Since the pK of OH^- would be larger than the pK_a of the amino group of the X^+-nucleotide complex the observed broadening by ligand binding would be insensitive to changes in this $-NH_2$ pK_a value, which might be associated with the particular nature of the cation. This would represent a distinct advantage over other nucleotide–cation binding studies because there is no need to saturate the nucleotide with cation to obtain measurable spectral changes. The $-NH_2$ line width is sensitive to a small equilibrium concentration of the complex and would provide the X^+-nucleotide dissociation constant in one measurement. Several potential cations are attractive candidates for studying interactions at the acidic ring nitrogen of nucleotides and possibly polynucleotides. Some of these, in addition to metal cations, might be active forms of mutagenic carcinogens, particularly those whose activity can be associated with an incipient or formal positive charge that can lead to substitution of the basic ring nitrogens (Irving, 1973).

Acknowledgments

I wish to thank Pat Seawell for her excellent technical assistance in the exploratory aspects of this work and Mrs. Katherine Cramer for her skillful assistance in the collection of the bulk of the pmr data.

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Chromophoric Labeling of Yeast 3-Phosphoglycerate Kinase with an Organomercurial†

Robert A. Stinson

ABSTRACT: Phosphoglycerate kinase, purified and crystallized from yeast, was reacted with the organomercurial, 2-chloromercuri-4-nitrophenol. The reaction products whether formed with the native enzyme or with enzyme denatured by 5 M urea or by 3.5 mM sodium dodecyl sulfate, followed by renaturation, appeared identical and were fully active. The modified enzyme appeared identical with the native enzyme in its catalytic properties. Only 1 mol of mercurial or 1 mol of 5,5′-dithiobis(2-nitrobenzoic acid) reacted per mol of enzyme. The pK_a of the phenolic hydroxyl of the free mercurial is 6.60 but became 8.30 when the compound associated with the single thiol residue of the enzyme. This association also resulted in shifts in the wavelength of maximum extinction (red shift) and decreased the extinction at these maxima (hypochromicity) for the acid and base forms of the mercurial. The slower electrophoretic mobility of the modified enzyme was readily reversed by β-mercaptoethanol. The mercurial dissociated from the enzyme during polyacrylamide gel isoelectric focusing to yield an isoelectric point of 7.01, identical with that of native enzyme. Interactions between the mercurial and enzyme were exemplified in a skewed titration curve and a slope between 1 and 2 for the linear representation of the Henderson–Hasselbalch equation. The phenolic hydroxyl of the mercurial bound to phosphoglycerate kinase had a pK_a of 8.72 in the presence of the substrate 3-phosphoglycerate and 8.54 in the presence of $MgATP^{2-}$. The rate of reaction between the mercurial and the enzyme is increased by $MgADP^-$ and $MgATP^{2-}$ and is decreased by 3-phosphoglycerate and phosphate. This reaction in a mixture of $MgADP^-$ and 3-phosphoglycerate had a rate intermediate between the rates in the two substrates added separately. The relevance of these findings to the mechanism of the enzyme is discussed.

Phosphoglycerate kinase (EC 2.7.2.3) is an important enzyme in the glycolytic pathway in that the reaction it catalyzes provides much of the driving force for the preceding steps in the sequence and particularly for the glyceraldehyde-3-phosphate dehydrogenase reaction (Mahler and Cordes, 1966). Until recently, investigations concerning this enzyme lagged well behind those on other glycolytic enzymes. The enzyme is the only monomer in the glycolytic pathway and has a molecular weight of 47,000 (see Scopes,

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1973 for a review). Very recent reports on the yeast enzyme present a high resolution X-ray crystallographic structure (Bryant *et al.*, 1974) and give evidence for the involvement of a glutamyl residue in the phosphoryl transfer (Brevet *et al.*, 1973).

In 1969 McMurray and Trentham reported on a new class of chromophoric organomercurials. They demonstrated with glyceraldehyde-3-phosphate dehydrogenase the usefulness of 2-chloromercuri-4-nitrophenol as a sulfhydryl reagent, as a protein structure probe, and as a compound suitable for heavy atom isomorphous replacement. A recent report has shown how the spectrum of this mercurial linked to creatine kinase changes when substrates bind (Quiocho and Thomson, 1973).

My primary interest is in the mechanism and chemistry of phosphoglycerate kinase and to this end enzyme structural probes were investigated. The present report demonstrates how the single sulfhydryl group of yeast phosphoglycerate kinase can be reacted with 2-chloromercuri-4-nitrophenol and how information is obtained about enzyme conformation when, among other things, the spectrum and pK_a value of the mercurial are monitored subsequent to substrate binding.

Materials and Methods

"Active dried yeast" was obtained from Distillers Co. (UK) and stored at 4°. The 2-chloromercuri-4-nitrophenol was the generous gift of Dr. D. R. Trentham. Solutions of urea were passed through a mixed bed ion exchange resin (Rexyn I-300 (Fisher Scientific)) immediately prior to use. The apparatus for polyacrylamide gel isoelectric focusing was from Medical Research Apparatus, Boston, and the carrier ampholytes (pH 7–9 and 3–10) were purchased from LKB. The components for the polyacrylamide gels were obtained from Bio-Rad Laboratories. Unless indicated otherwise the buffer was 50 mM triethanolamine hydrochloride, pH 7.5 (NaOH).

Enzyme Preparation and Assay. Phosphoglycerate kinase was prepared from "active dried yeast" essentially as described by Scopes (1973) except that following the first crystallization the enzyme was applied to an 18 cm × 1 cm column of DEAE-cellulose equilibrated with 20 mM KH_2PO_4, pH 7.5 (NaOH). The protein eluted with the equilibrating buffer was recrystallized at least twice. This material consistently had a specific activity of at least 850 U/mg when assayed as described. The enzyme was stored at 4° in the crystalline state and was dialyzed against the appropriate buffer just before use. The enzyme was assayed at 30° in the reverse direction as described (Krietsch and Bücher, 1970) with minor changes. The glyceraldehyde-3-phosphate dehydrogenase (specific activity 120 U/mg) used to catalyze the reduction of 1,3-diphosphoglycerate formed in the reaction was prepared from rabbit muscle as a by-product of a lactate dehydrogenase preparation (Stinson and Gutfreund, 1971).

Polyacrylamide Gel Electrophoresis. This was accomplished with 5.5% acrylamide gels in Tris-glycine buffer at pH 8.3 (Chrambach and Rodbard, 1971). The sample in 20% sucrose was layered on the gel. Amido Schwarz was the protein stain. Specimens subjected to β-mercaptoethanol before electrophoresis were treated identically except that the thiol was added to a concentration of 6.3 mM just prior to running. Gels were aligned by calculation of R_F values from a Bromophenol Blue marker and by electrophoresing mixtures of individually run specimens.

Isoelectric Focusing in Polyacrylamide Gels. This was performed with a Medical Research Apparatus gel isoelectric focusing system in 4% acrylamide gels, chemically polymerized with ammonium persulfate (Righetti and Drysdale, 1971). The gels contained 1.5% ampholine (pH 7–9) and 0.5% ampholine (pH 3–10). Focusing was for 16 hr at 4°. The isoelectric point of the protein was determined by reading the pH of 0.5 ml of water that contained the 0.15-mm segment of gel into which the enzyme had focused.

Absorption Spectra. Spectra were obtained at room temperature with a Cary 16 spectrophotometer. A constant slit of 0.2 mm was used.

Determination of Mercurial pK_a Values. The contents of a cuvet in a Unicam SP800 spectrophotometer was constantly mixed with a magnetic stirring bar as aliquots of 0.10 N NaOH were added to the acid form of the mercurial (usually 20 μN) from a syringe buret. The cuvet was fitted with a micro electrode so that pH and absorbance could be read continuously without disturbing the system. The titrations were in 5 mM triethanolamine plus 100 mM NaCl. The volume was 2.9 ml and the amount of base added during the titration was less than 5% of the total volume. The end point of the titration was estimated and the relative amounts of the ionized and un-ionized forms of the mercurial were determined at each pH. The logarithm of this ratio was plotted against pH, and the pK_a was read from the point where the log function was zero. Replicate determinations gave very precise results with a relative error of usually less than 1%. Because of the uncertainty of pH readings in protein denaturants, especially in 5 M urea, certain pK_a values calculated in the above manner were checked by spectral scans of the mercurial in the denaturant but in a strong buffer solution. The relative proportions of the acid and basic forms were determined at pH 7.5 and 144 mM triethanolamine using the following absorptivities: for the acid form ϵ_{mM}(327 nm) 9.30 and ϵ_{mM}(410 nm) 0, and for the basic form ϵ_{mM}(327 nm) 2.15 and ϵ_{mM}(410 nm) 17.4. The pK_a values were then calculated using the Henderson–Hasselbalch equation.

Titration with 2-Chloromercuri-4-nitrophenol in Urea. Stock mercurial was quantitated spectrophotometrically at 410 nm by using 17.4 as the extinction of a 1 mM solution in 0.1 N NaOH (McMurray and Trentham, 1969). Phosphoglycerate kinase solutions were dialyzed against 50 mM triethanolamine (pH 7.5) and quantitated by using $\epsilon_{280\,nm}$(1%) 4.9 (Krietsch and Bücher, 1970). Thioglycollic acid was used as a standard and its concentration was determined by titration with standard NaOH to a phenolphthalein end point. The reactions of 25 μN phosphoglycerate kinase and 25 μN thioglycollic acid with mercurial were carried out in 25 mM triethanolamine (pH 7.5), 5 mM EDTA, and 5 M urea. Mercurial was added in steps of 2.5 μN each to either the protein or the thiol and the blanked 410-nm absorbance was recorded.

Reaction with Nbs_2.[1] Either 25 μN phosphoglycerate kinase or 25 μN thioglycollic acid was reacted with 100 μN Nbs_2 in 25 mM triethanolamine (pH 7.5) in 5 M urea. The liberated phenolate ion was measured at 412 nm.

The reaction rate of Nbs_2 with native or 3.5 mM SDS denatured phosphoglycerate kinase was measured at 30° at 412 nm in a Unicam SP800 recording spectrophotometer. Enzyme at 25 μN was reacted with 1.0 mM Nbs_2 in the

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

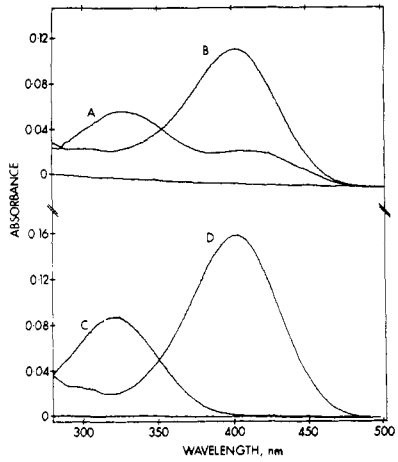

FIGURE 1: Absorption spectra of various forms of 2-chloromercuri-4-nitrophenol: (A) 10 μN phosphoglycerate kinase plus 7 μN mercurial in 50 mM triethanolamine buffer (pH 7.5) blanked against 10 μN phosphoglycerate kinase, also in buffer; (B) 7 μN mercurial in 50 mM triethanolamine buffer (pH 7.5) blanked against buffer. (C) 9.1 μN mercurial in 0.1 N HCl blanked against HCl; (D) 9.1 μN mercurial in 0.1 N NaOH blanked against NaOH.

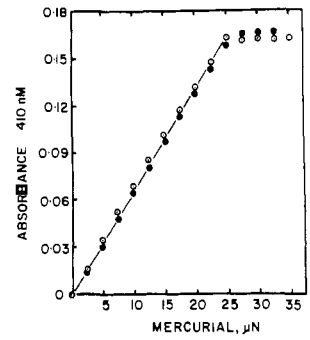

FIGURE 2: Titration of the thiol groups of thioglycollic acid (O) or phosphoglycerate kinase (●) with 2-chloromercuri-4-nitrophenol. Thioglycollic acid (25 μN) or enzyme (25 μN) were titrated in 5 M urea, 25 mM triethanolamine, and 5 mM EDTA (pH 7.5). Blanks were without thioglycollic acid or phosphoglycerate kinase.

presence of substrate and other constituents. Pseudo-first-order rate constants were determined by the Guggenheim method (Gutfreund, 1965) and converted to second-order constants.

Renaturation from Urea and SDS. Denaturation of 50 μN phosphoglycerate kinase was accomplished with either 5 M urea in 25 mM triethanolamine (pH 7.5) or 3.5 mM SDS in 50 mM triethanolamine (pH 7.5). To renature the enzyme, 5 μl of either the SDS or urea-treated material was diluted 1000-fold by addition to either 5.0 ml of 50 mM triethanolamine (pH 7.5) or 5.0 ml of the medium (minus the linking enzyme and β-mercaptoethanol) used to assay the enzyme. These dilutions were assayed immediately and then after various periods of incubation at 25.0°. Because renaturation continued to occur during the assay period, reaction rates often took 2–3 min to become linear. The activity was calculated from the linear portion of the rate curve.

Reaction Rates of Mercurial with Phosphoglycerate Kinase. A Durrum stopped-flow instrument was used to monitor, within 5 sec of mixing, the reaction between 5 μN enzyme and 100 μN 2-chloromercuri-4-nitrophenol at 410 nm in 50 mM triethanolamine (pH 7.5) in the presence and absence of substrates. The reactions at 22° were displayed on a storage oscilloscope and permanently recorded on Polaroid film. The pseudo-first-order reaction rates determined from the photos were converted to second-order constants.

Results

Enzyme Preparation. Consistent specific activities of yeast phosphoglycerate kinase of 850 U/mg of protein were obtained only if the preparation as described by Scopes (1971) was followed by DEAE-cellulose chromatography. The enzyme did not adsorb to the exchanger but at least two other components did, and these were therefore removed by the process.

Polyacrylamide Electrophoresis and Isoelectric Focusing. As judged by these two criteria the preparation appears homogeneous (Figure 4). The isoelectric point of yeast phosphoglycerate kinase determined in this way was 7.02 ± 0.07 SD for six determinations. The isoelectric point of "2-phosphoglycerate kinase-mercuri-4-nitrophenol" was found to be 7.01 ± 0.10 SD for five determinations. Presumably the procedure of isoelectric focusing has removed the mercurial from the protein and it now has the same isoelectric point as the native enzyme. An isoelectric point of 7.2 for yeast phosphoglycerate kinase was calculated by Krietsch and Bücher (1970) from the ratio of charged amino acids whereas Yoshida *et al.* (1972) determined a value of 7.9 for the erythrocyte enzyme.

Absorption Spectra. These are given for various forms of 2-chloromercuri-4-nitrophenol in Figure 1. The ionized form of the mercurial is highly colored (D) whereas the protonated form is not (C). Spectra A and B emphasize the considerable pK_a increase the phenolic hydroxyl undergoes when the mercury side chain associates with the thiol group of phosphoglycerate kinase. In addition, there is a red shift. The ionized form of the free mercurial absorbs maximally at 402 nm whereas maximum absorption is at 410 nm when complexed with phosphoglycerate kinase. Similarly, the protonated mercurial shifts its maximum absorbance from 322 to 327 nm when the side chain mercury associates with phosphoglycerate kinase.

Titration with 2-Chloromercuri-4-nitrophenol in Urea. The pK_a of the mercurial complexed with EDTA is 9.02, and, hence in pH 7.5 buffer there is little absorbance at 410 nm. Reaction with phosphoglycerate kinase in 5 M urea, however, results in an increase in absorbance at 410 nm (Figure 2), indicating that the pK_a of the mercurial complexed with protein in urea is considerably decreased. From an acid–base titration of this complex in urea, and a spectral scan, the pK_a was determined to be 7.35. The use of thioglycollic acid as a standard for this titration allowed a determination of the number of thiol groups on the enzyme that is independent of the mercurial concentration. A calculation based on the mercurial concentration yields 1.06 reactive sulfhydryl residues/mol of protein. Determination from the thioglycollic acid standard yields 1.10 sulfhydryl residues/mol. The pK_a values for the mercurial–thioglycollic acid complex in buffer and for the mercurial–protein complex in 5 M urea are 7.22 and 7.35, respectively, and therefore the maximum extinction changes when the two complexes are titrated with mercurial are similar (Figure 2). The pK_a of the mercurial bound to phosphoglycerate kinase in SDS was found by titration and spectral scans to be 8.35. The pK_a of 7.22 for mercurial–thioglycollic acid was unaltered by the presence of 3.5 mM SDS. The extinction at 410 nm of 2-chloromercuri-4-nitrophenol in 5 M urea or 3.5 mM SDS was increased 8 and 4%, respectively, relative to 0.1 N NaOH.

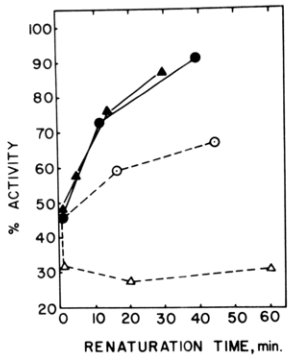

FIGURE 3: Phosphoglycerate kinase renaturation from urea or sodium dodecyl sulfate. Triangles are renaturation from urea in the presence (closed) and absence (open) of substrates. Circles are renaturation from sodium dodecyl sulfate in the presence (closed) and absence (open) of substrates. The buffer was 50 mM triethanolamine (pH 7.5) and the substrate mixture 12 mM 3-phosphoglycerate, 2.4 mM ATP, plus 8 mM $MgSO_4$.

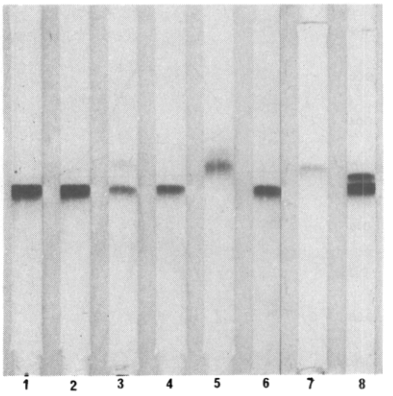

FIGURE 4: Polyacrylamide disc gel electrophoresis of phosphoglycerate kinase: (1) native enzyme; (2) native enzyme plus 6.3 mM β-mercaptoethanol; (3) mixture of native and mercurial enzyme; (4) native and mercurial enzyme plus 6.3 mM β-mercaptoethanol; (5) mercurial enzyme; (6) mercurial enzyme plus 6.3 mM β-mercaptoethanol; (7) native enzyme denatured in 5 M urea, reacted with mercurial and then renatured; (8) mixture of sample 7 and native enzyme (sample 1). The cathode is at the top.

Reaction with Nbs_2. The thiol group of the kinase was further characterized by reaction with Nbs_2. The extinction change at 412 nm when phosphoglycerate kinase reacts with Nbs_2 in 5 M urea was standardized with a known thioglycollic acid solution. From this calculation the protein has 0.95 reactive sulfhydryl residue/mol or protein. There is no evidence that 5 M urea completely denatures phosphoglycerate kinase, but the enzyme loses all enzymatic activity within 5 min in 3 M urea and reacts with 2-chloromercuri-4-nitrophenol 400 times faster in 3, 4, and 5 M urea than in 2 M urea (R. A. Stinson, unpublished). Thus, it appears that the "buried" thiol residue is completely exposed in 5 M urea.

The rate of reaction with Nbs_2 of the native and SDS denatured enzyme in the presence of substrates was investigated. The second-order rate constant between native enzyme and Nbs_2 in 50 or 25 mM triethanolamine in sec^{-1} M^{-1} was 0.28 ± 0.03 SD for five determinations. Under the pseudo-first-order conditions used, the half-time for the reaction was approximately 45 min. The error associated with the determination of slower rates precluded the assignment of any significance to the difference between the rates in the presence of $MgATP^{2-}$ (2.4 mM), 3-phosphoglycerate (12 mM), or phosphate (20 mM). The average of these rates was 0.07 sec^{-1} M^{-1}, four times slower than the rate with native enzyme. Further, the decreased rates in the presence of $MgATP^{2-}$ and phosphate were not additive. In the presence of 3.5 mM SDS all reactions had very similar reaction rates, the average being 90 sec^{-1} M^{-1}. Thus the rates in SDS were approximately 300 times faster than the fastest rate with the native enzyme.

Renaturation from Urea and SDS. In order to be certain that the chromophoric mercurial could react with all sulfhydryl residues of native phosphoglycerate kinase, renaturation of the enzyme from urea and SDS was investigated (Figure 3). Renaturation by dilution into a mixture of substrates is shown to be possible and to yield almost 100% recovery of activity. However, the same dilution into buffer results in only 70% recovery of enzymatic activity from SDS denaturation and very low recovery from urea denaturation. The 45% activity that appears as the "starting point" in Figure 4 results from the renaturation that occurs during the assay. The reaction rate was taken from the linear portion of the tracing that resulted when renaturation was complete. The longer the renaturation time (incubation at 25° of the diluted sample), the more quickly the reaction rates became linear. From this type of experiment it appears that SDS denaturation is more suitable than urea with respect to the ability of the enzyme to renature. However, it was felt that because of the difficulty in removing bound SDS from proteins, urea renaturation should be investigated more thoroughly. Denaturation of phosphoglycerate kinase in 5 M urea, reaction with a twofold excess of 2-chloromercuri-4-nitrophenol, and attempted renaturation by passing the mixture through Sephadex G-25, resulted in very little recovery of enzymatic activity. However, if the mixture was renatured slowly by gentle dialysis against buffer, 75% of enzymatic activity was reproducibly recovered.

Characterization of Mercurial–Phosphoglycerate Kinase Complex. Electrophoretic mobility, isoelectric point, substrate K_m values, pK_a values, and spectra were done to characterize the complex. Modified enzyme made in 5 M urea followed by renaturation has identical properties to modified enzyme made by reacting mercurial with native phosphoglycerate kinase. This verifies that only one sulfhydryl group reacts in both the native and denatured enzyme. Figure 4 illustrates that both native and renatured enzyme have the same electrophoretic mobility (gels 7 and 8). The modified enzyme moves slower than the native enzyme, suggesting that it has less negative charge. Samples of enzyme known to be 100% mercurialated sometimes formed two bands when electrophoresed, one corresponding to the mobility of the native enzyme. This is probably because the mercurial partially dissociates from the enzyme during electrophoresis. The modified enzyme, after recrystallization, formed in either manner resulted in a product with a specific activity and substrate K_m values comparable to the native enzyme. Arvidsson and Larsson-Raźnikiewicz (1973) reported that native and *p*-hydroxymercuribenzoate modified phosphoglycerate kinase had identical kinetic properties. I determined the K_m for 3-phosphoglycerate to be 0.30 mM and that for $MgATP^{2-}$ to be 0.19 mM. These values are comparable to those previously reported (Krietsch and Bücher, 1970). Solutions of mercurial–enzyme can be quantitated by enzymatic assay, addition of the complex to 0.1 N NaOH and measuring an extinction at 410 nm (ϵ_{mM}(410 nm) 17.4), or by taking extinction readings of the

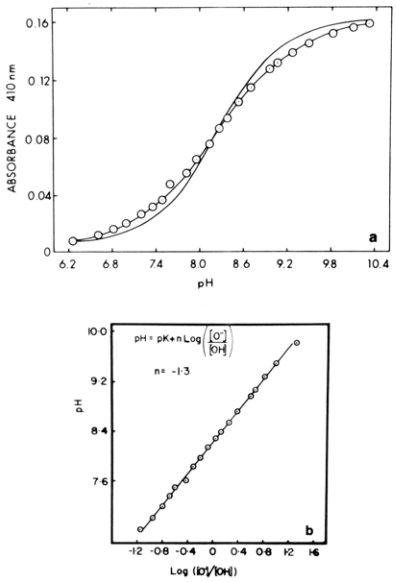

FIGURE 5: (a) Titration of 9 μN 2-chloromercuri-4-nitrophenol bound to phosphoglycerate kinase. The experimental data are shown as well as the theoretical line for the pK_a (8.24) determined from the best fit of the experimental data to the Henderson–Hasselbalch equation. (b) Linear plot, according to the Henderson–Hasselbalch equation, of the experimental data from Figure 5a.

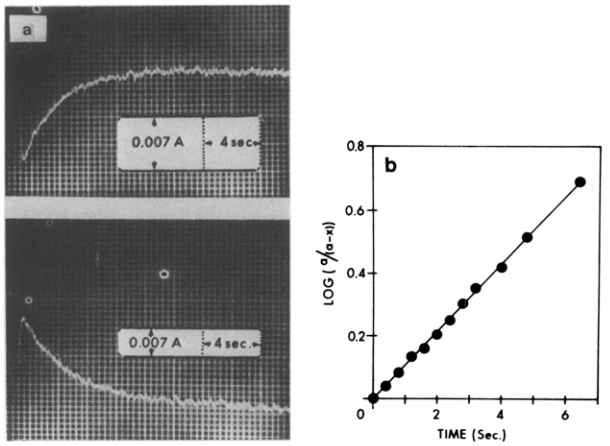

FIGURE 6: (a) Stopped flow reaction between 50 μN 2-chloromercuri-4-nitrophenol and 1.25 μN phosphoglycerate kinase in 50 mM triethanolamine (pH 7.5). The upper storage oscilloscope trace shows the decrease in optical density at 410 nm as the phenolic pK_a value increases from 6.6 to 8.3. The lower trace records the increase in optical density at 327 nm as a result of the increase in the protonated form of the mercurial. (b) Plot of the pseudo-first-order reaction monitored at 410 nm between 100 μN 2-chloromercuri-4-nitrophenol and 5 μN phosphoglycerate kinase in 50 mM triethanolamine (pH 7.5).

complex in pH 7.5 buffer at 410 and 322 nm and calculating the concentrations of the ionized and un-ionized forms of the mercurial present. From absorption spectra of mercurial–phosphoglycerate kinase at pH 4.4 in 100 mM sodium acetate and at pH 10.3 in 100 mM glycine, the millimolar absorptivities of the complex were found to be 8.55 at 327 nm and 15.32 at 410 nm, respectively, for the acidic and basic forms of the phenolic hydroxyl. The spectra also confirmed a shift from 402 to 410 nm and from 322 to 327 nm, for the basic and acidic forms, respectively, when the mercurial associates with the enzyme. Since only 1 mol of mercurial reacts/mol of enzyme, the enzyme concentration is the mercurial concentration.

Difference spectra of the enzyme–mercurial complex were done at pH 10.3 and pH 4.4. These spectra allowed an evaluation of the shift in the wavelength of maximum absorbance when the mercurial binds to the enzyme, independent of the pK_a of the mercurial. At pH 10.3 the maximum positive and negative absorbances were at 442 and 384 nm, respectively, with the isosbestic point at 418 nm. At pH 4.4, the maximum positive and negative absorbances were at 355 and 286 nm, respectively, with an isosbestic at 333 nm.

Verification that the altered electrophoretic mobility of the 2-chloromercuri-4-nitrophenol-treated enzyme was in fact due to the presence of bound mercurial was obtained by adding β-mercaptoethanol to the native and complexed enzymes (Figure 4). The thiol had no effect on the native enzyme (gels 1 and 2) but converted a mixture of mercurial and native enzyme into one band with the mobility to native enzyme (gels 3 and 4), and converted mercurialated enzyme into one band equivalent to native enzyme (gels 5 and 6). Mercurial does not stay bound to the enzyme during isoelectric focusing and hence it appears to have the same isoelectric point as native enzyme.

Acid–Base Titration of Mercurial–Phosphoglycerate Kinase. 2-Chloromercuri-4-nitrophenol alone, titrated normally with NaOH and the pK_a of the phenolic hydroxyl from ten titrations, was found to be 6.60 ± 0.04 SD. However, the mercurial bound to enzyme did not titrate normally (Figure 5a). A best fit of the experimental data to the Henderson–Hasselbalch equation allowed a pK_a of 8.24 to be determined. However, the theoretical line corresponding to this pK_a was in considerable disagreement with the experimental points. A plot of the experimental points according to the linear representation of the equation explains the anomaly (Figure 5b). For a standard titration, the slope of this plot is normally 1, and was found to be so for ten titrations of free mercurial (slope = 1.04 ± 0.07 SD). When a complex of mercurial and nondenatured enzyme was titrated, the slope was always 1.3 or greater. The consistency of the deviation from the normal relationship is revealed in the fact that the same pK_a value is obtained whether a plot is such as is shown in Figure 5b is used or whether a best fit is obtained such as is shown in Figure 5a. Although the presence of certain of the enzyme's substrates significantly altered the pK_a of the mercurial bound to the enzyme, there was no significant difference in the slopes of the linear plots constructed from the experimental data. Nine titrations in the presence of various substrates yielded a mean slope of 1.37 ± 0.08 SD. Slopes greater than 1 were only obtained when the enzyme was in its "native" state. Titration of enzyme–mercurial in 5 M urea of 3.5 mM SDS gave a slope of 1.0.

In the presence of 12.0 mM 3-phosphoglycerate or 2.4 mM ATP plus 8.0 mM $MgSO_4$, the phenolic hydroxyl of the mercurial–enzyme complex exhibited pK_a values of 8.72 or 8.54, respectively. The comparable pK_a for modified enzyme alone is 8.30. The magnitude of these pK_a differences between native enzyme and enzyme with substrate bound was consistent. In addition to these pK_a changes the substrates also caused wavelength shifts in the absorption maximum of the mercurial as revealed by difference spectra (R. A. Stinson, unpublished).

Reaction Rates of Mercurial with Phosphoglycerate Kinase. A decrease in the ionized form of the mercurial when it reacts with phosphoglycerate kinase is coincident with an increase in the protonated form. Thus, the reaction could be monitored either by a decrease in the 402-nm absorbance or an increase in the 327-nm absorbance (Figure 1). Figure 6a

TABLE I: Reaction Rates of Mercurial with Phosphoglycerate Kinase.[a]

Addition (mM)	Rate Constant (sec^{-1} M^{-1})[b] $\times 10^{-3}$
Control	3.3
ATP (2.4) plus $MgSO_4$ (8.0)	3.7
ADP (2.0) plus $MgSO_4$ (8.0)	3.9
3-Phosphoglycerate (12)	1.7
ADP (2.0) plus $MgSO_4$ (8.0) plus 3-phosphoglycerate (12)	2.4
ADP (2.0) plus ATP (2.4) plus $MgSO_4$ (8.0)	3.7
KH_2PO_4 (20.0), pH 7.5	2.9

[a] Phosphoglycerate kinase, 100 μN, was reacted with 5 μN 2-chloromercuri-4-nitrophenol in 50 mM triethanolamine (pH 7.5). [b] Values are the average of at least two determinations that were within 10%.

illustrates the same reaction at these two wavelengths. Figure 6b shows a first-order plot of the reaction between mercurial and enzyme monitored at 402 nm. The latter wavelength was used routinely. It may be significant that the reaction monitored at 327 nm is 30% faster than the reaction monitored at 402 nm; this may reflect subtle interactions between mercurial and protein when they associate, but the matter requires further clarification.

Table I lists the second-order rate constants for the reaction of mercurial with enzyme in the presence of various independent substrates, mixtures of substrates, and other constituents. The nucleotide substrates $MgATP^{2-}$ and $MgADP^{-}$ both result in a small but consistent increase in the rate of the reaction, whereas 3-phosphoglycerate decreases the rate by more than 50%. It is interesting that the reaction rate in $MgADP^{-}$ plus 3-phosphoglycerate is almost intermediate between the rates attained with the two substrates independently. The reaction rate in the presence of a mixture of $MgADP^{-}$ and $MgATP^{2-}$ is indistinguishable from the rates when the nucleotides are added separately. The reaction rate was also determined in 20 mM phosphate because of the effect that this ion had on the reaction of Nbs_2 with the enzyme. The reaction of the enzyme with the mercurial is definitely slower when 20 mM phosphate is added to the reaction.

Discussion

When the mercurial associated with phosphoglycerate kinase, there was an increase in the pK_a value of the phenolic hydroxyl from 6.60 to 8.30 and a change in the wavelength and magnitude of the absorbance maxima for the acidic and basic forms of the mercurial (Figure 1). McMurray and Trentham (1969) reported that the hydrophobic environment induced by 20% acetone increased the maximum extinction, at 410 nm, of the ionized form of 2-chloromercuri-4-nitrophenol by 20%. When the mercurial is bound to phosphoglycerate kinase at pH 10.4 there is a 12% decrease in the 410-nm extinction. At pH 4.4 the enzyme decreases the extinction maximum of the protonated form by 8% and shifts the maximum from 322 to 327 nm. A comparison of mercurial spectral changes induced by phosphoglycerate kinase, model thiol compounds, organic solvents, and denaturing solutions should allow deductions about the environment of the single thiol group of the enzyme. This will be the subject of a subsequent publication. A consideration of the pK_a increase of the mercurial to 8.30 and the relatively slow reaction of the enzyme with thiol reagents suggests a strongly hydrophobic (low dielectric constant) pocket for the reagent. However, the complex of EDTA and mercurial has a pK_a of 9.02 and implies that a high charge density could also bring about the increase.

Nbs_2 reacts 10^4 times slower with the thiol of phosphoglycerate kinase than does the mercurial (Table I). The experiments of Arvidsson and Larsson-Raźnikiewicz (1973) indicate a rate constant for the Nbs_2 reaction almost two orders of magnitude slower, under their conditions, than the fastest rate of 0.28 sec^{-1} M^{-1} reported here. This difference could at least partly be accounted for by the approximately 150 mM phosphate buffer used by these investigators.

In concurrence with Ikai and Tanford (1971), the results from the renaturation experiments suggest that during renaturation the enzyme passes through intermediate states that may or may not lead back to the native conformation. The correct pathway is favored by slowness of the transition steps and the presence of constituents, *i.e.*, substrates, that "lock" the correct conformation of the enzyme. From results reported here and those of others (Krietsch and Bücher, 1970; Arvidsson and Larsson-Raźnikiewicz, 1973), it appears that the sulfhydryl group of phosphoglycerate kinase is not in close proximity to the active site. These findings agree with the 3.5-Å resolution model of the enzyme (Bryant *et al.*, 1974) that places the cysteine residue some 30 Å from the catalytic site. At pH 8.3, the pH of electrophoresis, the phenolic hydroxyl should add one-half a negative charge to the protein-mercurial complex although the complex would also lose negativity, relative to the native enzyme, through reaction of the thiol residue. Barring any change in the conformation of the protein that would alter its surface charge and thereby its electrophoretic mobility, the pK_a of the thiol in the native protein must be less than 8.3. This value is at the lower end of the range suggested for the pK_a of protein sulfhydryl groups (Webb, 1963).

The rate of mercurial reaction with phosphoglycerate kinase in the presence of both $MgADP^{-}$ and 3-phosphoglycerate is intermediate between those rates obtained with independent additions of the two substrates. This suggests that both of these substrates are capable of binding simultaneously to the enzyme, a conclusion reached by Roustan *et al.* (1973) from direct difference spectrophotometry. These authors interpret their results as being consistent with three separate binding sites, one each for the two substrates and one for the phosphoryl group to be transferred. Could the effects of phosphate on the reaction rate of enzyme with Nbs_2 and mercurial (Table I) result from an association of the ion with the "phosphoryl binding site?" Unfortunately, the magnitudes of the effects of the two nucleotide substrates on the rate of reaction between mercurial and enzyme are similar, and hence the data are of no value in deciding whether there are two separate binding sites for the two nucleotides (Krietsch and Bücher, 1970). If the ability of both $MgATP^{2-}$ and 3-phosphoglycerate to alter the rate of reaction between enzyme and mercurial is the result of a specific binding to the enzyme, then a random addition of substrates is suggested for the catalytic mechanism. Kinetic analyses have also suggested a random order mechanism

(Larsson-Raźnikiewicz and Arvidsson, 1971). The enzyme thiol group is far enough removed from the active site such that its chemical modification does not alter catalytic efficiency and yet the enzyme's substrates do modify the reactivity of the thiol group with mercurial and with Nbs_2. These results and the effects of substrates on phosphoglycerate kinase bound mercurial pK_a values suggest that the enzyme undergoes conformational change(s) subsequent to substrate binding. Alternatively, certain of the results could be explained by steric blocking effects of substrates.

Structural changes could alter the reactivity of the thiol either by altering the accessibility of the group to the mercurial or by causing a change in the pK_a value of the sulfhydryl, if its reaction with mercurial is dependent on its ionization state.

The experiments reported here show that the spectral properties of 2-chloromercuri-4-nitrophenol bound to phosphoglycerate reflect different structural states of the enzyme. Phosphoglycerate kinase has only one sulfhydryl group and hence there is no problem with migration of the reagent from one thiol to another. The mercurial mentioned above is particularly useful since the pK_a of the hydroxyl is within the physiological range. Additional experiments utilizing this conformational probe are in progress.

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