A New Metal-Binding Site for Yeast Phosphoglycerate Kinase as Determined by the Use of a Metal-ATP Analog

Kameshwari M. Pappu, Baburaj Kunnumal, and Engin H. Serpersu
Department of Biochemistry, Cellular and Molecular Biology, University of Tennessee, Knoxville, Tennessee 37996-0840 USA

ABSTRACT Suicide substrate β, γ-bidentate Rh(III)ATP (RhATP) was used to map the metal ion-binding site in yeast phosphoglycerate kinase (PGK). Cleavage of the RhATP-inactivated enzyme with pepsin and subsequent separation of peptides by reverse-phase high-performance liquid chromatography gave two Rh-nucleotide bound peptides. One of the peptides corresponded to the C-terminal residues of PGK, and the other to a part of helix V. Of the four glutamates present in the C-terminal peptide, Glu 398 may be a likely metal coordination site. Therefore, importance of the C-terminal residues in PGK catalysis may be attributed, in part, to the coordination of metal ion of the metal-ATP substrate. Metal coordination may then align the C-terminal peptide to extend toward the N-terminal domain and form the "closed" active site. Results presented in this paper suggest that one or more side chains of the enzyme may be coordinated to the metal ion in the PGK·3-phospho-D-glycerate·RhATP complex, and that exchange-inert metal-ATP analogs could be used to determine metal coordination sites on kinases and other metal-ATP-utilizing enzymes.

INTRODUCTION

Phosphoglycerate kinase (PGK) is a monomeric enzyme with a mass of approximately 46 kDa. There is a high level of tertiary structure homology among the known PGK structures from horse muscle (Blake and Rice, 1981), yeast (Watson et al., 1982), pig muscle (Harlos et al., 1992), and *Bacillus stearrothermophillus* (Davies et al., 1994). Crystallographic studies show that these structures consist of two almost equal-sized domains, corresponding to the N- and C-terminal halves of the polypeptide chain.

3-Phospho-D-glycerate (3PGA) is found to bind to a cluster of basic amino acid residues in the N-domain of pig muscle PGK (Harlos et al., 1992). However, earlier crystallographic studies reported for yeast enzyme do not agree with this 3PGA-binding site (Watson et al., 1982). Both MgATP and MgADP are found to bind to the inner surface of the C-domain of PGK, in the region above the cleft. Watson et al. (1982) suggest that in the yeast enzyme the metal ion is hydrogen-bonded to the carboxyl group of aspartate 372, whereas a different location for the metal ion is described in the interaction of the two nucleotides with horse muscle PGK (Banks et al., 1979).

In all x-ray structures, the distance between the two substrate molecules was found to be too long to allow a direct transfer of the phosphoryl group between them. Hence a hinge-bending mechanism was proposed for bringing the substrates closer to each other within a "closed" active site (Watson et al., 1982; Blake and Rice, 1981; Banks et al., 1979). Earlier studies from this laboratory (Gregory and Serpersu, 1993) offer the first evidence that in the ternary complex of yeast PGK, substrates are located close enough to each other to allow for direct transfer of the phosphoryl group in solution. This is consistent with the inversion of configuration observed at the γ-phosphorus atom of MgATP in the reaction catalyzed by PGK (Webb and Trentham, 1980). We have also demonstrated that only the β, γ-bidentate RhATP (and not α, β, γ-tridentate RhATP) is a substrate for PGK, and both ATP and 3PGA are coordinated to the metal ion in the ternary complex (Pappu et al., 1994). Based on these observations, it was suggested that the entry of 3PGA into the coordination sphere of metal of the metal-ATP complex may initiate conformational changes to form a catalytically relevant "closed" active site between the two substrate-binding domains of the enzyme. However, it is not known whether the proximity of the substrates is due to the closure of the active site or simply to binding of substrates to proximal sites on the enzyme. To this end, distances determined between the metal ion and several histidine residues suggest that some of the histidines move at least 6 Å upon the formation of the ternary complex (Pappu and Serpersu, 1994). This observation is consistent with earlier data and is indicative of substrate-induced conformational changes in PGK. Accurate interpretation of these results requires that the metal-binding site be known precisely.

To investigate the role of Asp 372 in metal binding and kinase reaction, a D372N mutant yeast PGK was constructed by site-directed mutagenesis (Minard et al., 1990). In this study, the authors conclude that either the role of the metal-coordinating residue in phosphoryl transfer reactions is not well understood, or Asp 372 is not the metal coordination site in yeast PGK. Recently, the crystal structure of a mixed cocrystallized/soaked ternary complex of pig muscle PGK containing 3PGA and Mn-5′-adenylylimidodiphosphate (Mn-AMP-PNP) has been solved (May et al., 1996). In this ternary complex, the nucleotide is bound to PGK in

Received for publication 13 September 1996 and in final form 18 November 1996.

Address reprint requests to Dr. Engin H. Serpersu, Department of Biochemistry, Cell and Molecular Biology, M407, Walters Life Sciences Building, University of Tennessee, Knoxville, TN 37996-0840. Tel.: 423-974-2668; Fax: 423-974-6306; E-mail: serpersu@bionmr.bio.utk.edu.

0006-3495/97/02/928/08 $2.00

a manner similar to that of MgADP (Banks et al., 1979), with the exception that the metal ion is coordinated to all three α, β, and γ-phosphates of AMP-PNP, and not by the protein. However, the structure of a cocrystallized ternary complex of R65Q mutant of yeast PGK with Mg-AMP-PNP and 3PGA revealed that magnesium ion is not only coordinated to all phosphate groups of AMP-PNP, but may also be coordinated to the carboxyl group of Asp 372 (McPhillips et al., 1996). In both structures, the 3PGA molecule is observed to occupy the center of the basic patch region in the N-domain, in a manner similar to that in the binary complex (Harlos et al., 1992). However, both of the ternary complexes represent an "open" conformation of PGK, as the distance between the bound substrates is found to be too long for direct transfer of the phosphoryl group. While noting that the mechanism proposed by Pappu et al. (1994) cannot be ruled out, May et al. (1996) invoke a hinge-bending mechanism (Banks et al., 1979; Blake and Rice, 1981) to model the closure of the gap between the bound substrates in the psuedoternary crystal structure of pig muscle PGK. McPhillips et al. (1996) postulate that a "twisting" type motion around the interdomain region would bring the substrates together for catalysis. They also suggest that 3PGA may enter into the coordination sphere of metal ion to start the catalysis, reasserting what was shown earlier by Pappu et al. (1994).

It is clear that determination of the metal-binding site is essential to understanding the catalytic mechanism of this enzyme. Therefore, this paper describes attempts to determine the metal-binding site of PGK by using suicide substrate RhATP. A preliminary account of this work was presented earlier (Pappu and Serpersu, 1996).

MATERIALS AND METHODS

Materials and general procedures

Yeast phosphoglycerate kinase was isolated from yeast strain 20B12 containing the multicopy plasmid pCGY219. The plasmid and the yeast strain were kindly provided by Dr. Hitzeman (formerly from Genetech). The purification of the enzyme was done as described earlier by Scopes (1971). As determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the enzyme was greater than 98% pure. When assayed under saturating substrate concentrations in the absence of sulfate at 25°C and pH 7.5, a specific activity of ~550 μmol/min/mg was observed for the purified enzyme. Enzyme concentration was determined from its absorbance at 280 nm, using $\epsilon^{0.1\%}_{1\,cm} = 0.5$. RhATP complexes were prepared as described (Lin et al., 1984). Rhodium chloride was purchased from Aldrich Chemical Co (Milwaukee, WI). All other chemicals used were of the highest grade available commercially. Stock buffer solutions were passed over Chelex 100 to remove trace metals before use.

Inactivation of phosphoglycerate kinase

Inactivation of phosphoglycerate kinase by RhATP was performed at a concentration of 7 mg/ml enzyme in a total volume of 300 μl at 37°C. The final concentration of RhATP was 5 mM. The incubation medium also contained 4 mM 3PGA in 5 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.9. As described earlier by Pappu et al. (1994), at the beginning and the end of a 12-h incubation, 5-μl aliquots were withdrawn and the remaining enzyme activity was determined spectrophotometrically. The inactivated sample was stored in an ice bath for no more than 12 h.

Proteolytic digestion of the inactivated protein

pH of the solution containing RhATP-inactivated phosphoglycerate kinase (7 mg/ml) was adjusted to 4.7 and digested with pepsin for 30 min at room temperature. The substrate-to-enzyme ratio used was approximately 160:1 (w/w).

Separation of Rh-nucleotide containing peptide(s)

Digestion of the inactivated enzyme was stopped by the addition of 25 μM pepstatin, and the sample was kept on ice. Using a superPAC cartridge column (4.0 × 125 mm), the peptide fragments were separated by reverse-phase high-performance liquid chromatography (HPLC) (Pharmacia LKB Biotechnology). The solvent system used was 0.1% trifluoroacetic acid in 5% acetonitrile in water (solvent A) and 80% acetonitrile in water containing 0.1% trifluoroacetic acid (solvent B). A linear gradient was run from 0 to 60% solvent B at 60 min. The flow rate was 0.4 ml/min. Absorbance was monitored at 220, 290, and 315 nm for the detection of Rh-nucleotide bound peptide(s).

To improve the sensitivity of detection by HPLC, elution with a different mobile phase was also performed. The solvent system used was 6 mM HCl in water (solvent A) and 80% acetonitrile in water containing 6 mM HCl (solvent B). A linear gradient was run from 0 to 60% solvent B at 60 min. The flow rate was 0.4 ml/min. The isolated peak fractions were further purified by HPLC, with a linear gradient run from 0 to 35% solvent B at 10 min followed by a successive gradient to 45% solvent B at 40 min. The flow rate was 0.4 ml/min.

Absorption spectroscopy

The two peaks of interest, separated by reverse-phase HPLC, were collected, lyophilized, and each dissolved in 300 μl water. The absorption spectra of the peptides were recorded on a Hewlett-Packard Diode-array spectrophotometer, in the region of 220–350 nm.

Peptide sequencing

Sequencing of the isolated peaks was kindly performed by Dr. Carol Beach of the University of Kentucky's Macromolecular Structure Analysis Facility, using automated Edman degradation.

Mass spectrometry

Positive-ion electrospray mass spectra were obtained on a Quattro-II quadrupole instrument (Micromass, Manchester, England). A solution of peptide (~2 μM) in acetonitrile-water (1:1) containing 0.2% formic acid was sprayed into the API source at 5 μl/min while the quadrupole filter was scanned from 200 to 4000 Da at 400 Da/s.

^{1}H NMR spectroscopy

The two isolated peaks, separated by reverse-phase HPLC, were lyophilized and each dissolved in 400 μl of 99.8% ^2H_2O. ^{1}H NMR spectra of the peaks were recorded on a Bruker AMX wide-bore spectrometer operating at 400 MHz and equipped with a quad-nuclear (^{1}H, ^{11}B, ^{13}C, ^{31}P) probe. Quadrature phase detection and a 90° observation pulse (12.9 μs) were used at 27°C. 32K data points were collected over a spectral width of 6009 Hz with an acquisition time of 2.73 s. A total of 10,000 scans were collected. Chemical shift values were reported relative to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). 1-D spectra were processed with 3 Hz line broadening. No resolution enhancement was used.

RESULTS AND DISCUSSION

Previous work from this laboratory showed that the inactivation of yeast phosphoglycerate kinase by RhATP was a time-dependent pseudo-first-order reaction, which was slow and reached a steady state only after a few hours (Pappu et al., 1994). This is consistent with the mechanism (Morrison and Walsh, 1988)

$$E + I \leftrightharpoons EI \leftrightharpoons EI^*,$$

in which enzyme (E) and inhibitor (I) rapidly form EI, which then slowly isomerizes to EI*. The above observations suggest that at least one enzyme ligand is coordinated to Rh(III) in the inactive enzyme·RhATP complex. Although β, γ-bidentate RhATP is a substrate (Pappu et al., 1994), only a single catalytic turnover is observed. This is due to the extremely slow ligand-exchange rates of Rh(III) (Monsted and Monsted, 1989), which cause the reaction products to be trapped in the enzyme active site. Therefore, we decided to take advantage of this finding and attempted to isolate a Rh-nucleotide containing peptide from the inactivated enzyme to identify the metal coordination site(s).

PGK was inactivated by RhATP in the presence of 3PGA (residual activity was approximately 15%) and digested with pepsin as outlined under Materials and Methods. Peptide fragments were separated by HPLC using a reverse-phase column. Fig. 1 *A* is the HPLC trace of the digested enzyme as monitored at 220 nm. Fig. 1 *B* is the HPLC trace of the same digested sample as monitored at 290 nm. Two broad peaks (marked as 1 and 2) eluting at approximately 35% and 39% solvent B, respectively, were collected separately. Traces shown in Fig. 1, *A* and *B*, were reproducible qualitatively and quantitatively. This allowed for several HPLC separations, to accumulate sufficient amounts of peak fractions 1 and 2 (Fig. 1 *B*), for further analyses. Each peak fraction was separately lyophilized and dissolved in 300 μl of water. An aliquot from each fraction was subjected to automated Edman degradation. Sequence analysis by Edman degradation did not permit the identification of the residue coordinated by Rh(III), as the variation of pH during the process could cause dissociation of the metal ion from the peptide. As shown in Table 1, peaks 1 and 2 (as marked in Fig. 1 *B*) gave the same major amino acid sequences.

The peptide sequence beginning with residue 397 or 398 is the major component in one of the two peak fractions. As this sequence corresponds to the C-terminal segment of PGK, it can at most be 18–19 amino acids long, i.e., residues 397 or 398–415 (because residue 415 is the C-terminus of yeast PGK). This C-terminal peptide has four glutamates (residues 398, 401, 404, and 413). The second peptide sequence beginning with residue 174 is also common to both peaks. This peptide contains Asp 177 and may contain Glu 188 and Glu 190 if it is at least 18 amino acids long (maximum length of the C-terminal peptide starting with Glu 398). The above results suggest that either separation of the peptides by HPLC is incomplete or the metal ion may be coordinated to more than one residue, hence eluting with two different peptides in its coordination sphere.

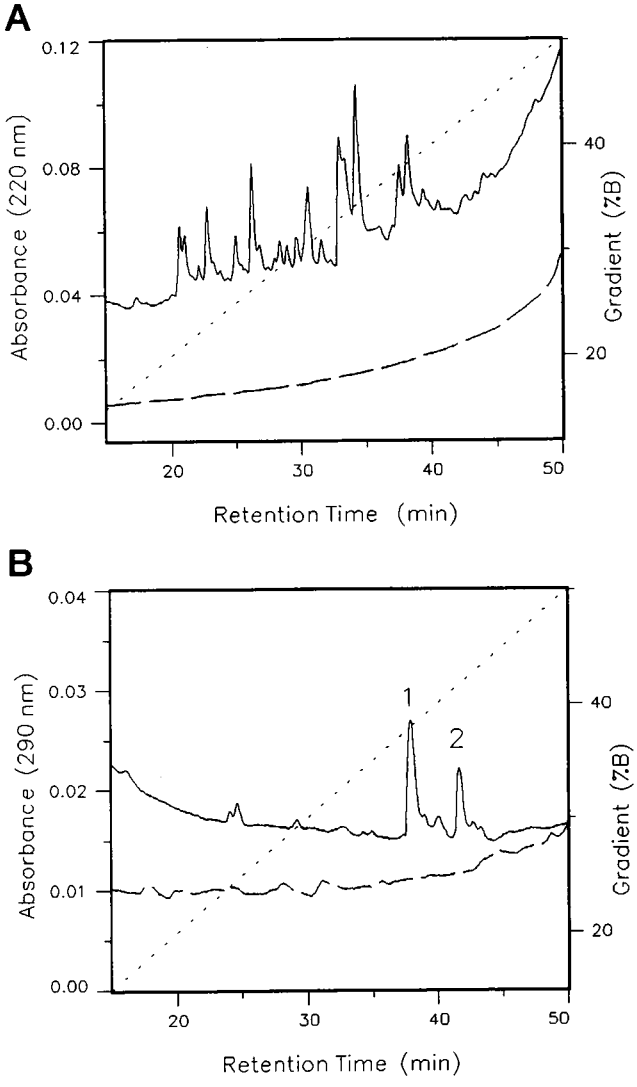

FIGURE 1 Separation of a pepsin digest of RhATP-inactivated phosphoglycerate kinase by reverse-phase HPLC. The enzyme was inactivated by 5 mM RhATP in the presence of 4 mM 3PGA for 12 h, digested with pepsin, and separated by elution with a linear gradient of 0.1% TFA in acetonitrile, as described under Materials and Methods. (*A*) Profile of the digest of the inactivated enzyme as monitored at 220 nm. (*B*) Profile of the digest of the inactivated enzyme as monitored at 290 nm. The two peaks labeled 1 and 2 were sequenced by Edman degradation. The long dashed line indicates the base line; the dotted line indicates the elution gradient.

To test the peptide separation by HPLC, dilute HCl was used as the mobile phase modifier. HCl is an alternative to TFA, because it has better optical clarity in UV. It also provides different separation selectivity for peptide mapping. Fig. 2 *A* is the HPLC trace detected at 290 nm of the digested enzyme separated in the presence of HCl as the mobile phase. Although HCl did not improve the resolution of the peptides, the sensitivity of detection was enhanced, as is apparent in the reduced baseline shift over the gradient.

TABLE 1 Sequence analysis by Edman degradation

Sample	Sequence*
Peak 1#	1) ^{398}ELLEGKE‖
	2) ^{174}VGFDLPQ‖
Peak 2§	1) ^{397}LELLEGK‖
	2) ^{174}VGFDLPQ‖

*Seven cycles of sequencing were performed.
#Small amount of contaminating peptide ^{331}IVWNGPP present.
§Small amount of contaminating peptide ^{284}IIADAFS present.
‖Peptide 1/peptide 2 ratios were 7/2.5 and 1/1 for the peaks 1 and 2, respectively.

The broad peak eluting between 41 and 44% eluant B was collected as two separate fractions (marked as 3 and 4 in Fig. 2 *A*). Peak fraction 3 was further purified on a shallower gradient as described under Materials and Methods. The HPLC trace as monitored at 290 nm is shown in Fig. 2 *B*. The two sharp peaks were collected separately, and an aliquot was lyophilized and sequenced. Despite the homogeneity of the peaks, the same peptides shown in Table 1 were obtained with slightly altered ratios. Analysis by mass spectrometry yielded a few major peaks, up to a molecular mass of 3500 Da, which is consistent with the above findings. Further analysis of the MS data was not straightforward, because of the presence of two peptides and possibly substrates (not necessarily in one-to-one ratio) in these samples. Therefore, these observations suggest that the metal ion may be coordinated to more than one site on the enzyme.

A major question to be answered was whether Rh-nucleotide was still associated with the purified peptides. RhATP exhibits maximum absorption in the visible region at 415 nm and at 327 nm, whereas RhADP exhibits maximum absorption at 409 nm and 315 nm (Lin et al., 1984). As the molar extinction coefficients (ϵ) of RhATP and RhADP in the visible region are very low, isolation of RhADP-bound peptide(s) while monitoring at 315 nm is very difficult. However, we were able to observe two peaks with a low signal-to-noise ratio in the HPLC traces (data not shown). Because the detection of absorption due to bound RhADP was difficult, it became necessary to use other techniques to demonstrate the presence of nonpeptide components associated with the purified peptides.

In Fig. 3, traces A and B represent the absorption spectra of peak fractions 1 and 2, respectively. Both the fractions have a maximum absorbance at 258 nm, suggesting the presence of a bound nucleotide. This would be possible only if the nucleotide were associated with the peptides through Rh(III). Sequencing data (Table 1) indicated that peak 1 contained a contaminating peptide that has a single tryptophan residue. Even if some of the absorbance at 258 and 290 nm was contributed by tryptophan, contribution of this single residue to the observed absorption will be less than 5% of the total absorbance. Likewise, peak 2 may have a tryptophan (Trp 308) only if the contaminating peptide beginning with residue 284 is at least 25 amino acids long.

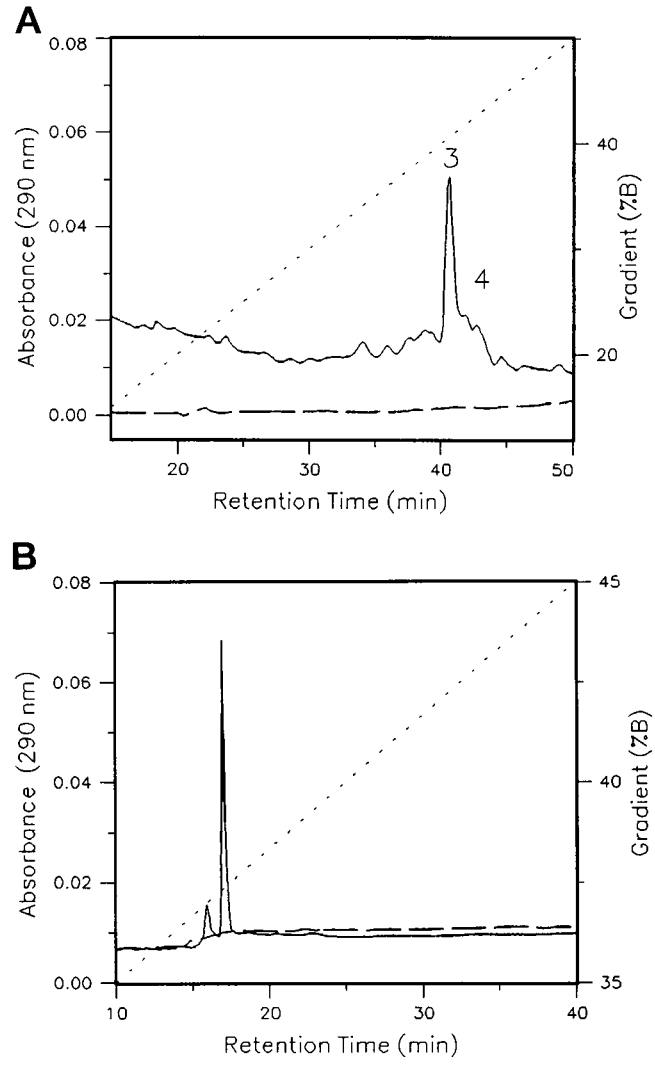

FIGURE 2 Separation of a pepsin digest of RhATP-inactivated phosphoglycerate kinase by reverse-phase HPLC. The enzyme was inactivated by 5 mM RhATP in the presence of 4 mM 3PGA for 12 h, digested with pepsin, and separated by elution with a linear gradient of 6 mM HCl in acetonitrile, as described under Materials and Methods. (*A*) Profile of the digest of the inactivated enzyme as monitored at 290 nm. (*B*) Profile of peak 3 (marked in *A*) repurified on a shallower gradient, as monitored at 290 nm. The two peaks obtained were sequenced by Edman degradation. The long dashed line indicates the base line; the dotted line indicates the elution gradient.

Contribution of this residue (and phenyl alanines) to the observed absorbance would again be insignificant. Thus a nucleotide must contribute significantly to the observed UV spectrum of the peptides.

Fig. 4, traces A and B, are the one-dimensional ^{1}H NMR spectra of peaks 1 and 2, respectively. In addition to aromatic resonances of the peptides, two proton resonances are seen in the downfield region at 8.6 and 8.5 ppm. Adenine H8 and H2 resonate in the downfield region at approximately 8.5 ppm and 8.3 ppm, respectively. The resonances at 8.6 and 8.5 ppm may represent an altered environment of adenine H8 and H2 resonances, clearly indicating the pres-

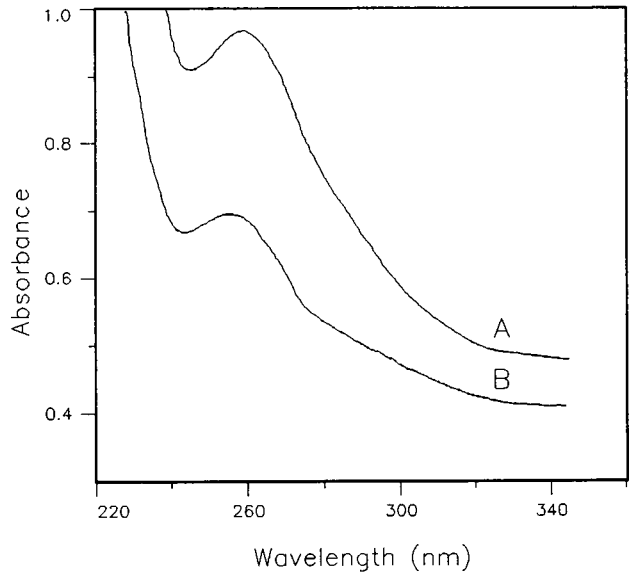

FIGURE 3 Absorption spectra of the isolated peaks. Traces *A* and *B* are the spectra of peaks 1 and 2 (marked in Fig. 1 *B*), respectively.

ence of adenine nucleotide attached to the peptide(s). In these spectra, the broad H8 proton resonance is barely visible and the doublet of the H1′ resonance of ribose ring is not observable, because of the low signal-to-noise ratio. This observation confirms the presence of a bound adenine nucleotide in the peptides and strongly suggests that the association of a nucleotide with the purified peptides can only be possible through coordination by Rh(III). Thus the above data suggest that these peptides are likely to contain the metal coordination site(s) of yeast PGK.

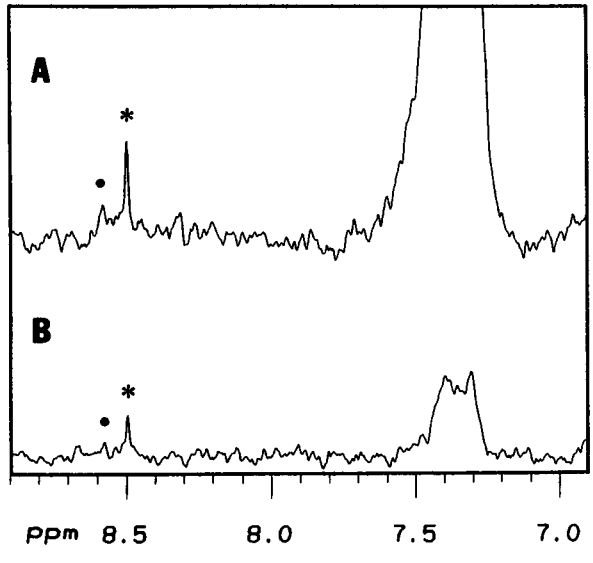

FIGURE 4 ^{1}H NMR spectra of the isolated peaks. Traces *A* and *B* are the spectra of peaks 1 and 2 (marked in Fig. 1 *B*), respectively. Resonances marked by * and ● in both traces represent C2 and C8 protons of the adenine ring, respectively. A total of 10,000 scans per spectrum were recorded. The spectra were processed with 3-Hz line broadening.

The presence of two major peptides could be attributed to the fact that the metal ion may be changing its coordination from one residue to another during the catalytic cycle. Alternatively, metal ion could coordinate more than one side chain of the protein, simultaneously. Although our data cannot distinguish between the two possibilities, we are currently suggesting that one or more enzyme side chains may be coordinated to metal ion in the PGK·3PGA·RhATP complex. Our data cannot exclude the possibility of metal ion changing its coordination during proteolysis. This is, however, unlikely, because ligand exchange rate of Rh is extremely slow, and the same peptides are reproducibly isolated, even under different chromatographic conditions. Fig. 5 shows the expected coordination of RhATP to one of the peptides. Because it is known that 3PGA remains in the coordination sphere of Rh(III) (Pappu et al., 1994), 3PGA was shown in the inner coordination sphere of Rh(III) in this figure. Fig. 6 is the crystal structure of yeast PGK determined by Watson et al. (1982), highlighting the side chains involved in substrate binding along with the four glutamate residues in the C-terminal peptide. Glu 398 is closest to the "basic patch" residues (especially to His 167) and to the invariant triple glycine loop that is suggested to anchor the carboxyl group of 3PGA in the active-site cleft (Watson et al., 1982). Glu 401 is close, but seems to face away from the cleft between the two domains of the enzyme, whereas glutamates 404 and 413 are farther away. Therefore, either Glu 398 or Glu 401 may be the likely metal coordination site. The x-ray structure of wild-type PGK in a ternary complex is not available. The structure of R65Q mutant in the ternary complex, which is still in an open conformation, shows Glu 398 and Glu 401 to be near the nucleotide-binding pocket (McPhillips et al., 1996). This is also consistent with either one of these glutamates being in the coordination sphere of the metal ion.

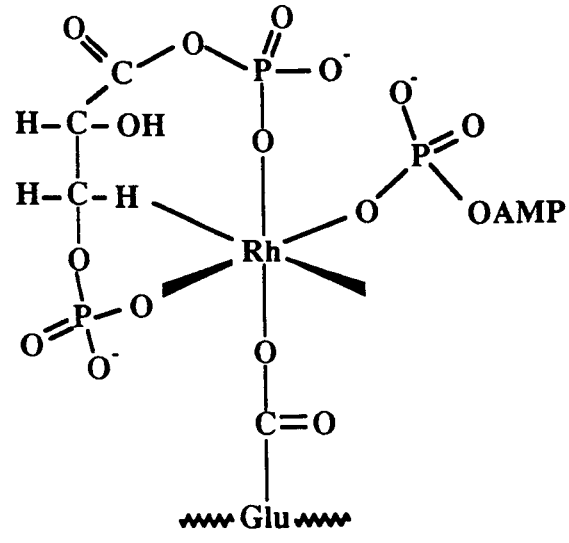

FIGURE 5 A possible coordination scheme for Rh-ATP in the active site of yeast phosphoglycerate kinase.

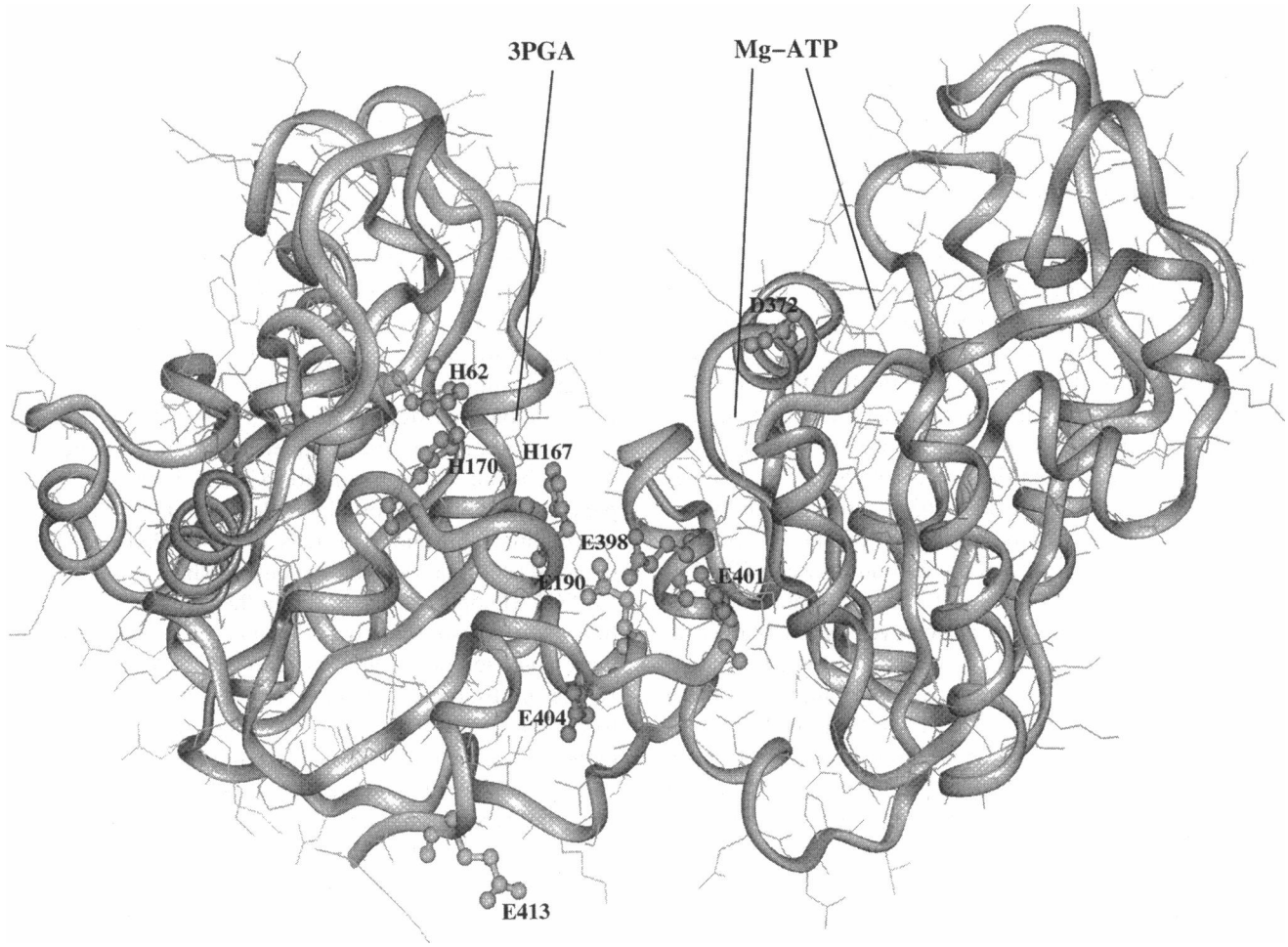

FIGURE 6 Crystal structure of yeast phosphoglycerate kinase. A ribbon diagram of PGK in an open conformation (Watson et al., 1982) is shown. The side chains of some active site residues along with Glu 190 in helix V and the four glutamate residues present in the C-terminal peptide are illustrated. The two lines from Mg-ATP mark the approximate boundaries of the binding site of this substrate, on the inner surface of the C-domain of PGK, above the cleft. 3PGA is proposed to bind to the N-domain of PGK.

However, preliminary results of mutations at Glu 398 and Glu 401 in PGK showed that the E398K and E398A mutants have lost activity by at least an order of magnitude, whereas E401K mutant exhibited activity similar to that of wild-type enzyme (details to be published later). This suggests that Glu 398, not Glu 401, may be involved in metal ion coordination. Additional data consistent with this hypothesis are obtained from the chemical modification studies of the carboxyl groups of the wild-type and mutant enzymes. E401K mutant behaved exactly like the wild-type enzyme, whereas E398K is not inactivated as fast as these two enzymes (data not shown). Longer incubation caused all enzymes to be inactivated after several hours. These observations are also consistent with Glu 398 functioning as a metal coordination site in PGK. Another relevant and interesting observation worth noting is that the metal ion-to-His 167 distance is lengthened by about 1.2 Å, upon binding of 3PGA to PGK·metal-ATP, as determined by NMR (Pappu and Serpersu, 1994). This observation is also consistent with the hypothesis that Glu 398 may be better positioned to coordinate the metal ion of the metal-ATP substrate. Therefore, to position 3PGA in the active site cleft, His 167 may have to be pushed away from the metal ion (Fig. 6).

The importance of the C-terminal residues of PGK has been shown earlier. Removal of the C-terminal peptide (12 to 18 residues) yielded mutant enzymes with approximately 0.1% activity (Ritco-Vonsovici et al., 1995; Mas and Resplandor, 1988), which is attributed to the loss of cooperativity between the N-terminal and C-terminal lobes, due to the loss of a helix formed by the C-terminal residues of PGK. Ritco-Vonsovici et al. (1995) suggest that the hydrophobic interactions between Leu 405 and Ala 395 and Leu 399 maintain helix XIII (residues 393–401) in the correct position for the catalytic process. This correct positioning of helix XIII would be critical if Glu 398 were the metal coordination site. Andrieux et al. (1995) showed that addition of the C-terminal complementary peptide (residues 404–415) to a mutant PGK (residues 1–403) increased its activity by 40-fold. Sherman et al. (1995) have also reported

that a L399W mutation resulted in a 50% loss in activity for PGK. Our results are in accord with these observations and suggest that the C-terminal residues may play more than just a structural role.

The second peptide sequence beginning with residue 174 may include Glu 188 and Glu 190, which are a part of helix V (residues 185–191) in the enzyme. Helix V is a covalent link between the two domains and is essential for enzyme activity and stability (Fairbrother et al., 1989). Asp 177 is not likely to be a coordination site, because of its position in the crystal structure of yeast enzyme; however, Glu 188 or Glu 190 may also be involved. Harlos et al. (1992) observed local conformational changes close to the 3PGA binding site in the crystal structure of a binary complex of pig muscle PGK with 3PGA. As a result of these changes, Phe 163 and His 167 were also shifted, although they themselves do not interact with 3PGA. Phe 163 is close to Glu 190 in the crystal structure, and the movement of Phe 163 seemed to affect Glu 190 as well. An interaction between Glu 190 and His 388 has been proposed to be important in hinge-bending (Watson et al., 1982). However, when Mas et al. (1987) used site-directed mutagenesis to evaluate the nature of this interaction and the relative contributions of these residues to domain movement, their results argued against a possible role of the hydrogen-bonding interactions between Glu 190 and His 388 in the conformational transition of PGK. Interestingly, E190D and E190Q mutants exhibited a seven- to eightfold decrease in activity (Mas et al., 1987).

CONCLUSIONS

The catalytically relevant "closed" conformation of PGK is still unknown, despite the availability of several crystal structures of the enzyme with substrates. In addition, substrate and metal-binding sites are not well resolved. Watson et al. (1982) suggest that the metal ion is hydrogen-bonded to the carboxyl group of Asp 372 in yeast PGK. In the recently determined structure of a ternary complex of yeast R65Q mutant PGK (McPhillips et al., 1996), metal ion is suggested to be coordinated to Asp 372. However, in the ternary complex of pig muscle PGK, metal ion is not coordinated to protein side chain (May et al., 1996). Our findings do not support Asp 372 as the metal coordination site. It is possible that the metal ion may be changing its coordination from one residue to another during the catalytic cycle, and coordination to Asp 372 may be an early event. Rh(III) exhibits extremely slow ligand exchange rates (Monsted and Monsted, 1989), making the above possibilty less likely. However, hydrogen bond formation between Asp 372 and a water molecule in the coordination sphere of Rh(III) cannot be excluded. Two Rh-nucleotide-bound peptides are consistently isolated; our data suggest that more than one side chain of the enzyme may be coordinated to the metal ion in the PGK·3PGA·RhATP complex. It is not clear whether the metal ion is simultaneously or sequentially coordinated to enzyme side chains during catalysis.

Results described in this paper agree well with earlier observations on the importance of C-terminal residues in catalysis. Because Glu 398 is on this peptide, truncation of the C-terminal residues may affect metal coordination, which is important in aligning the substrates. Metal coordination may then cause the C-terminal peptide to extend toward the N-terminal domain to form the "closed" active site. Data published earlier (Pappu et al., 1994) showed that RhATP arrests the enzyme in a conformation where the substrates are not exposed to solvent, thus indicating the formation of a closed enzyme active site during catalysis. Such a closed active site has not been observed in crystallographic studies of the ternary enzyme·3PGA·metal-AMP-PNP complexes (McPhillips et al., 1996; May et al., 1996). This may be, in part, a consequence of the tridentate metal-ATP substrate being bound to PGK. In both ternary complexes, the authors reported that the metal ion is coordinated to all three phosphoryl groups of AMP-PNP. Earlier we demonstrated that only the bidentate RhATP is a substrate for PGK (Pappu et al., 1994). On the basis of this observation, we suggested that the tridentate metal-ATP would be more compact and unable to reach 3PGA for coordination. Hence the tridentate complex may not be able to start the formation of a closed active site. Both reported crystallographic structures are consistent with this prediction.

It is known that tridentate metal-ATP binds PGK tighter than the bidentate complex (Dunaway-Mariano and Cleland, 1980). Therefore it is not suprising that both crystal forms of PGK have tridentate metal-AMP-PNP at the active site, because in a solution of Mg(II) or Mn(II) and AMP-PNP, both bidentate and tridentate complexes would be present. Exchange-inert stable metal-ATP complexes like CrATP and RhATP, on the other hand, can be prepared in a stable bidentate form (Lin et al., 1984). Therefore, results presented in this paper suggest that RhATP may be a suitable analog for studying the metal coordination site of PGK, and may be extended to other metal-ATP-utilizing enzymes.

We thank Dr. Jorge Churchich of this department for allowing us the use of his HPLC. We also thank Drs. Kelsey Cook and Al Tuinman of the Chemistry Department for analyzing peptide samples by mass spectrometry.

The National Science Foundation Chemical Instrumentation Program contributed to the acquisition of the Quattro-II mass spectrometer (grant BIR-94-08252). This work was supported by National Institutes of Health grant R29 GM 42661 (EHS).

REFERENCES

Andrieux, M., E. Leroy, E. Guittet, M. Ritco-Vonsovici, B. Mouratou, P. Minard, M. Desmadril, and J. M. Yon. 1995. Transferred nuclear Overhauser effect study of the C-terminal helix of yeast phosphoglycerate kinase: NMR solution structure of the C-terminal bound peptide. *Biochemistry.* 34:842–846.

Banks, R. D., C. C. F. Blake, P. R. Evans, R. Haser, D. W. Rice, G. W. Hardy, M. Merrett, and A. W. Phillips. 1979. Sequence, structure and activity of phosphoglycerate kinase: a possible hinge-bending enzyme. *Nature.* 279:773–777.

Blake, C. C. F., and D. W. Rice. 1981. Phosphoglycerate kinase. *Philos. Trans. R. Soc. Lond. Biol.* 293:93–104.

Davies, G. J., S. J. Gamblin, J. A. Littlechild, Z. Dauter, K. S. Wilson, and H. C. Watson. 1994. Structure of the ADP complex of the 3-phosphoglycerate kinase from *Bacillus stearothermophilus* at 1.65 Å. *Acta Crystallogr.* D50:202–209.

Dunaway-Mariano, D., and W. W. Cleland. 1980. Investigations of substrate specificity and reaction mechanism of several kinases using chromium(III) adenosine 5′-triphosphate and chromium(III) adenosine 5′-diphosphate. *Biochemistry.* 19:1506–1515.

Fairbrother, W. J., D. Bowen, L. Hall, and R. J. P. Williams. 1989. One- and two-dimensional NMR studies of yeast phosphoglycerate kinase. *Eur. J. Biochem.* 184:617–625.

Gregory, J. D., and E. H. Serpersu. 1993. Arrangements of substrates at the active site of yeast phosphoglycerate kinase. *J. Biol. Chem.* 268: 3880–3888.

Harlos, K., M. Vas, and C. F. Blake. 1992. Crystal structure of the binary complex of pig muscle phosphoglycerate kinase and its substrate 3-phospho-D-glycerate. *Proteins Struct. Funct. Genet.* 12:133–144.

Lin, I., W. B. Knight, S.-J. Ting, and D. Dunaway-Mariano. 1984. Preparation and characterization of rhodium(III) polyphosphate complexes. *Inorg. Chem.* 23:988–991.

Mas, M. T., and Z. E. Resplandor. 1988. Structure-function relationships in 3-phosphoglycerate kinase: role of the carboxy-terminal peptide. *Proteins Struct. Funct. Genet.* 4:56–62.

Mas, M. T., Z. E. Resplandor, and A. D. Riggs. 1987. Site-directed mutagenesis of glutamate 190 in the hinge region of yeast 3-phosphoglycerate kinase: implications for the mechanism of domain movement. *Biochemistry.* 26:5369–5377.

May, A., M. Vas, K. Harlos, and C. Blake. 1996. 2.0 Å resolution structure of a ternary complex of pig muscle phosphoglycerate kinase containing 3-phospho-D-glycerate and nucleotide Mn adenylylimidodiphosphate. *Proteins Struct. Funct. Genet.* 24:292–303.

McPhillips, T. M., B. T. Hsu, M. A. Sherman, M. T. Mas, and D. C. Rees. 1996. Structure of the R65Q mutant of yeast 3-phosphoglycerate kinase complexed with Mg-AMP-PNP and 3-phospho-D-glycerate. *Biochemistry.* 35:4118–4127.

Minard, P., D. J. Bowen, L. Hall, J. A. Littlechild, and H. C. Watson. 1990. Site-directed mutagenesis of aspartic acid 372 at the ATP binding site of yeast phosphoglycerate kinase: over-expression and characterization of the mutant enzyme. *Protein Eng.* 3:515–521.

Monsted, L., and O. Monsted. 1989. Mechanism of thermal and photochemical ligand substitution reactions of chromium(III) and other octahedral metal complexes. *Coord. Chem. Rev.* 94:109–150.

Morrison, J. F., and C. T. Walsh. 1988. The behavior and significance of slow-binding inhibitors. *Adv. Enzymol.* 61:201–301.

Pappu, K. M., J. D. Gregory, and E. H. Serpersu. 1994. Substrate activity of Rh(III)ATP with phosphoglycerate kinase and the role of the metal ion in catalysis. *Arch. Biochem. Biophys.* 311:503–508.

Pappu, K. M., and E. H. Serpersu. 1994. Proton NMR studies of a large protein. pH, substrate titrations, and NOESY experiments with perdeuterated yeast phosphoglycerate kinase containing [^{1}H]histidine residues. *J. Magn. Res.* 105:157–166.

Pappu, K. M., and E. H. Serpersu. 1996. A new metal-ATP binding site for phosphoglycerate kinase. *FASEB J.* 10:A1236. (Abstr.)

Ritco-Vonsovici, M., B. Mouratou, P. Minard, M. Desmadril, J. M. Yon, M. Andrieux, E. Leroy, and E. Guittet. 1995. Role of the C-terminal helix in the folding and stability of yeast phosphoglycerate kinase. *Biochemistry.* 34:833–841.

Scopes, R. K. 1971. An improved procedure for the isolation of 3-phosphoglycerate kinase from yeast. *Biochem. J.* 122:89–92.

Sherman, M. A., J. M. Beechem, and M. T. Mas. 1995. Probing intradomain and interdomain conformational changes during equilibrium unfolding of phosphoglycerate kinase: fluorescence and circular dichroism study of tryptophan mutants. *Biochemistry.* 34:13934–13942.

Watson, H. C., N. P. C. Walker, P. J. Shaw, T. N. Bryant, P. L. Wendell, L. A. Fothergill, R. E. Perkins, S. C. Conroy, M. J. Dobson, M. F. Tuite, A. J. Kingsman, and S. M. Kingsman. 1982. Sequence and structure of yeast phosphoglycerate kinase. *EMBO J.* 1:1635–1640.

Webb, M. R., and B. R. Trentham. 1980. Analysis of chiral inorganic [^{16}O, ^{17}O, ^{18}O] thiophosphate and the stereochemistry of the 3-phosphoglycerate kinase reaction. *J. Biol. Chem.* 255:1775–1779.