0.40 g of H_2O/g of protein for the native enzyme is in reasonable accord with that value (0.46 g of H_2O/g of protein) given by Spragg et al. (1976) using the NMR technique.

Substrate Binding to Yeast 3-Phosphoglycerate Kinase. (1) Spectrophotometric Study. Binary Complex Formation. The binding of substrates to 3-phosphoglycerate kinase induces the characteristic spectral effect described earlier (Roustan et al., 1973). Difference spectra observed with 3-phosphoglycerate display three negative peaks at 287, 279, and 230 nm. The perturbation of the phenolic chromophore of the enzyme induced by this substrate was used for the determination of the amount of bound 3-phosphoglycerate (Figure 5). The graphical treatment of the data shows that there is one binding site for 3-phosphoglycerate per mole of protein (Figure 5, Table III).

Difference spectra observed with ATP-Mg at 278 and 252 nm are related to a perturbation of the adenine ring (Figure 6). Similarly, there is one binding site for ATP-Mg per mole of enzyme corresponding to this effect (Figure 6 and Table III).

Inactive Ternary Complex Formation. The simultaneous

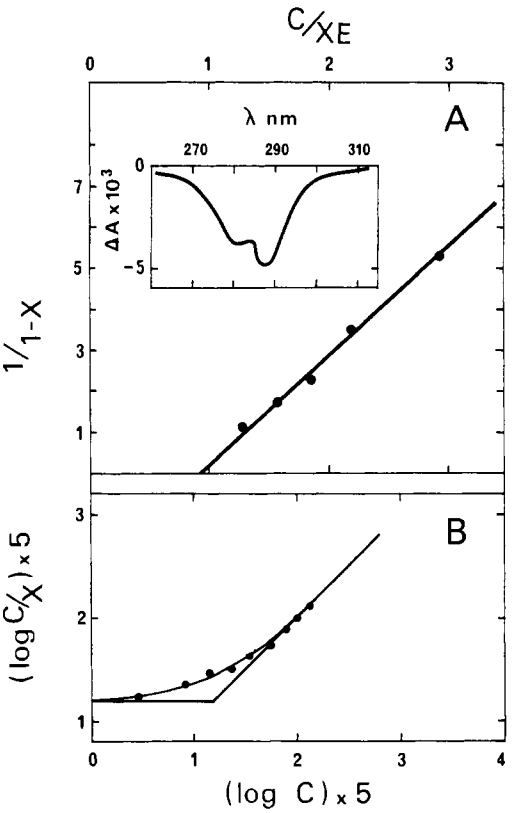

FIGURE 5: Spectrophotometric determination of the number of PGA binding sites. 0.01 M Tris–acetate buffer, pH 7.5, temperature 19 °C, 145 μM enzyme, 0.028–1.3 mM substrate, 0.15-cm light path cells. Fractional spectral effect X was determined at 287 nm (A), $1/(1-X)$ vs. $C/(XE)$. Inset: difference spectrum of PGA–enzyme complex, for 0.22 mM substrate. (B) Log C/X vs. log C.

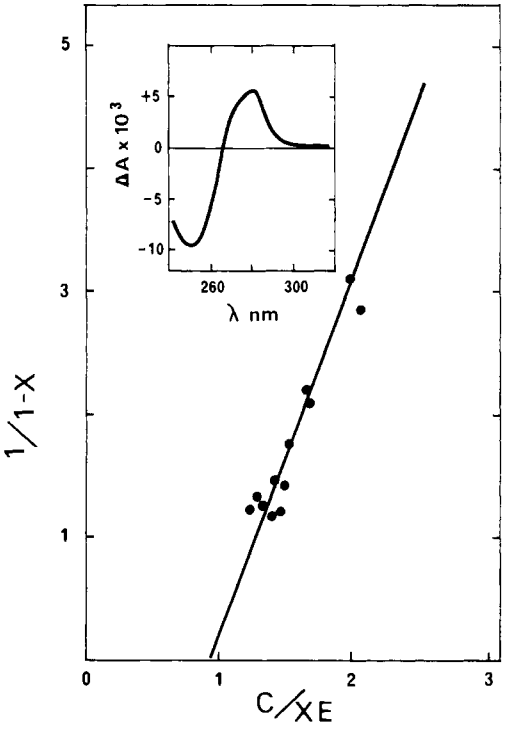

FIGURE 6: Spectrophotometric determination of the number of ATP binding sites. 0.01 M Tris–acetate buffer, pH 7.5, temperature 19 °C, 125 μM enzyme, 14–172 μM substrate, 0.15-cm light path cells. Fractional spectral effect X was determined at 252 and 278 nm. Inset: difference spectrum of ATP–enzyme complex, for 58 μM substrate.

binding of ATP and 3-phosphoglycerate to the enzyme in 0.02 M Tris-HCl and 10^{-4} M EDTA buffer, pH 7.5 (Figure 7A),

Table III: Substrate Binding to 3-Phosphoglycerate Kinase[a]

substrates	λ (nm)	SO_4^{2-} (10 mM)	$\Delta\epsilon_M$	K_s (μM)	n
PGA	287	−	240	150	1
	230	−	1400	150	
PGA	287	+	170	130	
	230	+	980	120	
ATP-Mg	252	−	2200	9	1
ATP-Mg	252	+	1400	9	

[a] The binding parameters of substrates with or without sulfate were determined from the characteristic difference spectra at the wavelengths indicated (see Materials and Methods).

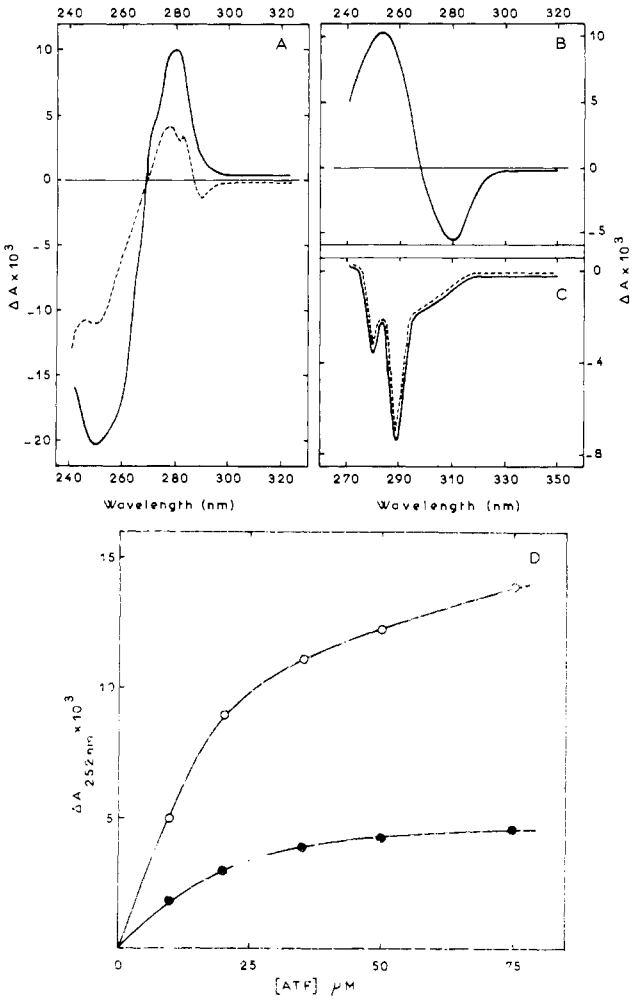

FIGURE 7: Simultaneous interaction of ATP and PGA with PGK. Difference spectra in 0.02 M Tris-HCl and 10^{-4} M EDTA buffer, pH 7.3, temperature 19 °C, 0.437-cm light path cells. (A) 38 μM enzyme, addition of 100 μM ATP (—) followed by 1 mM PGA (---). (B) The base line is made with 22 μM enzyme in the four compartments. 100 μM ATP and 1 mM PGA were added together in one compartment of the sample cell and separately in the two compartments of the reference cell. (C) 75 μM enzyme, addition of 1 mM PGA (—) followed by 100 μM ATP (---). (D) Magnitudes of difference spectra of ATP–enzyme complex (○) are compared with those of ATP–PGA–enzyme complex (●). 38 μM enzyme, 1 mM PGA. ΔA_{252nm} is plotted vs. ATP concentration.

yields an inactive complex which does not display additive spectral effects.

In fact, 3-phosphoglycerate produces a difference spectrum and by inference a conformational change that is not further altered by ATP (Figure 7C). On the other hand, the difference spectrum produced by ATP is further changed by 3-phosphoglycerate at the level of its magnitude ($\Delta\epsilon_{M,252nm}$ decreases from 2200 to 700) (parts B and D of Figure 7). A similar result has been previously obtained for the active ternary complex ATP-Mg–3-phosphoglycerate–enzyme (Roustan et al., 1973).

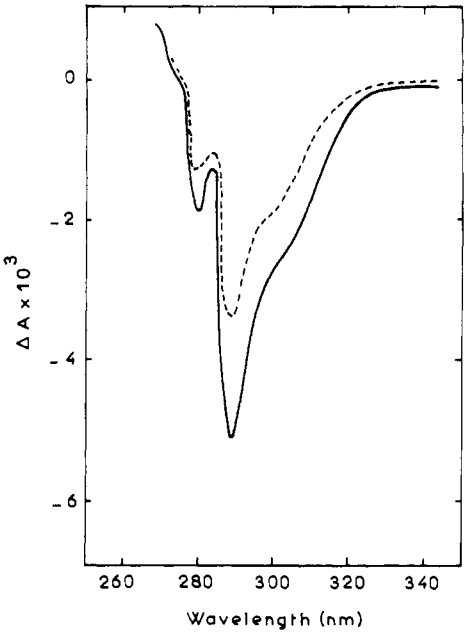

FIGURE 8: Effect of sulfate on the PGA–enzyme interaction. Difference spectra were obtained with 36 μM enzyme in 0.02 M Tris-HCl buffer, pH 7.5. Temperature 19 °C, 0.437-cm light path cells; (—) effect of 1 mM PGA; (---) effect of 1 mM PGA in the presence of 10 mM sulfate.

Table IV: Protection by Substrates and Sulfate against Inactivation by CMC and NTEE of 3-Phosphoglycerate Kinase

substrate or effector	concn (mM)	protection[a] (%)
PGA	10	4
ATP-Mg	2.5	10–20
PGA + ADP-Mg	5 + 2.5	10
PGA + ATP-Mg	5 + 2.5	95
PGA + ATP + EDTA	5 + 2.5 + 0.5	75
SO_4^{2-}	10	5
PGA + SO_4^{2-}	5 + 10	60
ATP-Mg + SO_4^{2-}	2.5 + 10	70
ADP-Mg + SO_4^{2-}	2.5 + 10	60
ADP-Mg + PGA + SO_4^{2-}	2.5 + 5 + 10	70

[a] Protection is related to the ratio of constant rates of inactivation with or without ligand at the indicated concentrations.

Effect of Sulfate on the Substrate Binding. As shown in Figure 8 and Table III, the spectrum in the near-UV characteristic of 3-phosphoglycerate binding is decreased when sulfate is present in the sample. However, the corresponding value of the K_s seems not to be sulfate dependent (Table III). A similar effect is observed in the case of ATP or ATP-Mg (Table III).

(*2*) *Protection by Substrates and Sulfate against Modification of the Critical Glutamyl Residue by CMC and NTEE.* In order to characterize the formation of some complexes between either enzyme and substrates or enzyme, substrates, and sulfate in relation with the conformational state of the protein, we tested the protective effect of these ligands used at a saturating concentration toward inactivation by CMC and NTEE (Table IV). Substrates or sulfate were tested separately and do not afford any significant protection. In contrast, when sulfate is added to 3-phosphoglycerate–enzyme, ATP-Mg–enzyme, or ADP-Mg–enzyme complexes, 60–70% pro-

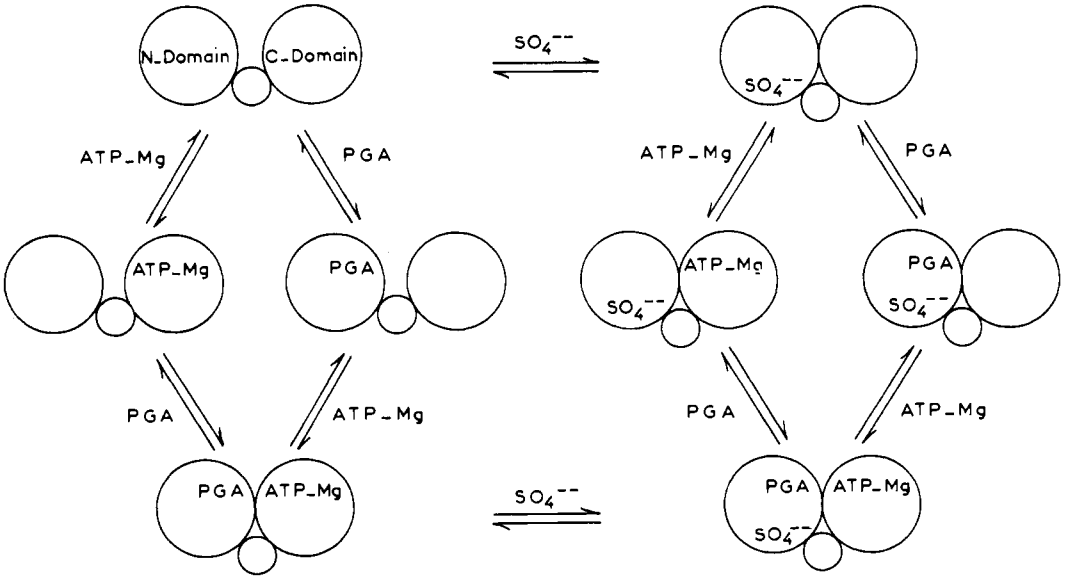

FIGURE 9: Tentative scheme showing the different conformational states of the ligand 3-phosphoglycerate kinase complex.

Table V: Effect of Substrates on the Sedimentation Coefficient of 3-Phosphoglycerate Kinase

ligands	concn (mM)	s_{20} (S)
without substrates		3.10
SO_4^{2-}	10	3.85
PGA	1	3.15
	10	3.16
ATP + Mg	0.5 + 0.5	3.07
PGA + ADP + Mg	0.5 + 0.5 + 1	3.20
PGA + ATP + Mg	0.5 + 0.5 + 0.5	3.95
PGA + ATP + EDTA	0.5 + 0.5 + 0.1	3.89
PGA + SO_4^{2-}	1 + 10	3.87
ATP + Mg + SO_4^{2-}	0.5 + 0.5 + 10	3.71

tection is observed. On the other hand, while the abortive substrate combination (ADP-Mg + 3-phosphoglycerate) does not afford protection, the inactive substrate combination (ATP + 3-phosphoglycerate) and the active substrate combination (ATP-Mg + 3-phosphoglycerate) produce important protective effects.

(*3*) *Analytical Centrifugation.* 3-Phosphoglycerate kinase is found in two conformational states (Figure 9), the unliganded enzyme with a s_{20} of 3.10 S and the sulfate–enzyme with a s_{20} of 3.85 S. The enzyme incubated with 3-phosphoglycerate, or ATP-Mg, or 3-phosphoglycerate + ADP-Mg exhibits the same s_{20} value as the free enzyme (Table V). In contrast, the ATP-Mg–3-phosphoglycerate–enzyme or ATP (in the presence of EDTA)–3-phosphoglycerate–enzyme complex affords a s_{20} of respectively 3.95 or 3.89 S (Table V). Comparable values were obtained with 3-phosphoglycerate–sulfate–enzyme and ATP-Mg–sulfate–enzyme (Table V).

Discussion

Tanswell et al. (1976) have reported that in the presence of 15 mM sulfate, the accessibility of the protons of two histidines to solvent was modified in the yeast 3-phosphoglycerate kinase.

In view of localizing the area where sulfate could bind, we studied the relation of this anion to some critical residues which were specifically modified by chemical reagents (Wrobel & Stinson, 1978; Philips et al., 1978; Roustan et al., 1979; Desvages et al., 1980); except for cysteinyl residue, the modification of these amino acids results in a total loss of the activity of 3-phosphoglycerate kinase.

The effect of protection by sulfate observed toward modification of cysteinyl, arginyl, glutamyl, and tyrosyl residues appeared quite different according to the residue modified. The accessibility of DTNB to the thiol group and that of phenylglyoxal to arginyl residues are prevented when the anion binding occurred. The dissociation constants calculated for sulfate are respectively 1 and 2.8 mM. A higher concentration of sulfate is required for protection toward modification of the tyrosyl residue by NBD-Cl; in this case the dissociation constant for sulfate is 25 mM. At last, no protection by sulfate is observed toward modification of the glutamyl residue. The values of the dissociation constants obtained for sulfate in the case of the protection of cysteinyl and arginyl residues are of the same order as that calculated when studying the effect of the anion on the environment of the intrinsic aromatic residues of the protein, by means of difference spectrophotometry. It also corresponds to that obtained by studying the decrease of $\Delta\alpha_{233}$ by means of optical rotatory dispersion.

Furthermore, these values are in agreement with those obtained by Tanswell et al. (1976), Wrobel & Stinson (1978), and Scopes (1978a). In this range of concentrations, sulfate is known to activate 3-phosphoglycerate kinase. In contrast, the dissociation constant of 25 mM, obtained for protection of the tyrosyl residue, is much higher. In fact, at high concentration, sulfate is an inhibitor of the catalytic reaction (Larsson-Raznikiewicz & Jansson, 1973) and the corresponding value of $s_{20,w}$ takes the value of the unliganded protein.

From these results, two classes of sites for sulfate could exist. If we assume along with Wrobel & Stinson (1978) that the activating anion site is different from the active site, we may suggest that it should be located not too far from cysteines, arginines, and histidines in the N-terminal domain. This hypothesis is indeed supported by the fact that the glutamyl residue which is located in a helical portion near the waist region (Desvages et al., 1980) is not protected against modification. Finally, a second class of sulfate sites of weaker affinity should exist; the binding of the anion to this site inhibits the enzyme, protects the tyrosyl residue, and reverses the conformational change induced by the binding of sulfate to the first activating site.

In view of studying the hydrodynamic properties of the free and sulfate-liganded enzyme in solution, analytical ultracen-

trifugation appeared to be a useful tool.

The data for sedimentation experiments (Table II) which are obtained by UV scanning for the free enzyme are in good accord with those reported by others using the Schlieren procedure (Smith & Spragg, 1979; Larsson-Raznikiewicz, 1970).

For the sulfate-liganded enzyme, the $s^0_{20,w}$ value is raised from 3.30 to 3.90 S. Such a variation could result either from a conformational change or from an association such as dimerization. This last hypothesis is unlikely for the following reasons. First, as shown on Figure 4, the nondependence of s vs. enzyme concentration for the sulfate-liganded 3-phosphoglycerate kinase is not in accord with a self-associating monomer–dimer system (Gilbert & Gilbert, 1973). Second, the molecular weight estimated by ultracentrifugation equilibrium is identical for the unliganded and liganded species. So, the decrease of the frictional ratio f/f_0 observed for the liganded species should be related to a change of the shape of the molecule.

The value obtained for the preferential hydration of unliganded enzyme using the method of Katz & Schachman (1955) is decreased in the liganded species. If we consider that this method is available, in the sense that no self-association may be caused by concentrated sucrose on the liganded species, the value obtained may reflect a decrease in the effective surface area of the enzyme. This variation in relation to the decrease of the calculated frictional ratio f/f_0 could then be interpreted as a transition of the molecule from an asymmetric to a more compact and globular shape. The binding of sulfate slightly alters the intrinsic Cotton effect at 233 nm; if we correlate this slight alteration with some others much more important, observed, for example, in the case of the diiodination of tyrosine residues in 3-phosphoglycerate kinase (Roustan et al., 1976), one may assume that the gross secondary structure of the enzyme has not been altered by the conformational change. Thus, the two domains of the enzyme seem to possess a stable globular structure. These data give evidence for the presence of two conformational states in equilibrium upon sulfate control. The change in the shape of the molecule would correspond to a motion of the two domains closing together (Banks et al., 1979; Anderson et al., 1979). Such a motion would induce a decrease in the hydration of the molecule which becomes more compact.

On the other hand, the data of Pickover et al. (1979) which refer to a change in the shape of the molecule induced by substrate binding are supported by our own results obtained from studies of the binary and ternary complexes of 3-phosphoglycerate kinase, with and without sulfate.

The differential spectra observed during the binding of 1 mol of ATP or 3-phosphoglycerate to the enzyme are related to the interaction of these substrates at the so-called "first" or "high-affinity" subsite, that is, the catalysis sites.

The binding of 3-phosphoglycerate produces a conformational change of the enzyme detectable as shown not only by the perturbation of some tyrosyl residues by difference spectrophotometry (Roustan et al., 1973) or by circular dichroism (Markland et al., 1975) but also by the perturbation of histidyl residues as shown by NMR (Tanswell et al., 1976). By use of these methods, the amplitude of such a conformational change cannot be evaluated, but it is to be related to the observations of Banks et al. (1979), according to which the binding of 3-phosphoglycerate to the crystal of phosphoglycerate kinase produces extensive perturbations. In contrast, the binding of ATP-Mg does not seem to alter the conformational state of the protein. In fact, the only minute alteration observed in crystals is related to the movement of the side chain of one glutamyl residue which is hydrogen bonded to the ribose of the nucleotide substrate (Banks et al., 1979).

These binary complexes (E–ATP-Mg or E–PGA) are unable to induce a significant conformational change in the shape of the molecule as judged by ultracentrifugation. In contrast, there is evidence of a rearrangement of the molecular structure in the working enzyme (E–ATP-Mg–PGA) or in the inactive ternary complex (E–ATP–PGA).

First, a drastic change is observed in the environment of the bound nucleotide by differential spectrophotometry. Second, these substrate combinations provide considerable protection against modification of the glutamyl residue by CMC and NTEE. This protection would not be directly related to the substrate binding since substrates taken separately as well as the combination ADP-Mg–PGA do not significantly afford protection. Third, the value of the sedimentation coefficient is increased from 3.3 S to about 3.9 S. It may be noticed that the conformational change occurs even in the absence of Mg^{2+} and thus appears to take place before the transphosphorylation step.

On the other hand, the value of 3.9 S is identical with that obtained for the sulfate-liganded enzyme and could then be related to the cleft-closing structural change reported by Pickover et al. (1979). Our data are in agreement with theirs and differ from those proposed by Banks et al. (1979) concerning muscle 3-phosphoglycerate kinase in the crystalline form where the conformational change would be induced by only 3-phosphoglycerate. The addition of ATP-Mg or 3-phosphoglycerate to the sulfate-liganded enzyme does not modify the value of the sedimentation coefficient, suggesting that the shape of the molecule is conserved.

These combinations, however, as in the case of the working enzyme and in contrast to those where sulfate or substrates are taken alone, afford an important protection toward the glutamyl residue. This would imply, first, that the conformational change induced by sulfate is unable by itself to protect this critical residue and, second, that when the conformational change induced by sulfate has occurred, either ATP-Mg or 3-phosphoglycerate is in a different environment and is then able to protect the glutamyl residue.

This suggestion is supported by the observed effect of sulfate on the differential spectra of either ATP-Mg or 3-phosphoglycerate; the environment of the adenine ring of ATP-Mg is perturbed by sulfate binding in the same way as that in the ternary complex (E–ATP–PGA); a specific effect of sulfate is also observed at the 3-phosphoglycerate site. This anion produces a significant change in the environment of 3-phosphoglycerate as shown as well by modification of its characteristic spectrum as by ^{31}P NMR studies (Rao et al., 1978). However, the K_s values of these two substrates are not significantly altered, indicating that they interact with the same affinity on the enzyme, whatever its structural state may be. Figure 9 summarizes the results obtained and presents a tentative scheme of the conformational states of 3-phosphoglycerate kinase during sulfate or substrate binding.

The non-Michaelian kinetics of the enzyme and its activation by anions, reported by Larsson-Raznikiewicz (1967, 1970) and Scopes (1978a), may be explained by the "hinge-binding" conformational change. In fact, it may be postulated that the transition is one limiting step of the reaction. The motion which brings the two domains together could be fast because it requires the rotation of only one peptide bond (Banks et al., 1979). So, the sulfate displaces the equilibrium toward the closed form of the enzyme and thus enhances the transfer

reaction rate. An effect of sulfate on the dissociation rate constant of 1,3-diphosphoglycerate (Scopes, 1978b) is not excluded. Further investigation by rapid kinetics is necessary to substantiate this hypothesis.

Finally, it is interesting to note that this large structural rearrangement which induces the in-line position of substrates needed for catalysis seems to be a general feature among kinases. This motion is required, for example, in the catalytic efficiency of hexokinase (Bennett & Steitz, 1978), adenylate kinase (Pai et al., 1977), pyruvate kinase (Stammers & Muirhead, 1975), and probably phosphagen kinases.

This phenomenon could be controlled not only by substrates but also by effectors and could be implicated in the regulation of these enzymes.

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