Arrangement of the Substrates at the Active Site of Brain Pyridoxal Kinase*

(Received for publication, June 12, 1991)

Willem F. Wolkers†, Jay D. Gregory, Jorge E. Churchich, and Engin H. Serpersu‡

From the Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996-0840

The distances between enzyme-bound paramagnetic CrATP (a stable, β,γ-bidentate complex of Cr³⁺ and ATP) at the active site of sheep brain pyridoxal kinase and the protons of bound inhibitor 4-dPyr (4-deoxy-pyridoxal) were determined in the ternary enzyme-CrATP-4-dPyr complex by measuring the paramagnetic effects of Cr³⁺ on the longitudinal relaxation rates (1/T₁p) of the protons of 4-dPyr. The correlation time for the Cr³⁺-4-dPyr dipolar interaction on the enzyme was estimated as 1.59 ns by the frequency dependence of 1/T₁p of water protons. Temperature dependence of 1/T₁p values indicated the fast exchange of 4-dPyr from the paramagnetic enzyme-CrATP-4-dPyr complex; hence the measured 1/T₁p values can be used for metal-nucleus distance determinations. The distances from the Cr³⁺ of the enzyme-bound CrATP to the 2-methyl (7.19 Å), 4-methyl (7.18 Å), and H6 proton (6.18 Å) of the 4-dPyr are too great to permit a direct coordination of any group from 4-dPyr. However, these distances can be built into a model in which phosphorus of the γ-phosphoryl group of ATP is 4 Å away from the oxygen atom of the 5-CH₂OH group of the 4-dPyr. This suggests that phosphorylation of pyridoxal can occur via direct transfer of the phosphoryl group between the bound substrates at the active site of pyridoxal kinase.

The distances between enzyme-bound paramagnetic CrATP (a stable, β,γ-bidentate complex of Cr³⁺ and ATP) at the active site of sheep brain pyridoxal kinase and the protons of bound inhibitor 4-dPyr (4-deoxy-pyridoxal) were determined in the ternary enzyme-CrATP-4-dPyr complex by measuring the paramagnetic effects of Cr³⁺ on the longitudinal relaxation rates (1/T₁p) of the protons of 4-dPyr. The correlation time for the Cr³⁺-4-dPyr dipolar interaction on the enzyme was estimated as 1.59 ns by the frequency dependence of 1/T₁p of water protons. Temperature dependence of 1/T₁p values indicated the fast exchange of 4-dPyr from the paramagnetic enzyme-CrATP-4-dPyr complex; hence the measured 1/T₁p values can be used for metal-nucleus distance determinations. The distances from the Cr³⁺ of the enzyme-bound CrATP to the 2-methyl (7.19 Å), 4-methyl (7.18 Å), and H6 proton (6.18 Å) of the 4-dPyr are too great to permit a direct coordination of any group from 4-dPyr. However, these distances can be built into a model in which phosphorus of the γ-phosphoryl group of ATP is 4 Å away from the oxygen atom of the 5-CH₂OH group of the 4-dPyr. This suggests that phosphorylation of pyridoxal can occur via direct transfer of the phosphoryl group between the bound substrates at the active site of pyridoxal kinase.

Pyridoxal 5-phosphate is a required cofactor for numerous enzymes that catalyze a large variety of reactions. The formation of pyridoxal 5-phosphate from pyridoxal, ATP, and divalent cation (Zn²⁺) is catalyzed by pyridoxal kinase (1). Kinetic data are consistent with the hypothesis that the phosphoryl group is transferred to pyridoxal within a quaternary complex of enzyme, ATP, pyridoxal, and an activating cation (2). Affinity-labeling studies with adenosine tetraphosphate pyridoxal and adenosine tetraphosphate pyridoxine (AP,-PN) showed that adenosine tetraphosphate pyridoxine is a competitive inhibitor of the enzyme with respect to both ATP and pyridoxal; however, pyridoxal offered only partial protection against the labeling of the enzyme by adenosine tetraphosphate pyridoxal (3). This suggests that the pyridoxal-binding site may be close to the nucleotide-binding site; however, little is known about the mechanism and the structure of this enzyme.

* This work was supported by National Institutes of Health Grant R29 GM422661 (to E. H. S.) and by National Science Foundation Grant BNS 8510237 (to J. E. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Dept. of Biochemistry, Agricultural University, 6703 BC Wageningen, The Netherlands.
‡ To whom correspondence should be addressed. Tel.: 615-974-2668; Fax: 615-974-0878.

EXPERIMENTAL PROCEDURES

Materials

Sheep brain pyridoxal kinase was purified by a procedure developed by Henry et al. (12) that includes a step of affinity chromatography on pyridoxyl-Sepharose and elution with 50 mM pyridoxal at pH 5. ATP, pyridoxal, and 4-deoxypyridoxine were obtained from Sigma. β,γ-Bidentate CrATP³⁺ is a stable, substitution inert, metal-ATP analog, which has been successfully used as a paramagnetic probe for intersubstrate distance measurements (6, 7), and it was also used as a metal-ATP analog for various kinases and ATPases (8–11). This work describes an attempt to determine the proximity of the bound substrates at the active site of pyridoxal kinase by measuring the metal-nucleus distances between the substrate molecules using CrATP as a paramagnetic probe.

Methods

Kinetic Studies—The inhibition of pyridoxal kinase activity by the substrate analogs CrATP and 4-deoxypyridoxine was measured by following the rate of formation of pyridoxal 5-phosphate from the increase in absorbance at 388 nm, at which pyridoxal-5-P has a molar absorptivity of 4900 M⁻¹ cm⁻¹ at pH 7.0. The assays were performed essentially as described before (3). The assay medium contained 70–100 mM KCl in 50 mM MES, pH 6.0, with 0.1 mM (CH₃COO)₂Zn or 0.75 mM MnCl₂. For the inhibition studies with CrATP, ATP concentration was varied from 0.25 to 0.5 mM in the presence of 0.1 mM pyridoxal. CrATP concentrations were 0, 140, 290, and 420 μM. In kinetic experiments where 4-dPyr was used as inhibitor, ATP concentration was held constant at 0.372 mM, and pyridoxal concentration was varied from 15 to 516 μM. Initial rate measurements were carried out by monitoring the change in absorbance at 388 nm for 1–2 min in a Beckman DU-70 spectrophotometer at 28 °C. The initial velocity data were fitted by using Enzfitter software of Elsevier-Biosoft.

¹ The abbreviations used are: CrATP, exchange inert β,γ-bidentate Cr(H₂O)₂ATP complex, MES, 2-(N-morpholino)ethanesulfonic acid, 4-dPyr, 4-deoxypyridoxine.
Magnetic Resonance Studies—The longitudinal relaxation rate of water protons was measured with a Seimco pulsed NMR instrument at 24.3 MHz, as described previously (14, 15), by using the 180°-τ-90° pulse sequence of Carr and Purcell (14, 16). The observed enhancement of the relaxation rate is defined as \( r = (1/T_{1p})/(1/T_{1}) \), where \( 1/T_{1p} \) is the paramagnetic contribution to the observed relaxation rate in the presence of paramagnetic species and \( 1/T_{1} \) in the absence of such species. The enhancement value for the binary CrATP-4-dPyr was obtained at 169 μM CrATP by extrapolation of the observed enhancement values at 5–25 mM 4-dPyr concentration to infinite 4-dPyr concentration. The samples also contained 50 mM MES, pH 6.0, and 75 mM KCl in a total volume of 40 μL. The determined enhancement value \((s)\) was then used to calculate the fraction of CrATP bound to 4-dPyr, which then yielded the dissociation constant of the binary CrATP-4-dPyr complex.

The correlation time for the dipolar electron nucleus interaction in the binary enzyme CrATP and ternary enzyme-CrATP-4-dPyr complexes was determined by studying the frequency dependence of \( 1/T_{1p} \) of water protons at 15, 24.3, 40, and 59.8 MHz on a Seimco pulsed NMR spectrometer equipped with a variable frequency probe, at 200 MHz on a Nicolet NT-200 spectrometer, and at 250 MHz on a Bruker AC250 spectrometer by inversion recovery, as previously described (14, 15). Evaluation of \( r \), the frequency-dependent \( 1/T_{1p} \) value of enzyme, was performed with the NMR spectrometer (14, 15, 17). Samples contained 50 mM MES, pH 6.0, 75 mM KCl, 95.9 μM pyridoxal kinase (191.8 μM in active sites), 67 μM CrATP with and without 250 μM 4-dPyr. The observed relaxation rates were corrected for the paramagnetic effects of free CrATP.

The paramagnetic effect of CrATP on the relaxation rates of the proton bands of enzyme-bound 4-deoxyxypiridoxine was determined in 500-μL samples in \( ^{2} \)H2O. Buffer and 4-dPyr were exchanged in \( ^{2} \)H2O by three cycles of lyophilization and resolubilizing. Pyridoxal kinase was exchanged in \( ^{2} \)H2O after concentrating by centrifugation with Amicon microcentrifuge filters and diluted in \( ^{2} \)H2O. CrATP was prepared immediately before use, concentrated by rapid rotatory evaporation, and diluted in \( ^{2} \)H2O. The solution was then titrated with CrATP, and the increases in the longitudinal \((1/T_{1})\) relaxation rates of the protons of 4-dPyr was measured. The data were analyzed by plotting the relaxation rates against the concentration of CrATP bound in the ternary enzyme-CrATP-4-dPyr complex. The slopes of these curves were then corrected by subtracting the contribution of the paramagnetic effects of CrATP in the binary CrATP-4-dPyr complex that was based on the known distribution of CrATP among the binary and ternary complexes, as calculated from the respective dissociation constants and the measured paramagnetic effects of CrATP on 1/T of 4-dPyr protons in the binary CrATP-4-dPyr complex. The corrected slopes obtained in this way, when multiplied by the total 4-dPyr concentration, yielded the normalized relaxation rates \((1/T_{1p})\), where \( f \) is defined as bound \( [\text{paramag}] / [\text{CrATP}] \).

\(^{1} \)H NMR spectra were obtained at 200 MHz (Nicolet NT-200 spectrometer) and at 250 MHz (Bruker AC250 spectrometer) by using 12-bit A/D conversion, collecting 16K data points over a spectral width of 2500 Hz. The longitudinal relaxation rates were determined by inversion recovery. 8–64 transients were collected by using a 12-s delay to obtain fully relaxed spectra. The 90° pulse was typically 15 μs at 200 MHz and 7 μs at 250 MHz.

The temperature dependence of \( 1/T_{1p} \) in the binary and ternary complexes of enzyme CrATP and 4-dPyr were done at 16°, 21°, and 26° C at 200 MHz.

RESULTS

Kinetic Studies—Substrate analog CrATP was tested for active site binding. Fig. 1 shows the Lineweaver-Burk plot of the inhibition of pyridoxal kinase by CrATP in the presence of 0.1 mM pyridoxal and 0.1 mM zinc acetate and ATP as the variable substrate with 0, 140, 280, and 420 μM CrATP present. A secondary plot of the data (Fig. 1, inset) clearly indicates that CrATP is a linear competitive inhibitor of pyridoxal kinase with respect to ATP, with a \( K_{i} \) value of 83 μM. Thus, CrATP can be used as a paramagnetic probe for the determination of metal-nucleus distances in the bound substrates.

Inactive substrate analogs are necessary for paramagnetic distance determination studies; otherwise the presence of enzyme will produce a mixture of various complexes of the enzyme with the substrates and products. Although CrATP was not hydrolyzed by the enzyme, the use of a second substrate analog was necessary because the presence of pyridoxal at high concentrations caused precipitation of the enzyme in the NMR tube in our conditions, which are necessary for the paramagnetic metal-nucleus distance measurements. Therefore, we used 4-deoxyxypiridoxine, a pyridoxal analog in which the aldehyde group of pyridoxal is replaced by a methyl group, which would not cause precipitation of the enzyme when present at high concentrations. Kinetic studies revealed that 4-dPyr is also a linear competitive inhibitor of the enzyme with respect to pyridoxal with a \( K_{i} \) value of 17.3 μM (data not shown).

Magnetic Resonance Studies—A \(^{1} \)H NMR spectrum of 4-dPyr is shown in Fig. 2. The resonances were assigned by the chemical shifts and by comparison with the pyridoxal spectrum (Fig. 2). The peak at 8.0 ppm was assigned to the ring proton at position 6, and the resonances at 2.58 and 2.36 ppm were assigned to the methyl protons at positions 2 and 4, respectively. The proton resonance of 5-CH₂O₂H was obscured by the residual \(^{1} \)H2O. Fig. 2, spectrum c, shows that, in the presence of pyridoxal kinase 4-dPyr, resonances broadened and shifted upfield, indicating the formation of a binary enzyme-4-dPyr complex.

CrATP was titrated into a solution containing pyridoxal kinase and 4-dPyr, and 1/T, \( [\text{ATP}] \) values of the proton resonances of 4-dPyr were monitored (Fig. 3). The 1/T, \( [\text{ATP}] \) values increased linearly with respect to added CrATP. Fig. 3 shows the results of such titration in which the observed 1/T, \( [\text{ATP}] \) values are plotted against the concentration of CrATP bound to the ternary enzyme-CrATP-4-dPyr complex. The slopes of such titrations yielded the paramagnetic effects of CrATP on the longitudinal relaxation rates \((1/T_{1})\) of enzyme-bound 4-dPyr protons. To provide a correction for the contribution of free CrATP and the binary CrATP-4-dPyr complex that formed at the micromolar concentrations during the titrations to 1/T, a control experiment was performed by titrating comparable amounts of CrATP into an identical sample that lacked the enzyme. The result of such titration is also shown in Fig. 3. Comparison of the paramagnetic effects of CrATP on 1/T, 4-dPyr resonances in ternary and binary systems shows that, in the presence of pyridoxal kinase, the paramagnetic effects of CrATP are enhanced, indicating the formation of a ternary complex.
NMR Studies of Pyridoxal Kinase

FIG. 2. NMR spectra of pyridoxal and 4-deoxypyridoxine in the presence and absence of pyridoxal kinase. The samples contained (a) 60 mM pyridoxal, (b) 60 mM 4-deoxypyridoxine, or (c) 43.3 μM pyridoxal kinase and 10.2 mM 4-deoxypyridoxine in 1 mM MES, pH 6.0, with 70 mM KCl in a volume of 500 μl. NMR spectra were obtained at 250 MHz by using 64 transients with 16K data points, an acquisition time of 2.7 s, a spectral width of 2500 Hz, a 5-s delay with a 45° pulse with the preirradiation of the residual HOD resonance for 1 s 30 db below 0.2 watt (no enzyme) or a 15-s delay with a 90° pulse (with the enzyme), and a 12-bit A-D conversion at 22 °C.

enzyme-CrATP-4-dPy complex. The slope of the paramagnetic effects observed in binary titration was subtracted from the slopes of the respective ternary titration curves in order to determine the paramagnetic effects of CrATP on the protons of 4-dPy in the ternary complex. The corrected and normalized paramagnetic effects (1/Tp) of CrATP on the protons on 4-dPy in the ternary enzyme-CrATP-4-dPy are used in metal-nucleus distance determination after justifying that they contain distance information as described below.

Also an attempt has been made to displace CrATP from the ternary complex by the addition of diamagnetic substrate; however, addition of MgATP in millimolar concentrations caused the precipitation of the enzyme in the NMR tube.

The metal-nuclear distances can be determined from the normalized relaxation rates (1/Tp) of the bound substrate protons with the following equation (4).

\[ r = C[ (1/T_p)/(1/T_{pp}) ]^{1/2} \]

where C is a constant, equal to 705 Å/s1/3 for interactions between Cr³⁺ and protons. The correlation function is defined as follows

\[ f(\tau_c) = \frac{3\tau_c}{1 + \omega^2\tau_c^2} + \frac{7\tau_c}{1 + \omega^2\tau_c^2} \]

**FIG. 3.** Paramagnetic effects of CrATP on the protons of free and enzyme-bound 4-deoxypyridoxine. The samples contained 10.2 mM 4-dPy, 70 mM KCl in 1 mM MES, pH 6.0, in the presence (Δ, ○, □) and in the absence (○, +, ×) of 43.3 μM pyridoxal kinase. The lines represent the least squares fit to the data points for 2-CH₃ (○, ×), 4-CH₃ (□, +), and H6 (○, Δ) protons.
where \( \omega_e \) and \( \omega_n \) are the nuclear and electron precession frequencies and \( \tau_e \) is the correlation time. The correlation time was determined by measuring the \( 1/T_{1p} \) of water protons of the samples at six frequencies, as described under "Experimental Procedures." The paramagnetic contribution to the relaxation rates (\( 1/T_{1p} \)) observed at frequencies 15.0, 24.3, 40.0, 59.8, 200, and 250 MHz for the binary and ternary complexes of CrATP are listed in Table I. The measured relaxation rates of bound CrATP were corrected for the contribution of free CrATP to the observed relaxation rates. The results of such an experiment for the ternary enzyme-CrATP-4-dPyr complex, which are listed in Table II, reflect the weighted average of their positions with respect to Cr\(^{3+}\). The distances shown in Table II are from the best fitted curve to the data points, which also yields \( B = 4.68 \times 10^{10} \) s\(^{-2}\) and \( \tau_e = 7.76 \times 10^{-13} \) s.

\[ \frac{1}{T_{1p}} = \left( \frac{C}{r} \right) \left( \frac{1}{\gamma_e \tau_e} \right) \]

where \( r = 2.50 \) Å for the Cr\(^{3+}\) to proton distance, as determined by x-ray studies (24). The correlation time at 250 MHz for the ternary enzyme-CrATP-4-dPyr was estimated to be \( 1.59 \times 10^{-10} \) s, which is used with the determined paramagnetic effects of CrATP on the protons of enzyme-bound 4-dPyr (Fig. 2) to estimate the metal-proton distances.

The use of the above equations to calculate metal-nucleus distances requires a high rate of exchange of 4-dPyr out of the paramagnetic enzyme-CrATP-4-dPyr complex, compared with \( 1/T_{1p} \) values shown in Fig. 5. \( 1/T_{1p} \) values decrease with increasing temperature, indicating fast exchange conditions for the protons of 4-dPyr since the exchange rates (1/r\(_m\)) would increase with increasing temperature. Therefore, the observed \( 1/T_{1p} \) values may be a result of the paramagnetic enzyme-CrATP-4-dPyr complex which are shown in Table II. Since the protons of methyl groups are not distinguishable from each other, the distances shown in Table II reflect the weighted average of their positions with respect to Cr\(^{3+}\). The distances shown in Table II can be built into a model in which the phosphorus atom of the \( \gamma \)-phosphoryl group of ATP is 4 Å away from the oxygen atom of the 5-

![Fig. 4. Frequency dependence of the proton relaxation time of pyridoxal kinase-CrATP-4-dPyr complex.](image)

**Table I**

Frequency dependence of the corrected and normalized relaxation rates and correlation times in the ternary pyridoxal kinase-CrATP-4-deoxypyridoxine complex

<table>
<thead>
<tr>
<th>Frequency (MHz)</th>
<th>( 1/T_{1p} )</th>
<th>( 1/T_{2p} )</th>
<th>( \tau_e )</th>
<th>( f(\tau_e) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>8.30</td>
<td>8.37</td>
<td>0.56</td>
<td>1.66</td>
</tr>
<tr>
<td>24.3</td>
<td>8.66</td>
<td>8.42</td>
<td>0.56</td>
<td>1.68</td>
</tr>
<tr>
<td>40</td>
<td>10.20</td>
<td>8.59</td>
<td>0.58</td>
<td>1.71</td>
</tr>
<tr>
<td>59.8</td>
<td>8.96</td>
<td>8.86</td>
<td>0.62</td>
<td>1.76</td>
</tr>
<tr>
<td>200</td>
<td>5.52</td>
<td>5.46</td>
<td>1.24</td>
<td>1.09</td>
</tr>
<tr>
<td>250</td>
<td>3.19</td>
<td>3.31</td>
<td>1.59</td>
<td>0.66</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 \( 1/\tau_e = B(\omega_e^2 + \omega_n^2) + 4\gamma_e/(1 + 4 \omega_n^2) \) and the equations given in the text, where \( \tau_e \) is the dipolar correlation time, \( \omega_n \) is the electron precession frequency, \( \tau_e \) is the longitudinal electron spin relaxation time, \( B \) is the zero field splitting parameter, and \( \gamma_e \) is a time constant for ligand motion that modulates B. The correlation times shown above are from the best fitted curve to the data points, which also yielded \( B = 4.68 \times 10^{10} \) s\(^{-2}\) and \( \tau_e = 7.76 \times 10^{-13} \) s.

* Calculated from the fitted curve by using the equation \( 1/T_{1p} = (C/r)/(\gamma_e \tau_e) \), where \( r = 2.50 \) Å for the Cr\(^{3+}\)-proton distance and \( C = 705 \) Å/m\(^{-1}\) for the Cr\(^{3+}\)-proton interactions.

![Fig. 5. Temperature dependence of the proton relaxation rates of pyridoxal kinase-CrATP-4-deoxypyridoxine complex.](image)

**Table II**

Corrected relaxation rates and Cr\(^{3+}\)-proton distances in the pyridoxal kinase-CrATP-4-deoxypyridoxine complex

<table>
<thead>
<tr>
<th>Proton</th>
<th>1/T(_{1p})</th>
<th>Distance* (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-CH(_3)</td>
<td>537</td>
<td>7.19 ± 0.4</td>
</tr>
<tr>
<td>4-CH(_3)</td>
<td>543</td>
<td>7.18 ± 0.4</td>
</tr>
<tr>
<td>H6</td>
<td>1335</td>
<td>6.18 ± 0.36</td>
</tr>
</tbody>
</table>

* The errors shown in the absolute distances include 7.5-9.5%, 15.6%, and 30% due to experimental errors in the measurements of 1/T\(_{1p}\), \( \tau_e \), and the dissociation constant of CrATP from the ternary complex, respectively.
CH$_2$OH group of 4-deoxypyrdoxine. Hence, direct transfer of a phosphoryl group between the substrates is likely to be the mechanism of phosphorylation of pyridoxal by pyridoxal kinase. One of the possible arrangements of the substrates at the active site of pyridoxal kinase that satisfies the determined Cr$^{3+}$-proton distances is shown in Fig. 6.

**Discussion**

Kinetic studies$^2$ indicated that the stable $\beta,\gamma$-bidentate CrATP is a linear competitive inhibitor of brain pyridoxal kinase; therefore, it was used as a paramagnetic substrate analog for the determination of metal-nucleus distances of the bound substrates. The paramagnetic effect of CrATP on the protons of 4-dPyr is greatly enhanced when pyridoxal kinase was present (Fig. 3), indicating the formation of a ternary enzyme-CrATP-4-dPyr complex. The increases observed in the relaxation rates of 4-dPyr protons indicate stronger interaction between CrATP and 4-dPyr protons when both substrate analogs are bound to the catalytic site of the enzyme.

For structural data obtained from the paramagnetic studies to be reliable, a reasonable estimate of the contribution of $\tau_M$, the exchange rate of substrate from the paramagnetic complex, to the observed relaxation rates should be made. We have determined the contribution of $\tau_M$ by measuring the temperature dependence of 1/$T_1p$ values. As shown in Fig. 5, 1/$T_1p$ values decreased with increasing temperature, which suggests that $\tau_M$ does not contribute to the observed relaxation rates, since 1/$\tau_M$ would be increasing at higher temperature. Therefore, the measured paramagnetic effects of CrATP on the protons of 4-dPyr can be used to determine Cr$^{3+}$-proton distances in the ternary enzyme-CrATP-4-dPyr complex. Moreover, $T_1p$ depends on frequency, as shown in Fig. 4, whereas $\tau_M$ does not.

The determined correlation time for the electron-nuclear dipolar interaction in the ternary enzyme-CrATP-4-dPyr complex is in reasonable agreement with the values determined for CrATP proton interactions with other enzymes (19). In addition, even an error of 50% in the determined correlation time would cause only 8% error in the absolute distances due to the inverse sixth power relationship shown in the first equation (4).

The distances to the methyl protons and H6 from Cr$^{3+}$ are too great to permit coordination of any group from 4-dPyr to the metal ion. However, by using the distances shown in Table II, one can build a possible arrangement of CrATP and 4-dPyr at the active site, which allow a distance of 4 Å between the $\gamma$-phosphorus of CrATP and the oxygen atom of the 5-CH$_2$OH group of 4-dPyr. It should be noted that the 5-CH$_2$OH group of pyridoxal is the acceptor of the phosphoryl group from ATP during catalysis. The important feature of this arrangement is that it suggests a direct transfer of a phosphoryl group between two substrates bound to the catalytic site of pyridoxal kinase. Some kinases, like nucleoside diphosphate kinase and nucleoside phosphotransferase, are known to form chemically and catalytically competent phosphoenzyme intermediates (20). It seems that brain pyridoxal kinase, like the majority of kinases (20), catalyzes the phosphoryl transfer without the formation of a phosphoenzyme intermediate. Earlier efforts to detect and/or isolate phosphorylated enzyme have failed,$^3$ which is consistent with the direct transfer of a phosphoryl group.

Although the arrangements of the substrates shown in Fig. 6 are not the only possible combination, it should be noted that 4-dPyr is not a flexible molecule and there are not many arrangements that would satisfy the observed distances. The approximate equidistances of the methyl protons and H6 proton restrict the position of the aromatic ring of 4-dPyr with respect to Cr$^{3+}$ in such a way that 4-dPyr can be moved only either on a surface of a hemisphere with Cr$^{3+}$ at the center or 4-dPyr can be rotated about an axis passing through the center of the ring and perpendicular to the plane of the ring. Some of the orientations resulting from these movements, as well as rotation of the C-5-CH$_2$OH bond, will result in longer reaction coordinate distances, which will allow the formation of a phosphorylated enzyme intermediate. However, kinetic data and the failure to observe any phosphorylated enzyme intermediate provides an indirect support for the model, which suggests direct transfer of a phosphoryl group between ATP and pyridoxal.

The enzyme has been crystallized only recently (21), and the x-ray structure is not available at this time. Hence the docking of the substrates in the conformation shown in Fig. 6 to the active site of pyridoxal kinase is not yet possible. This would provide a test for the fit for the arrangement of the substrates shown in Fig. 6 or at least it would limit the number of possible arrangements due to the conformation of the active site itself.

In summary, the findings presented in this paper provide the first structural information regarding the relative positions of the substrates at the active site of pyridoxal kinase and possible mechanistical significance of this arrangement.

---

$^2$We should note that pyridoxal kinases from various tissues and species have low activities and, therefore, one unit of enzyme activity is defined as nanomoles of product formed/min at 37 °C (1, 22, 23). Therefore, the rates (at 25 °C) reported in this paper agree well with the rates reported in the literature (1, 2, 22, 23).

$^3$F. Kwok and J. E. Churchich, unpublished observations.
REFERENCES