CHARACTERIZATION OF THE ACTIVE SITE STRUCTURES OF ARGININE KINASE-SUBSTRATE COMPLEXES

WATER PROTON MAGNETIC RELAXATION RATES AND ELECTRON PARAMAGNETIC RESONANCE SPECTRA OF MANGANOUS-ENZYME COMPLEXES WITH SUBSTRATES AND OF A TRANSITION STATE ANALOG

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SUMMARY

The apparent number, n, of water ligands coordinated to bound Mn(II) in the ternary arginine kinase-MnADP or ATP complexes, in the abortive quaternary complex E-MnADP-arginine and in the transition state analog complex formed by addition of nitrate to the quaternary complex was estimated from the frequency dependence of the longitudinal proton relaxation rate of water (PRR) of lobster (Homarus americanus) muscle arginine kinase complexes. The apparent number of residual water ligands becomes progressively smaller as successive sites on the enzyme become occupied and is approximately 2 for the ternary nucleotide complexes, 1 for the abortive quaternary complex and considerably less than 1 for the transition state analog complex. The low values of n may be ascribed to the successive substitution of water ligands by protein ligands as each substrate and anion is successively bound to the enzyme or alternatively to an apparent rather than real disappearance of water ligands. The latter explanation implies that conformational changes induced by successive occupation of the binding sites on the enzyme are of such a nature that the rate of exchange of Mn(II)-bound water with solvent water becomes so slow for some Mn(II) water ligands that only a fraction of the total Mn(II)-bound water exchanges sufficiently rapidly to contribute to the PRR of bulk solvent.

From the frequency dependence of the PRR of the ternary and quaternary complexes, it has been established that T₁ₑ, the electron spin relaxation time, is the dominant term in the correlation time which modulates the interaction between the Mn(II) electron spin and the water proton nuclear spin. Values of the electron spin relaxation times, T₁ₑ, estimated from PRR experiments are consistent with the lower limits of T₁ₑ obtained from line widths of the EPR spectra of the same complexes.

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No change is observed in the position of the peaks in the EPR spectra in proceeding from the binary MnADP complex to the ternary E-MnADP to the abortive quaternary E-MnADP-arginine or from MnATP to E-MnATP, and it may be inferred that there has been no gross modification in the symmetry of the electronic environment of Mn(II). Small changes in the line widths of the EPR spectra for these complexes do indicate that the accessibility of the Mn(II) liganded structure to collision with solvent molecules has been modified consistent with changes in the number of exchangeable water protons determined from PRR. Addition of nitrate to the abortive quaternary complex to form the transition state analog, as in the case of rabbit muscle creatine kinase, induces significant changes in the active site structure of arginine kinase as reflected in the altered EPR line shape. In this case altered line positions of the EPR spectrum are also observed. The latter indicates an asymmetry of the electronic environment of Mn(II) which may result from a substitution of ligands. The corresponding transition state analog for creatine kinase exhibits a much more anisotropic EPR spectrum.

The EPR spectra are much more sensitive than PRR values to subtle changes in active site structure, e.g. spectral changes induced by D-arginine differ markedly from those produced by L-arginine in the presence of nitrate. Thiocyanate, nitrite, and formate at relatively high concentrations also modified the active site structure of arginine kinase, as evidenced by the alterations in the EPR spectrum of the quaternary complex upon addition of these anions. Modifications of the EPR spectra caused by these anions were qualitatively similar to those of nitrate but produced smaller changes in peak positions.

Arginine kinase catalyzes the reversible transfer of the γ-phosphoryl group of ATP to the guanidino nitrogen of L-arginine (1). The enzyme may utilize the paramagnetic manganous ion as an activator (2, 3) and binds MnADP to form a ternary complex
with a highly enhanced PRR (3, 4). A large decrease in PRR is observed upon binding of L-arginine to this complex (3, 4). A further decrease in PRR as well as a marked increase of L-arginine binding affinity results from the interaction of the nitrate ion with the abortive quaternary complex (4). The present investigation was initiated in order to elucidate the nature of the structural modification produced at the active site by the binding of substrates and of nitrate which is reflected in the PRR changes.

It has been pointed out in detailed discussions of the theory of the contribution of Mn(II) complexes to the PRR of water (3-8) that \( n \), the number of water ligands in the first coordination sphere of Mn(II) which are exchangeable with bulk water, and \( \tau_s \), the correlation time which modulates the nuclear-electron spin interaction, may be evaluated from the magnitude and frequency dependence of water PRR. A modification in the number of exchangeable water ligands will reflect an alteration in the interaction of the metal ion with the enzyme, substrates, or solvent water molecules. An alteration in the value of the correlation time in itself indicates a structural modification of the enzyme in the vicinity of the metal ion.

Additional information concerning the structure of the active site is obtained from EPR spectra of the Mn(II) enzyme complexes. Changes in the position and shape of EPR lines of bound Mn(II) can provide information regarding alterations in the symmetry of the electronic environment of the metal ion, from which alterations in the ligand composition of Mn(II) may be inferred, and in the dynamic interaction between solvent and bound Mn(II) (9-11). Both EPR and water PRR techniques have proved useful in elucidating structural differences among various complexes of pyruvate kinase (5, 6, 11) and of creatine kinase (7, 10, 12).

**EXPERIMENTAL PROCEDURE**

**Materials**—Crystalline arginine kinase was isolated from lobster (Homarus americanus) tail muscle by the method of Blethen and Kaplan (13). The concentration of arginine kinase was determined spectrophotometrically at 280 nm using a value of 6.7 for the extinction of a 1% solution (2) and a molecular weight of 40,000 (23). ADP and ATP were purchased from P L Biochemicals. Hepes, L-arginine, and n-arginine were purchased from Calbiochem. All other compounds were reagent grade.

**EPR and PRR Measurements**—The longitudinal proton relaxation time, \( T_1p \), of water was measured at 8, 13, 15.0, 24.3, 40.0, and 60.0 MHz by a pulsed nuclear magnetic resonance procedure described previously (5). The EPR spectra were recorded at 9.1 GHz with a Varian E-3 spectrometer. Base-line corrections were made by subtracting a scan of the buffer solution from the original spectrum employing a Varian C-1024 computer of average transients (9). The temperature for all measurements was controlled with a Varian model V-4340 variable temperature accessory.

**RESULTS**

**Frequency and Temperature Dependence of PRR**

**Ternary E-Mn-Nucleotide Complexes**—The binding of MnADP to arginine kinase is approximately 7-fold greater than that of ADP (4). Consequently, under conditions of high enzyme concentration and a severalfold excess of ADP over Mn(II), most of the Mn(II) will be found in the ternary E-MnADP complex. Under the experimental conditions, 0.51 mM enzyme, 0.1 mM MnCl₂, 0.46 mM ADP, and 25 mM Hepes-KOH buffer (pH 8), approximately 94% of the total Mn(II) in solution was bound in the ternary complex at 22°. Hence, the observed \( 1/T_1p \) value of 13.6 s\(^{-1} \) for this solution (24.3 MHz and 22°) was almost equal to 13.6 s\(^{-1} \), the \( 1/T_1p \) value for the ternary complex which was calculated from a complete titration (4). At lower temperatures, an even higher fraction of Mn(II) is in the bound form.

The value of \( T_1p \) for the ADP ternary complex is plotted as a function of the square of the frequency in Fig. 1B at three temperatures, 0, 11, and 22°. The relevant frequency dependent term, \( \tau_e/(1 + \omega^2\tau_e^2) \) in the Solomon-Bloembergen equation for \( T_1M \) (14, 15) predicts a linear relation between \( T_1p \) and \( \omega^2 \), but as in a number of other ternary enzyme complexes (7) the plots of \( T_1p \) versus \( \omega^2 \) pass through a minimum in the low frequency region at all temperatures (Fig. 1B). The value of \( T_1p \), at 8 MHz in each case is greater than that at 24.3 or 40 MHz, indicating that the correlation time, \( \tau_s \), itself is frequency-dependent. Since \( 1/\tau_e = 1/\tau_n + 1/\tau_M + 1/\tau_s \) and since both the rotational correlation time, \( \tau_n \), and the residence time for a water molecule in the first coordination sphere of Mn(II), \( \tau_M \), are independent of frequency, the dominant term in \( \tau_e \) must be the spin relaxation time, \( \tau_1M \), which may be frequency-dependent. The frequency dependence of \( T_1p \) for the ATP ternary complex (Fig. 2A) follows a similar pattern and consequently \( \tau_1M \) is again the dominant term in \( \tau_e \).

Calculation of \( n \) for Ternary E-Mn-Nucleotide Complexes—The calculation of \( n \), the number of exchangeable water ligands coordinated to Mn(II), from the Solomon-Bloembergen equation for \( T_1M \) is predicated on the assumption that the observed paramagnetic contribution to the relaxation rate, \( 1/T_1p \), is a true average of the relaxation rates of the free and bound water protons, not limited to any extent by the exchange rate between them. Since the relationship between \( T_1M \) and \( T_1p \) may be expressed as (16-18)

\[
1/T_1p = n\rho_i/(T_1M + \tau_M)
\]

(1)

![Fig. 1](http://example.com/fig1.png)

**Fig. 1.** A, temperature dependence of molar relaxivity of the arginine kinase-MnADP complex. The solution contained enzyme, 0.51 mM; MnCl₂, 0.1 mM; ADP, 0.46 mM; and Hepes-KOH buffer, 25 mM, pH 8.0. B, frequency dependence of molar relaxivity of the arginine kinase-MnADP complex at 0, 11, and 22°. Same data as in A.
The assumption of fast exchange is equivalent to $r_M < H_{\text{EPR}}$, where $H_{\text{EPR}}$ is the EPR spectrum at $3^\circ$ (cf. Fig. 6B), consistent with an equivalence of $\tau_M$ and $\tau_T$. The absence of any contribution from $\tau_M$ to the ternary ADP complex results in the formation of the abortive quaternary $E$-MnADP-L-arginine complex and an accompanying large decrease in FRR (3, 4). Nevertheless, under conditions in which the ternary complex is saturated with L-arginine, the general features of the frequency-dependent behavior of $T_{1p}$ are quite similar to those observed for the ternary complexes (cf. Figs. 1B, 2A, and 5). For the quaternary complex as well as the ternary complexes, the dominant term in $\tau_T$ is $T_{1p}$ and the observed relaxation rate is not exchange-limited. The values of $\tau_T$ in the quaternary complex estimated graphically from Fig. 3 are $3.48, 2.98,$ and $2.32 \times 10^{-8}$ s at $0, 11,$ and $22^\circ$, respectively; $\tau_T$ at $0^\circ$ is longer than the lower limit of $T_{1p}$ obtained from the EPR spectrum (cf. Fig. 6C), again consistent with an equivalence of $\tau_T$ and $\tau_M$.

Since the values of $\tau_T$ for the ternary and quaternary ADP complexes are almost equal, the large decrease in FRR resulting from the addition of L-arginine to the ternary complex cannot be attributed to a decrease in $\tau_T$. Thus, it appears likely that this decrease in relaxation rate arises from a reduction in $n$, the number of exchangeable water ligands in the first coordination sphere of Mn(II). Utilizing the values of $\tau_T$ determined for the ternary and quaternary complexes, the values of $\tau_T$ for the Mn(II)-water proton distance, were calculated for different values of $n$ (Table 1). An $\tau_T$ value of $2.86$ A was determined by Morgan (20) for the aquo-Mn(II) complex, and a lower limit of $2.815$ A and an upper limit of $2.923$ A was estimated (5) for this distance from crystallographic data (21–24). The only distance values in Table 1 that fall within these limits are those obtained with $n = 2$ for the ternary ADP complex and $n = 1$ for the quaternary complex. The value $n = 2$ for the ternary ADP complex yields a distance slightly higher than the usual range, i.e. $2.95$ A.

**Effect of Nitrate—**The addition of nitrate to the quaternary $E$-MnADP-L-arginine complex lowers the IRR by a factor of 2 (4) and alters its frequency dependence pattern (cf. Figs. 3 and 4B). With nitrate present, $T_{1p}$ was a linear function of $\omega^2$ for the quaternary complex containing either L-arginine (Fig. 4B) or D-arginine (Fig. 5) over the whole frequency range investigated. As in the case of other complexes, before attempting to calculate $\tau_T$ and $n$, it must first be ascertained that $1/T_{1p}$ is determined only by $T_{1M}$ without any contribution from $\tau_T$ (cf. Equation 1).

The temperature dependence of the FRR for the L-arginine complex plus nitrate is shown in Fig. 4A. Since the highest PRR values and thus the shortest values of $T_{1M}$ are observed at $8$ MHz, the maximum contribution from $\tau_T$ would be expected at this frequency. Thus, the occurrence of a positive slope in the plot of $1/T_{1p}$ versus $1/T$ at $8$ MHz clearly indicates that $\tau_M$ does not contribute significantly to the observed PRR. The
Distances of Mn(II) to water protons as function of n, number of water ligands, in ternary and quaternary arginine kinase complexes

<table>
<thead>
<tr>
<th>Complex</th>
<th>τ_e (× 10^9 s)</th>
<th>1/T_1p (× 10^9 s^-1)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>n = 1</td>
<td>n = 2</td>
</tr>
<tr>
<td>E-MnADP</td>
<td>2.96</td>
<td>3.06</td>
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<tr>
<td>E-MnATP</td>
<td>2.90</td>
<td>2.93</td>
</tr>
<tr>
<td>E-MnADP-L-arginine</td>
<td>2.98</td>
<td>2.85</td>
</tr>
</tbody>
</table>

* Determined from the ratios of slope to intercept of the plots of T_1p versus ω_l^2. The intercept was obtained from the extrapolation of the linear region at 11°, in Figs. 1, 2A, and 3, respectively.

\[ F(T_1p) = (T_1p + 0)r_c \]

\[ r_c = \frac{1}{k_B T} \frac{1}{\omega_l^2} \left( \frac{1}{\omega_1} + \alpha \right)^{1/2} \]

Values of τ_e at several temperatures were calculated for the nitrate forms of the L-arginine and n-arginine quaternary complexes from the linear T_1p versus ω_l^2 plots, and the results are shown in Table II. The value of τ_e at 11° increased almost 3-fold as a result of the addition of nitrate to the L-arginine quaternary complex (cf. Table I). At three temperatures studied, the value of τ_e was somewhat longer for the L-arginine quaternary complex than for the n-arginine complex (cf. Table II). In the presence of either quaternary complex, the observed values of 1/T_1p are considerably less than the theoretical values of 1/T_1p calculated for a single exchangeable water ligand remaining in the first coordination sphere of Mn(II), and a Mn(II) to water distance of 2.86 Å. The value of 1/T_1p is a function of \( (r_e/(1 + \alpha \omega l^2 - \omega l) \), where \( \omega l \) is the Larmor precession frequency of the proton.

a Calculated from data in Figs. 1, 2, and 3 at 24.3 MHz and 11°.

Comparison of observed and calculated molar relaxation rates at 24.3 MHz for E-ADP-Mn-L-arginine-NO_3 and d-arginine complexes

<table>
<thead>
<tr>
<th>Temperature</th>
<th>τ_e (× 10^9 s^-1)</th>
<th>1/T_1p (× 10^9 s^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
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<td></td>
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<td>10.6</td>
<td>8.3</td>
</tr>
<tr>
<td>11</td>
<td>8.2</td>
<td>9.1</td>
</tr>
<tr>
<td>22</td>
<td>6.2</td>
<td>9.3</td>
</tr>
<tr>
<td>d-Arginine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.9</td>
<td>9.2</td>
</tr>
<tr>
<td>11</td>
<td>6.7</td>
<td>9.3</td>
</tr>
<tr>
<td>22</td>
<td>4.0</td>
<td>8.3</td>
</tr>
</tbody>
</table>

A comparison of the spectra of free MnATP and enzyme-bound MnATP (Fig. 7, A and B) also reveals no fine structure in either complex, only a small increase in line width in the enzyme complex but no change in line position, i.e. a small increase in electron spin relaxation rate but no change in the symmetry of the electronic environment of Mn(II) or the absence of a unique distortion of symmetry in this environment (10).

The binding of L-arginine to the ternary E-MnADP complex also produces a minor alteration of spectral line shape, consisting of a small decrease in line width, without an accompanying fine structure splitting (cf. Fig. 6, B and C). It is highly unlikely that a significant amount of Mn-L-arginine was present in the solution containing the quaternary complex (Fig. 6C) since, at the concentrations used in the enzyme experiments, L-arginine in the absence of enzyme does not form a complex with Mn(II) to any observable extent as measured by EPR. An increase in the concentration of free MnADP would not produce the spectral change observed with L-arginine (cf. Fig. 6, A and C).
Fig. 6. EPR spectra for various MnADP complexes. All solutions contained MnCl₂, 0.4 mM; ADP, 2.0 mM; Hepes-KOH buffer, 25 mM, pH 8.0. Additional components were arginine kinase, 0.96 mM; L-arginine, 20 mM; KNO₃, 5 mM. Spectra were recorded at 3°C.

Hence, the decrease in spectral line width reflects a small increase in electron spin relaxation time for Mn(II) bound at the active site in the ternary MnADP complex. The binding of L-arginine to the ADP ternary complex also decreases the spectral line width (cf. Figs. 6B and 8A).

Fig. 7. EPR spectra for various MnATP complexes. All solutions contained MnCl₂, 0.4 mM; ATP, 2.0 mM; Hepes-KOH buffer, 25 mM, pH 8.0. Additional components were arginine kinase, 1.12 mM; L-arginine, 10 mM. Spectra were recorded at 3°C.

The addition of L-arginine to the E-MnATP complex leads to the formation of the equilibrium mixture, and it is difficult to estimate the relative amounts of the many species which coexist under these conditions. Since the EPR spectrum (Fig. 7C) undoubtedly results from a superposition of several spectra any interpretation is open to question. However, it is apparent from an examination of the EPR spectrum (Fig. 7C) that no drastic changes have occurred and that the spectra of the active quaternary complexes E-MnATP-arginine or E-MnADP-P-arginine do not differ significantly from the abortive E-MnADP-arginine complex.

Effect of Nitrate—Addition of nitrate to the ternary E-MnADP complex had no significant effect on its structure by the criteria of PRR and EPR. At a high concentration (50 mM) nitrate gave rise only to a 10% decrease in the PRR of the ternary complex, and even at a concentration as high as 100 mM the anion did not alter the EPR spectrum. In contrast, both the PRR (4) and EPR spectrum of the L-arginine quaternary complex (Fig. 6D) were markedly altered by additions of low concentrations of nitrate. The addition of nitrate to the quaternary complex produced changes in spectral line positions and a decrease in both line amplitudes and line widths (cf. Fig. 6, C and D).

In the spectrum with nitrate (Fig. 6D), fine structure components characteristic of a powder spectrum (9) are found on the low field side of the normal hyperfine sextet of Mn(II). The change in spectral line position brought about by nitrate reflects an increase in the zero field splitting caused by asymmetry of the Mn(II) environment. The EPR spectrum for the L-arginine quaternary complex is also markedly altered by the addition of nitrate (cf. Fig. 8, A and B). However, the spectra for the nitrate forms of the N- and L-arginine quaternary complexes are quite different (cf. Fig. 8, B and C).

The narrowing of the spectral lines resulting from the inter action of nitrate with the quaternary complex reflects an increase in the electron spin relaxation time of Mn(II). This observation...
is consistent with the large increase in $\tau_e$ for the Mn(II)-water proton interaction that results from the addition of nitrate (cf. Tables I and II). Since the principal mechanism for electron spin relaxation has been ascribed to distortion of the Mn(II) octahedral complex by outer sphere solvent molecules (16, 25), it is likely that the collision frequency of solvent water molecules with bound Mn(II) is reduced for the transition state analog complex.

**Effects of Other Anions**—High concentrations (100 to 300 mm) of chloride did not induce the changes observed with nitrate, i.e. the EPR spectrum of the abortive quaternary complex was not altered and the PRR was lowered only slightly. On the other hand, the planar anions, formate, thiocyanate, and nitrite, are intermediate in effectiveness. They bind much more weakly than nitrate, lower the PRR to a lesser extent (4), and affect the EPR spectrum of the abortive quaternary complex to a lesser extent (cf. Figs. 8C and 9). In contrast to nitrate, these anions changed the line positions very slightly but at 200 mm concentration significantly decreased the spectral line widths. Apparently these anions, as was the case for nitrate, induced structural alterations at the active site that protect the Mn(II) complex from collision with solvent water but do not induce the asymmetry in the environment of Mn(II) that leads to the anisotropic EPR spectrum observed with nitrate.

**DISCUSSION**

The two spectroscopic properties used to investigate arginine kinase-metal-substrate complexes, EPR and FRR, are sensitive to different structural aspects of the local environments of Mn(II). For example, the EPR spectra of the binary MnADP complex and ternary $E$-$E_{-}$MnADP complex reveal no significant spectral differences between them but the FRR values differ greatly. The same observations have been made for the creatine kinase-MnADP complex; the FRR enhancement and the EPR spectrum is the same as for arginine kinase (10, 26). In that case it was concluded (7, 10) that there was no change in the environment of Mn(II) upon addition of enzyme to MnADP and that the increase in PRR by a factor of almost 20 was due to a change in correlation time, $\tau_e$. Such a change in correlation time is not anticipated since in the binary MnADP complex the dominant correlation time is the rotational correlation time $\sim 10^{-10}$ s, but in the ternary complex the rotational correlation time becomes very long and the electron spin relaxation is the dominant correlation time. Thus, the structure of the ternary complex which was inferred from this interpretation was $E$-$E_{-}$MnADP with the nucleotide forming a bridge between the enzyme and the metal ion and the ligands of Mn(II) remaining unchanged, i.e. $n$, the number of water ligands remaining in the first coordination of Mn(II), was the same in the binary MnADP complex and the ternary $E$-$E_{-}$MnADP complex. Support for the conclusion that the protein donated no ligands to Mn(II) in the ternary complex is derived from the finding that the binding constant of MnADP to creatine kinase is only twice that of ADP.

The interpretation discussed above for the creatine kinase system must be modified somewhat in view of the additional information available for arginine kinase from the present investigation. In the arginine kinase system, because the metal-nucleotides bind sufficiently tightly to the enzyme, experimental conditions were easily attainable so that all the Mn(II) existed in the enzyme-bound form. Consequently, for the first time it has been possible to estimate $n$, the number of exchangeable water ligands in the first coordination sphere of Mn(II), in each of the complexes from its PRR frequency dependence. Thus, for the ternary $E$-$E_{-}$MnADP and $E$-$E_{-}$MnATP complexes, $n$ equals 2 and apparently only 2 residual water ligands remain in the first coordination sphere of the metal. Since the binary MnADP complex has 3 or 4 water ligands, this result is most readily explained by the substitution of at least 1 water ligand by a ligand from the protein. An alternative explanation is that 1 Mn(II) water ligand now exchanges so slowly with the bulk water over the temperature range investigated that it contributes nothing to the PRR. The factor of 7 favoring the binding of metal-ADP over ADP is rather small to be considered unequivocal evidence for the substitution of a water ligand by a protein ligand. The lack of change in the EPR spectrum upon substitution of a water ligand by a protein ligand would imply that the ligand or ligands donated by the enzyme do not impose an electronic environment for Mn(II) significantly different from that imposed by the water ligands they replace. In fact, Mn(II) bound to a protein such as pyruvate kinase (11) can yield an EPR spectrum very similar to the $E$-$E_{-}$MnADP complex of either arginine or creatine kinase.

The finding that the number of residual water molecules in the Mn(II) coordination sphere is the same in $E$-$E_{-}$MnADP and in $E$-$E_{-}$MnATP is somewhat puzzling. The same conclusions discussed in detail above which led to the conclusion that the Mn(II) ligands remained unchanged when Mn-nucleotide binds to enzyme would also predict that $E$-$E_{-}$MnADP would have one more Mn(II)-water ligand than $E$-$E_{-}$MnATP, since in the absence of enzyme the residual number of water molecules in the Mn(II) coordination sphere has been found to be 4 for MnADP and 3 for MnATP. There are three possible explanations of the experimental result that the apparent values of $n$ are the same for the di- and triphosphate complexes: (a) Mn(II) is coordinated to

*W. K. Cheng, unpublished results*
only two phosphate groups on either nucleotide when the nucleotides are bound to the enzyme, (b) one additional Mn-protein ligand is formed in the \(E\)-MnADP than in the \(E\)-MnATP complex, and (c) the ligands to Mn(II) are unchanged in binding either Mn-nucleotide to enzyme, but the conformations of the two ternary complexes at the active site differ in such a way that the active site is less open in the ADP complex so that an additional molecule of coordinated water can not exchange rapidly with water. Evidence may be adduced for each of the three possibilities, particularly by analogy to the creatine kinase system. For example, it has been concluded from high resolution NMR experiments that, in the quaternary creatine kinase complexes containing creatine, Mn(II) is coordinated only to the \(\alpha\) and \(\beta\) phosphates when ATP or ADP is the fourth component. Thus, there is a precedent for the first possibility considered. The 10-fold greater affinity of MnADP relative to MnATP to arginine kinase may be used to bolster the second possibility of an additional protein ligand to Mn(II) in the diphosphate complex.

The third possibility which implies decreased access of solvent water to the bound Mn(II) is lent plausibility from a comparison of the EPR spectra of the two ternary complexes (cf. Figs. 6B and 7B) which show that the electron spin relaxation is longer for the ADP complex and thus Mn(II) in that complex is less accessible to solvent water. However, it is obvious that at this stage a definitive choice cannot be made among the proposed explanations.

As more subsites on the enzyme become occupied first by the addition of the second substrate to form an abortive quaternary complex and then by the further addition of nitrate to form a transition state analog complex, the number of residual water molecules exchangeable with solvent water decreases. In fact, in the transition state analog, \(n < 0.5\), as it is in the analogous creatine kinase (7) complex and in the Mn-pyruvate kinase-P-enolpyruvate complex (6). As discussed in the latter cases, there are several possible interpretations of such a low value of \(n\). Regardless of interpretation the observation taken in conjunction with the finding that the lines in the EPR spectra are narrowed implies that the active site becomes less and less accessible to solvent as successive binding sites are occupied.

The addition of L-arginine to the \(E\)-MnADP spectrum does not change the position of the lines in the EPR spectrum and it may be inferred that there is no change induced in the symmetry of the electronic environment of Mn(II). In the analogous experiment with creatine kinase, changes are observed in the line positions of the EPR spectrum upon addition of creatine due to introduction of asymmetry in the electronic environment of Mn(II). It was suggested that the asymmetry might be ascribed to the substitution of one or more water ligands by a protein ligand. In the case of arginine kinase, the number of Mn(II) water ligands is reduced by one upon addition of arginine but there is no evidence from EPR that ligand substitution has occurred. Either ligand substitution in this case does not change the symmetry or there is no ligand substitution but rather the induced conformational change prevents a Mn(II) water ligand from exchanging with solvent water.

When nitrate is added to the \(E\)-MnADP-L-arginine complex to form the transition state analog, there is a change in the line positions of the EPR spectrum. However, the change is small compared to the analogous creatine kinase complex, indicating that the asymmetry introduced at the arginine kinase active site is not as great. The inhibition of the forward reaction by nitrate (4, 27) is also greater for creatine kinase than for arginine kinase. It should be noted that, unlike the FRR parameter, the EPR spectrum is sensitive to a change in anion structure. Similarly, the EPR spectrum is sensitive to stereospecificity as evidenced by differences between L-arginine and D-arginine complexes. Thus, the two spectroscopic techniques used are complementary to one another; the FRR values respond primarily to the number of subsites occupied at the active site of arginine kinase, and the EPR spectra respond to the detailed structure of the ligands occupying the subsites.

Whether the active site structures of the various enzyme-metal-substrate complexes of the two guanidino kinases investigated by magnetic resonance methods, i.e. rabbit muscle creatine kinase and lobster (Homarus americanus) muscle arginine kinase, differ basically remains to be determined. Future experiments with \(^{31}\text{P}\) should establish the coordination of the metal ion to the nucleotides in the various complexes. The observation of an intermediate nuclear Overhauser effect between bound formate and a lysine residue of creatine kinase in the transition state analog complex (28) supplies strong evidence that lysine is the binding site of the transferable phosphate and experiments in progress with arginine kinase should determine whether a lysine residue of the latter enzyme plays the same role. Mapping of the active site by the same methods used for creatine kinase with Mn(II) as a probe (29) may reveal whether the alignment of the substrates is the same for both enzymes.

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