Arrangement of Substrates at the Active Site of Yeast Phosphoglycerate Kinase

EFFECT OF SULFATE ION*

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Previously structural studies of phosphoglycerate kinase have been ambiguous regarding the relative orientation of the substrates when bound at the active site. Crystals of yeast PGK were grown in solutions containing high concentrations of ammonium sulfate, but would not grow in the presence of MgATP or MgADP (10, 11). Therefore, the binding sites and the relative orientation of substrates were obtained from crystal soaking experiments that yielded very low occupancy with MgATP and no occupancy with 3PGA (10). The results of x-ray studies suggested that the γ-phosphoryl group of MgATP was 10-12 Å away from 3PGA (12). Previous studies have shown that phosphoryl transfer catalyzed by PGK occurs with inversion of configuration at phosphorus, suggesting a direct transfer mechanism (13). Additionally, several studies have shown that substrate binding induces large conformational changes in the enzyme (14-16). On this basis, a hinge-bending mechanism for closing the active site and bringing the two substrates into catalytically relevant orientation was proposed (11, 12, 14). The structure of the closed active site and the relative conformations of the bound substrates are not known. The x-ray structure of a binary pig muscle PGK-3PGA complex crystallized from polyethylene glycol solution has been recently published (17); the results of this study do not support the proposed location of the 3PGA-binding site suggested for the yeast enzyme. Furthermore, studies with site-specific mutations of several residues failed to confirm their specific roles for sulfate activation or metal binding as suggested by x-ray studies (18, 19). Studies from our laboratory† have also shown that a fluorescent ATP analog, pyridoxal 5'-diphospho-5'-adenosine, specifically labeled Lys-385 instead of lysine 213 or 217, which were suggested by crystallographic studies to interact with the polyphospho chain of ATP.

In this study, we have used a paramagnetic substrate analog, exchange-inert β,γ-bidentate CrATP, as a probe to determine various metal-to-nucleus distances between Cr²⁺ and 3PGA in the ternary PGK-CrATP-3PGA complex in the presence and absence of activating concentrations of sulfate ion. Metal-ADP complexes have been used with 3PGA and PGK in paramagnetic metal-to-phosphorus distance measurements...
Preparation of [U-13C]3PGA—[U-13C]PGA synthesis was achieved by combination and modification of two methods described previously for preparation of dihydroxyacetone phosphate + glycer-aldehyde 3-phosphate from fructose 1,6-diphosphate (29) and preparation of Ba-3PGA from yeast (30). In the first step, [U-13C]glycerol was incubated with 250 units of glycerol kinase, 500 μg of glycerophosphate dehydrogenase, and 50 μg of triose-phosphate isomerase in 1 M hydrazine-1,2-dimethyl-2-silapentane-5-sulfonate. To avoid the interference of Tris protons, the sample was composed of 0.35-0.45 mM PGK, 8.3 mM 3PGA, and either 60 mM NaCl or 20 mM sulfate in acetate buffer, pH 5.9. The paramagnetic effect of CrATP on the relaxation rates of 3PGA carbon atoms was measured at 62 MHz on a Bruker AC-250 spectrometer. T1 values were determined using the progressive saturation method (31) due to the extremely long delays needed for complete relaxation of the carbonyl carbon. Separate experiments showed that T1 values for the carbons of 3PGA were identical when determined with a 90° pulse followed by a 1 s delay to allow for complete relaxation and with 90° pulses longer than 1 s. The T1 values are referenced to external dioxane (67 ppm). Five to seven variable delays were used, ranging from 1 ms to 30 s. 90° pulse widths were around 5.2 μs. The paramagnetic effect of CrATP on the relaxation rates of 3PGA carbon atoms was measured at 161 MHz on a Bruker AMX-400 wide-bore spectrometer equipped with a 5-mm quad-nuclear (1H, 13C, 19F, and 31P) probe. Relaxation rates were determined by inversion recovery method with 500-μs pulses in H2O and 16 variable delay values ranging from 2 ms to 15 s. 90° pulse widths were around 5.2 μs. Chemical shifts are referenced to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate at 0 ppm. The 1H spectrum of 3PGA and either 60 mM NaCl or 20 mM sulfate in acetate buffer, pH 5.9. The paramagnetic effect of CrATP on the relaxation rates of 3PGA carbon atoms was measured at 62 MHz on a Bruker AC-250 spectrometer. T1 values were determined using the progressive saturation method (31) due to the extremely long delays needed for complete relaxation of the carbonyl carbon. Separate experiments showed that T1 values for the carbons of 3PGA were identical when determined with a 90° pulse followed by a 1 s delay to allow for complete relaxation and with 90° pulses longer than 1 s. The T1 values are referenced to external dioxane (67 ppm). Five to seven variable delays were used, ranging from 1 ms to 30 s. 90° pulse widths were around 5.2 μs. The binary titration sample consisted of 10.5 mM [U-13C] 3PGA in 50 mM MES, pH 5.9, with 20% H2O. Samples for the ternary titrations contained 0.3-0.4 mM PGK, 9.9 mM [U-13C]PGA, and either 60 mM NaCl or 20 mM sulfate in 50 mM MES, pH 5.9, with 20% H2O.

Heteronuclear 1H-13C correlated spectroscopy was done on a Bruker AMX-400 spectrometer using standard Bruker software. The temperature dependence of 1/T1 of the binary and ternary complexes was examined with [U-13C]CrATP, [U-13C]PGA, and either 60 mM NaCl or 20 mM sulfate in 50 mM MES, pH 5.9, at 200 MHz. Sample composition was as described above for analysis of the paramagnetic effect of CrATP on the protons of 3PGA.

Longitudinal relaxation rates of water protons were determined with a Seimco pulsed NMR spectrometer equipped with a variable frequency probe as previously described (32, 33) using the 180°-90° pulse sequence of Carr and Purcell (34) and a delay of 5 T, to allow for complete relaxation. T1 values, the paramagnetic contribution to the relaxation rate (1/T1), and evaluation of enhancements of paramagnetic effects of CrATP were determined as described (32, 33, 35). The correlation time for the dipolar electron-nucleus interaction in the ternary PGK-CrATP:3PGA complex in the 1H spectrum of 3PGA contains 20 mM sulfate in the ternary titration of water protons at 15, 24.3, 42, and 59 MHz with the Seimco NMR spectrometer using the null-point method (32, 33) and at 250 MHz on a Bruker AC-250 spectrometer and at 400 MHz on a Bruker AC-250 spectrometer and at 400 MHz on a Bruker AC-250 spectrometer.
RESULTS

Kinetic Studies—CrATP is a paramagnetic exchange-inert MgATP analog that is stable at pH 5.90 (28). To use CrATP as an NMR probe with PGK, it was first necessary to characterize the kinetics at pH 5.90 to assure that the kinetic behavior of the enzyme was unchanged from that observed in earlier studies at pH 7.5–7.8 (36, 37). As expected, the kinetic findings clearly showed that the kinetic behavior of PGK at pH 5.90 is unchanged when compared to the findings at pH 7.5–7.8 (36, 37).

Kinetic analysis of the inhibition of PGK by CrATP showed that CrATP is a linear competitive inhibitor with respect to MgATP (Fig. 1, a and b). Interestingly, however, the apparent $K_{i}$ for MgATP increased as the concentration of the fixed substrate (3PGA) was increased between 70 and 700 $\mu$M 3PGA (Fig. 1c). Linear extrapolation of the $K_{i}$ values observed in the presence of various concentrations of 3PGA yielded a $K_{i}$ of 70 ± 14 $\mu$M in the absence of 3PGA (Fig. 1c). In the presence of sulfate, CrATP was still a linear competitive inhibitor of the enzyme with respect to MgATP (data not shown), with a $K_{i}$ value of 725 ± 178 $\mu$M. However, the observed $K_{i}$ for CrATP was independent of the concentration of 3PGA (Fig. 1c). These findings suggest that the conformation of the enzyme active site is different in the presence of sulfate ion.

Mixed-type inhibition by CrATP was observed when 3PGA was the variable substrate (data not shown), with $K_{i}$ values of 40 ± 20 $\mu$M and $K_{i}$ (data not shown), with $K_{i}$ values of 570 ± 130 $\mu$M and 450 ± 100 $\mu$M.

Binding Studies—In order for the results to be easily interpretable, a 1 mol/1 mol stoichiometry of paramagnetic probe/enzyme must be established. Previous studies in this laboratory had shown a 1 mol/1 mol stoichiometry for the binding of [γ-32P]CrATP to PGK and the PGK-3PGA complex (24, 25), with no indication of CrATP binding to a second site even at ratios of CrATP to enzyme far exceeding those used in this study. These findings are in agreement with the earlier binding studies using MgATP, which showed that the dissociation constant of MgATP from the second nucleotide site is 30–50-fold higher than its dissociation constant from the active site (2, 38). The determined dissociation constants for binding of CrATP to the PGK-3PGA complex were 550 and 900 $\mu$M in the absence and presence of sulfate ion, respectively.

The formation of the ternary enzyme-CrATP-3PGA complex was monitored by NMR at 24.3 MHz by titrating the enzyme-CrATP complex with 3PGA. Fig. 2a shows the results of such titrations starting from various CrATP/enzyme ratios (from 1:3 to 4:3). The enhancement of the observed relaxation rates ($\gamma$) increased at low CrATP concentrations and began to decrease at higher 3PGA concentrations. Similar titrations performed in the presence of sulfate yielded curves that were almost mirror images of those observed in the absence of sulfate (Fig. 2b), where an initial de-enhancement at low

**FIG. 1.** Inhibition of phosphoglycerate kinase by CrATP. A, double-reciprocal plot of initial velocity versus MgATP concentration. CrATP concentrations were 0.25 (□), 0.13 (△), 0.08 (■), and 0.0 (○) mM. The assay medium also contained 50 mM MES, pH 5.90, 120 mM NaCl, 0.07 mM 3PGA, 150 $\mu$g of glyceraldehyde-phosphate dehydrogenase, and 1 mM excess of ATP concentration, and NADH sufficient to give 1–1.3 A units at 340 nm. The reaction in a total volume of 1 ml at 25 °C was started by the addition of 0.25 $\mu$g of PGK. B, secondary plot of slopes versus CrATP concentration, which yielded $K_{i}$ (data not shown), with $K_{i}$ values of 0.069 ± 0.008 mM. C, plot of $K_{i}$ for CrATP versus 3PGA concentration obtained from different experiments (data not shown), which yielded $K_{i}$ values of 0.670 ± 0.014 mM in the absence of sulfate (○) and 0.725 ± 0.178 mM in the presence of sulfate (□).
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3PGA concentration was followed by enhancement as 3PGA concentration increased. These results clearly show the formation of the ternary enzyme-CrATP-3PGA complex in both cases. The results further suggest that the environment of metal at the active site is different in the presence and absence of sulfate. Attempts were made to obtain an enhancement factor for the ternary complex ($e^*$) by curve-fitting as described earlier (33, 39). The data could not be explained by a single $e$ value, suggesting multiple conformational changes during the formation of the ternary complex, resulting in different environments of metal at the active site.

Paramagnetic Effects of CrATP on Protons of 3PGA—A $^1$H NMR spectrum of 3PGA is shown in Fig. 3a. The resonances were assigned by two-dimensional heteronuclear $^1$H-$^1$C correlated spectroscopy (data not shown). The multiplet centered at 4.24 ppm was assigned to the proton attached to carbon 2 (designated H-2), and the multiplets centered at 4.01 and 4.13 ppm were designated H-3 and H-3', respectively. Under the conditions of this study, the 3PGA/PGK ratios used were sufficient to give >92% of the enzyme in the binary PGK-3PGA complex at the beginning of the titrations. Titration of CrATP into samples containing PGK and 3PGA resulted in an increase in the $1/T_1$ values of the protons of 3PGA in the presence or absence of sulfate. Fig. 4a shows the observed $1/T_1$ values plotted against the concentration of CrATP bound in the ternary PGK-CrATP-3PGA complex. $1/T_1$ values varied linearly with bound CrATP concentration under both salt conditions. Despite the multiplicity of the resonances, the inversion recovery plots were not multiphasic within the time range used to determine $T_1$. To correct for the effect of free CrATP and the binary CrATP-3PGA complex on the longitudinal relaxation rates of 3PGA protons, the same titration was performed in the absence of PGK (Fig. 4b). Comparison of the paramagnetic effects of CrATP on $1/T_1$ of 3PGA protons in binary and ternary systems shows that the presence of PGK results in an enhancement of the paramagnetic effect.
FIG. 3. NMR spectra of 3PGA and [U-^{13}C]3PGA. Sample composition and data acquisition parameters were as follows: a, 20 mM 3PGA in 99 atom % ^2H_2O, spectrum obtained at 400 MHz using 16 transients, spectral width of 2200 Hz, 5-s acquisition time, and 90° pulse with 10-s delay; b, 150 mM 3PGA in 20% ^2H_2O, spectrum obtained at 100 MHz using 3800 transients, 17-kHz spectral width, 0.95-s acquisition time, 45° pulse with 12-s delay, and bi-level proton decoupling for nuclear Overhauser effect enhancement of signals; c, 10 mM [U-^{13}C]3PGA in 20% ^2H_2O, spectrum obtained at 400 MHz using 3300 transients, 26-kHz spectral width, 0.6-s acquisition time, 45° pulse with 15-s delay, and proton decoupling. All spectra were recorded with 32,000 data points and 16-bit A-D conversion at 27 °C.
of CrATP, indicating the formation of a ternary PGK-CrATP-3PGA complex. Furthermore, the presence of sulfate causes significant changes in the relative and absolute paramagnetic effect of CrATP on the three protons (note the different scales on the CrATP axis in Fig. 4, a and b). The corrected and normalized paramagnetic effects ($1/T_{1p}$) of CrATP on the 3PGA protons in the ternary PGK-CrATP-3PGA complex are given in Table I and were used for distance determinations after justifying that they contain distance information as described below.

**Paramagnetic Effects of CrATP on $^{31}P$ of 3PGA**—The proton-decoupled $^{31}P$ NMR spectrum of 3PGA yields a singlet 4.17 ppm downfield from H$_3$PO$_4$. Addition of the enzyme causes a 3-ppm upfield shift of the 3PGA resonance as well as significant broadening, indicating binary complex formation. Similar to $^1H$ NMR studies, titration of CrATP into this sample resulted in an enhancement of the paramagnetic effects of CrATP on $1/T_{1p}$ of 3PGA phosphorus as compared to the same experiment without enzyme (Fig. 5). The presence of 20 mM sulfate anion reduced the paramagnetic effect of CrATP on $1/T_{1p}$ in the PGK-CrATP-3PGA complex (Fig. 5). The addition of 5 mM ATP removed the observed paramagnetic effects, indicating that the outer sphere contribution to the observed relaxation rates was negligible. Table I contains the $1/T_{1p}$ values determined from these experiments. In the ternary complex, the $1/T_{1p}$ values of phosphorus exceeded all of the $1/T_{1p}$ values by more than an order of magnitude (Table I), which is consistent with fast exchange conditions.

**Paramagnetic Effects of CrATP on $^{13}C$ Longitudinal Relaxation Rates of 3PGA**—The proton-decoupled $^{13}C$ spectra of both natural abundance and [U-$^{13}C$]3PGA are shown in Fig. 3 (b and c, respectively). The resonances centered at 68, 73, and 179 ppm were assigned as C-3, C-2, and C-1, respectively, based on chemical shifts and two-dimensional heteronuclear $^1H$-$^13C$ correlated spectroscopy with the unlabeled compound. Determinations of metal-to-proton distances had indirectly indicated the positions of C-2 and C-3. Since the position of C-1 was undetermined and since this carbonyl group represents the acceptor for the transferred phosphoryl group in the reaction catalyzed by phosphoglycerate kinase, the data acqui-

**Table I**

Corrected relaxation rates and Cr$^{3+}$-to-nucleus distances in the PGK-CrATP-3PGA complex

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>$1/T_{1p}$ (s$^{-1}$)</th>
<th>$r^*$</th>
<th>$1/T_{1p}$ (s$^{-1}$)</th>
<th>$r^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-2</td>
<td>6.052 ± 0.51</td>
<td>6.82</td>
<td>7.51 ± 0.21</td>
<td>7.26</td>
</tr>
<tr>
<td>H-3</td>
<td>4.031 ± 0.26</td>
<td>5.27</td>
<td>7.36 ± 0.71</td>
<td>10.1</td>
</tr>
<tr>
<td>H-3'</td>
<td>20.406 ± 1.592</td>
<td>4.02</td>
<td>170.2 ± 0.20</td>
<td>9.09</td>
</tr>
<tr>
<td>P</td>
<td>250.9 ± 32</td>
<td>6.22</td>
<td>407.7 ± 3.3</td>
<td>8.61</td>
</tr>
<tr>
<td>C-1</td>
<td>78.7 ± 20</td>
<td>6.46</td>
<td>61.3 ± 0.7</td>
<td>6.88</td>
</tr>
<tr>
<td>C-2</td>
<td>220 ± 140</td>
<td>5.23</td>
<td>48 ± 0.24</td>
<td>7.02</td>
</tr>
<tr>
<td>C-3</td>
<td>841 ± 420</td>
<td>4.35</td>
<td>7.86 ± 0.55</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^*$The errors shown in the absolute distances include ±2-5% error in the measurement of $1/T_{1p}$ from the binary PGK-CrATP titration, 6.5-50% error in the measurement of $1/T_{1p}$ from the ternary titrations, 8.8% accuracy in the estimation of $r$, and either 21.8 or 7.8% error in the measurement of the dissociation constant of CrATP from the ternary complex in the absence or presence of sulfate.

$^*$Not determined.

**Fig. 4.** Paramagnetic effects of CrATP on protons of free and enzyme-bound 3PGA in absence and presence of sulfate. The samples contained 0.108 mM PGK, 10 mM 3PGA, and 60 mM NaCl (a) or 0.338 mM PGK, 7 mM 3PGA, and 20 mM Na$_2$SO$_4$ (b) in acetate buffer, pH 5.9, and 99 atom % $^1$H$_2$O in a total volume of 500 µl. Shown are the $1/T_{1p}$ values determined from these experiments. (a) CrATP ternary PGK-CrATP complex concentration.

**Fig. 5.** Paramagnetic effects of CrATP on phosphorus of free and enzyme-bound 3PGA in absence and presence of sulfate. The samples contained 8.3 mM 3PGA, 50 mM MES, pH 5.9, and either 0.35 mM PGK and 60 mM NaCl (C) or 0.45 mM PGK and 20 mM Na$_2$SO$_4$ (b) in a total volume of 500 µl with 20% $^1$H$_2$O for locking. Solid lines represent the least-squares fit to the data points; dashed lines represent the binary contributions. Identical binary contributions were used in both (a) and (b) MHz using the inversion recovery method as described under "Experimental Procedures." [CrATP]$_{tern}$, ternary CrATP complex concentration.
Optimization parameters were optimized for determination of the metal-to-C-1 distance.

Fig. 6 shows the effect of titrating CrATP into samples containing either 3PGA alone or 3PGA and PGK in the presence and absence of sulfate. The enhancement of 1/T₁ in the presence of enzyme as well as the decreased slope of the 1/T₁ versus [CrATP] curves in the presence of sulfate ion as compared to the no-sulfate case are both clearly visible. These findings agree well with the observations shown in Figs. 4 and 5. A much larger effect was observed with C-2, which is shown in the inset in Fig. 6 due to large differences in T₁ values as compared to C-1. This is in excellent agreement with the observed effect of sulfate on the paramagnetic effect of CrATP on H-2. The C-3 peak was obscured by an impurity when sulfate was present; and therefore, the paramagnetic effect of CrATP on this resonance was not determined under these conditions. This does not have a large effect on the determination of the conformation of 3PGA since the position of this atom was determined by known distances to the directly bound protons. The 1/T₁ values obtained from this data are given in Table I.

**Determination of Correlation Times and Evaluation of Temperature Dependence of 1/T₁ of 3PGA Protons**—The temperature dependence of 1/T₁ of the protons of 3PGA was examined to assure that fast exchange conditions were being met. In this experiment, fast exchange is indicated by a decrease in relaxation rate with increasing temperature; increasing relaxation rates with increasing temperature are indicative of exchange limitation since exchange rates (1/τₑ) increase at higher temperatures. Fig. 7 shows the positive slope of 1/T₁ versus 1000/T plots for all protons of 3PGA, indicating that the observed relaxation values can be used for distance determinations.

Determination of metal-to-nucleus distances from observed 1/T₁ values requires a measurement of τₑ, the correlation time for the dipolar Cr³⁺-nucleus interaction. τₑ was evaluated by measuring the 1/T₁ of solvent water protons of samples at six frequencies as described under “Experimental Procedures.” The paramagnetic contributions to the relaxation rates (1/T₁) observed at 15, 24.3, 42.5, 59, 250, and 400 MHz for the binary and ternary complexes of CrATP in the presence and absence of sulfate are listed in Table II. The measured relaxation rates were corrected to account for the contribution of free CrATP, and τₑ was determined by using a least-squares fitting computer program with an 8.8% error. The determined τₑ values, which were used in the calculation of metal-to-nucleus distances, are shown in Table II; these values are similar to those observed earlier for enzyme-bound CrATP (40–42). τₑ values for the various complexes were also calculated assuming τₑ to be independent of frequency; this analysis yielded slightly shorter distances, but did not affect the determined distances beyond the error limits shown in Table I.

**Determination of Cr³⁺-to-Nucleus Distances in Ternary Complexes**—Distances of the nuclei of 3PGA from Cr³⁺ of CrATP in the ternary enzyme-CrATP-3PGA complex in the presence and absence of sulfate were calculated according to Equation 1 (43):

\[
r = C((q/T₁)/f(τₑ))^{1/6}
\]

where r is the internuclear distance, C is the product of known physical constants, q is the stoichiometry, and f(τₑ) is given by Equation 2.

\[
f(τₑ) = \frac{3τₑ}{1 + ω²τₑ²} + \frac{7τₑ}{1 + ω²τₑ²}
\]
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50-20-10:

FIG. 7. Temperature dependence of $1/T_{1p}$ of 3PGA protons in ternary PGK-CrATP-3PGA complex. The sample contained 0.108 mM PGK, 10 mM 3PGA, 2.37 μM CrATP, and 60 mM NaCl in acetate buffer, pH 5.9, and 99 atom % $^2$H₂O in a total volume of 500 μl. Shown are H-2 (Δ), H-3 (☐), and H-3' (○) protons of 3PGA. NMR spectra were collected at 200 MHz as described for Fig. 2a.

TABLE II

Frequency dependence of the corrected and normalized relaxation rates and correlation times in the ternary PGK-CrATP-3PGA complex

<table>
<thead>
<tr>
<th>Frequency (MHz)</th>
<th>$1/T_{1p}'$</th>
<th>$\tau_d$</th>
<th>$(1/\tau_M)'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.0</td>
<td>1.54</td>
<td>2.83</td>
<td>8.54</td>
</tr>
<tr>
<td>24.3</td>
<td>1.92</td>
<td>2.83</td>
<td>8.50</td>
</tr>
<tr>
<td>42.0</td>
<td>2.66</td>
<td>2.84</td>
<td>8.48</td>
</tr>
<tr>
<td>59.0</td>
<td>2.45</td>
<td>2.85</td>
<td>8.47</td>
</tr>
<tr>
<td>250</td>
<td>2.16</td>
<td>3.33</td>
<td>7.84</td>
</tr>
<tr>
<td>400</td>
<td>1.71</td>
<td>4.08</td>
<td>5.97</td>
</tr>
</tbody>
</table>

$^*$ Determined from the frequency dependence of the normalized longitudinal relaxation rates ($1/T_{1p}'$) of water protons at the frequencies indicated and analyzed according to the equation $1/\tau_d = \frac{B r_d}{1 + r_d^2} + \frac{4 r_d}{1 + 4 r_d^2}$ and the equation given in text, where $\tau_d$ is the dipolar correlation time, $\omega$ is the electron precession frequency, $\tau_M$ is the longitudinal electron spin relaxation time, $\beta$ is the zero field splitting parameter, and $r_d$ is a time constant for ligand motion that modulates $\beta$. The correlation times shown above are from the best fitted curve to the data points that also yielded $\beta = 3.16 \times 10^3$ s$^{-1}$ and $\tau_M = 2.51 \times 10^{-13}$ s.

The $\beta$ values used were 705, 522, and 445 for protons, phosphorus, and carbon, respectively. The results of these calculations, which represent the Cr$^{3+}$-to-3PGA nuclear distances in the ternary complexes in the presence and absence of sulfate, are given in Table I. These distances can be built into a model that allows for direct transfer of the phosphoryl group under both ionic conditions (Fig. 8). The substrate arrangements shown in Fig. 8 were built using Desktop Molecular Modeller software from Oxford University Press.

DISCUSSION

The presence of phosphoglycerate kinase was observed to enhance the paramagnetic effect of CrATP on relaxation rates of 3PGA nuclei, showing the formation of a ternary PGK-CrATP-3PGA complex. To use this relaxation data for metal-to-nucleus distance determinations, it is necessary to estimate the contribution of $\tau_M$, the exchange rate of the substrate from the paramagnetic complex, to the observed relaxation rates. This was done by measuring the temperature dependence of $1/T_{1p}'$. Fig. 7 shows that $1/T_{1p}'$ values decreased as temperature increased, indicating fast exchange of 3PGA out of the ternary complex. Since $1/\tau_M$ would be expected to increase at higher temperatures, the data suggest that $\tau_M$ does not contribute to the observed relaxation rates. Additionally, frequency dependence of $1/T_{1p}'$ values (Table II) are consistent with fast exchange conditions since $\tau_M$ is independent of frequency. Therefore, the observed relaxation rates carry distance information and can be used in the determination of metal-to-nucleus distances.

Table I shows the determined metal-to-nucleus distances for the nuclei of 3PGA in the ternary complex. The distances were determined from data collected in three different types of experiments for each ionic condition using different enzyme and CrATP preparations. Models of the conformation of 3PGA at the active site of PGK with and without sulfate were constructed using the determined distances. In building the...
models, we arbitrarily restricted the position of the carboxyl oxygen of 3PGA such that it was optimally aligned for phosphoryl transfer. Under both sets of conditions, at least one conformation of 3PGA was found that would yield an arrangement of substrates that satisfied the determined metal-to-nucleus distances. The resulting models were checked to make sure that there was no van der Waals overlap between the 3PGA nuclei and the water ligands of Cr³⁺. The distances in Table I and Fig. 8 show that all 3PGA nuclei were farther from the metal when sulfate was present. In addition to an overall increase in the distance of 3PGA from Cr³⁺, sulfate causes relative conformational changes of 3PGA. Of the 3PGA protons, H-3' is closest to the metal center, followed by H-3 and H-2. With sulfate present, H-2 becomes the closest, followed by H-3' and H-3; and all are farther from Cr³⁺ than in the no-sulfate case. These results are confirmed by the determined metal-to-carbon distances that indicated significant lengthening of the Cr³⁺-to-C-2 distance in the presence of sulfate (Fig. 6 and Table I). Another significant change was observed in the metal-to-phosphorus distance, which was also longer when sulfate was present. If sulfate ion binds at the active site, electrostatic interactions may cause displacement of the phosphoryl group by sulfate, resulting in an altered arrangement of substrates. However, other explanations are also possible.

The kinetic and water proton relaxation data shown in Figs. 1 and 2 are consistent with and support the arrangement of substrates that satisfied the determined metal-to-carbon distances that indicated significant lengthening of the Cr³⁺-to-C-2 distance in the presence of sulfate (Fig. 6 and Table I). Also, the ternary complex of PGK and 3PGA also confirm our earlier suggestion at the "closed" active site in the absence of sulfate (25). The models shown in Fig. 8 also confirm our earlier suggestion based on the studies of interactions of various CrATP analogs with PGK (24, 25).

Despite significant changes in the conformation of 3PGA, the carboxyl carbon-to-metal distance remains fairly constant under both conditions, with only a slight increase observed with sulfate present. This suggests a small change in the distance between the attacking nucleotide and γ-phosphorus of ATP. Based on the models shown in Fig. 8, we determined oxygen-to-phosphorus distances of 4.43 and 4.85 Å in the absence and presence of sulfate, respectively. Cohn and coworkers (7) reported a 15% decrease in the rate of phosphoryl transfer in the presence of an activating concentration of sulfate; the observed distance increases, possibly along with a deviation from optimum transfer alignment, could be one explanation for their findings. However, it is unlikely that this difference would lead to a change in the reaction mechanism in the presence of sulfate.

The results described here offer the first evidence that the ternary complex of PGK and its substrates has the substrates located close enough to each other to allow for direct transfer of the phosphoryl group in solution. This is consistent with the proposed closing of the enzyme active site when both substrates are bound. However, we do not know whether the proximity of the substrates is due to closure of the active site or simply due to binding of substrates to proximal sites on the enzyme. Additionally, our results indicate that sulfate ion alters both the conformation of 3PGA and the relative arrangement of the substrates at the active site of PGK. This may be due to the formation of a less closed form of the active site when sulfate is present.

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REFERENCES