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Quantitation of movement of the phosphoryl group during catalytic transfer in the arginine kinase reaction: ^{31}P relaxation measurements on enzyme-bound equilibrium mixtures**

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Abstract

^{31}P nuclear spin relaxation measurements have been made on enzyme-bound equilibrium mixtures of lobster-muscle arginine kinase in the presence of substituent activating paramagnetic cation Co(II) (in place of Mg(II)), i.e., on samples in which the reaction, E•CoATP•arginine ⇌ E•CoADP•P-arginine, is in progress. The results have been analyzed on the basis of a previously published theory (Nageswara Rao, B.D. (1995) *J. Magn. Reson.*, **B108**, 289–293) to determine the structural changes in the reaction complex accompanying phosphoryl transfer. The analysis enables the determination of the change in the Co(II)-^{31}P (γ-P(ATP)) vector as the transferable phosphoryl group moves over and attaches to arginine to form P-arginine. It is shown that the Co(II)-^{31}P distance of ∼3.0 Å, representing direct coordination of Co(II) to γ-P(ATP), changes to ∼4.0 Å when P-arginine is formed in the enzyme-bound reaction complex. This elongation of the Co(II)-^{31}P vector implies an excursion of at least 1.0 Å for the itinerant phosphoryl group on the surface of the enzyme.

Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; E•M•S, enzyme-metal-substrate; E•S, enzyme-substrate; E•M•S_1•S_2, enzyme-metal-substrate1-substrate2; E•MP_1•P_2, enzyme-metal-product1-product2; E•MATP, enzyme-metal-ATP; E•MADP, enzyme-metal-ADP; Hepes, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; NMR, nuclear magnetic resonance.

Introduction

It is a generally accepted paradigm that the molecular basis of enzyme catalysis lies in the conformational arrangement of the enzyme-bound substrates and their amino-acid environment. As the enzyme-bound substrates and products interconvert on the surface of the enzyme, structural changes occur in the reaction complexes. For example, in the arginine kinase equilibrium mixture given by,

E•MATP•arginine ⇌ E•MADP•P-arginine, (1)

the terminal phosphoryl group (γ-P) of ATP[1] moves over and attaches to arginine in the forward reaction. In Equation 1, M represents the divalent cation, Mg(II) *in vivo*, which is an obligatory component of most reactions of ATP-utilizing enzymes. Thus, in the context of the arginine kinase reaction, one may ask the question: 'How far does the phosphoryl group move as the phosphoryl transfer occurs?' A determination of this distance is useful in visualizing the molecular events accompanying catalytic transfer and in elucidating the roles of the functional amino-acid residues and of the activating cation.

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Most of the ATP-utilizing enzymes are activated *in vitro* by substituent paramagnetic cations Mn(II) and Co(II). The relaxation rates of nuclei in the vicinity of paramagnetic cations are measurably enhanced proportional to the reciprocal sixth power of the cation-nucleus distances (Mildvan and Gupta, 1978; Villafranca, 1984). This fact has been extensively used to determine cation-nucleus distances in enzyme-substrate complexes of ATP-utilizing enzymes (Gupta et al., 1976; Jarori et al., 1985, 1989; Ray and Nageswara Rao, 1988; Ray et al., 1988, 1996; Raghunathan et al., 1999; Lin and Nageswara Rao, 2000). Experimental protocols for nuclear-relaxation-enhancement measurements require a small fraction (0.5–10%) of paramagnetic complexes (E•MS) in exchange with diamagnetic complexes (E•S). The observed relaxation rate, therefore, depends on the lifetime of the E•M•S complex, τ_M, leading thereby, to the requirement of fast exchange for relaxation data to be structurally relevant, i.e., $\tau_M << T_{1M}$, where T_{1M} is the relaxation time of the substrate nucleus. Relaxation rates in equilibrium mixtures are additionally complicated by the interconversion rate on the enzyme ($E\bullet MS_1\bullet S_2 \rightleftharpoons E\bullet MP_1\bullet P_2$), and this complexity has been an impediment to attempts to extract information on structural changes in the reaction complex accompanying enzyme turnover. However, it has been recently shown theoretically that the relaxation rates of paramagnetic-cation-activated enzyme-bound equilibrium mixtures are amenable to analysis under conditions that are experimentally realizable (Nageswara Rao, 1995).

If relaxation measurements can be made on enzyme-bound equilibrium mixtures containing activating paramagnetic cations, and the results are interpretable, it will be possible to acquire information on the structural changes accompanying substrate-product interconversion. Thus, for example, ^{31}P relaxation measurements leading to the determination of the cation-^{31}P (γ-P) and cation-^{31}P (P-arginine) distances of the itinerant ^{31}P nucleus, as the interconversion occurs in the enzyme-bound equilibrium mixture, are capable of providing an answer to the question posed above on the excursion of the phosphoryl group during catalytic transfer (Nageswara Rao, 1999a, b).

This paper presents ^{31}P relaxation measurements on enzyme-bound equilibrium mixtures of lobster muscle arginine kinase performed to determine the above structural changes in the reaction complex accompanying the phosphoryl transfer. These measurements were made with Co(II) as the substituent paramagnetic cation as the relaxation rates with Mn(II) are exchange limited ($\tau_M >> T_{1M}$) (Jarori et al., 1989). A concise description of the theoretical basis (Nageswara Rao, 1995) for the analysis of relaxation data in equilibrium mixtures is given below.

Theory

The various exchanges of a nucleus A in an enzyme-bound substrate S_1 (e.g., ^{31}P in the γ-P(ATP) in Equation 1) which converts to B in the enzyme-bound product P_2 (e.g., ^{31}P in P-arginine) are shown in Equation 2:

$$\begin{array}{ccc} A'(E\bullet S_1\bullet S_2) & & B'(E\bullet P_1\bullet P_2) \\ \updownarrow & & \updownarrow \\ A(E\bullet MS_1\bullet S_2) & \longleftrightarrow & B(E\bullet MP_1\bullet P_2) \end{array} \qquad (2)$$

where $E\bullet MS_1\bullet S_2$ and $E\bullet MP_1\bullet P_2$ are the interconverting active paramagnetic reaction complexes. The itinerant nucleus also exists in the corresponding inactive diamagnetic complexes $E\bullet S_1\bullet S_2$ and $E\bullet P_1\bullet P_2$ in which it is labeled as A′ and B′, respectively. Fast exchange implies that the dissociation rates of the paramagnetic complexes are much larger than the relaxation rates (R_A and $R_{B)}$ in these complexes, as well as the interconversion rate (ε) between them. If these conditions are valid, the recoveries of the superposed magnetizations ($m_A+m_{A'}$) and ($m_B+m_{B'}$) in an inversion-recovery experiment are, in general, biexponential, given by (Nageswara Rao, 1995):

$$\begin{pmatrix} m_A + m_{A'} \\ \\ m_B + m_{B'} \end{pmatrix} = -2M_0 \begin{pmatrix} e^{-\Omega_+ t}(\cos^2\varphi - \cos\varphi\sin\varphi) + \\ e^{-\Omega_- t}(\sin^2\varphi + \cos\varphi\sin\varphi) \\ \\ e^{-\Omega_+ t}(\sin^2\varphi - \cos\varphi\sin\varphi) + \\ e^{-\Omega_- t}(\cos^2\varphi + \cos\varphi\sin\varphi) \end{pmatrix}, \qquad (3)$$

where $m_i = M_{zi} - M_{0i}$, and M_{zi} and M_{0i} are, respectively, the instantaneous and equilibrium values of the longitudinal magnetization of nucleus i. M_0 is the total equilibrium magnetization of A and A′ (and of B and B′),

$$\tan 2\varphi = 2\varepsilon/R_A - R_B), \quad (R_A > R_B), \qquad (4)$$

and

$$\Omega_\pm = R_D + p[\varepsilon + (R_A + R_B)/2] \pm p[(R_A - R_B)^2]/4 + \varepsilon^2]^{1/2}, \qquad (5)$$

where R_D is the relaxation rate in the two diamagnetic complexes (assumed equal) and p is the fractional concentration of the paramagnetic complexes. For the sake of transparency, Equations 3 to 5 are given for the case where the equilibrium constant on the enzyme is unity, and for the same value of p for both sides of the reaction. Equations for more general conditions are available in Nageswara Rao (1995). The following special cases of Equation 3 are of interest:
(1) If $R_A \approx R_B$ or if $\varepsilon >> |R_A\text{-}R_B|/2$, $\cos\varphi = \sin\varphi = 1/\sqrt{2}$, both the A and B magnetizations recover with a single *average* rate given by

$$\Omega_- = R_D + p(R_A + R_B)/2. \tag{6}$$

(2) If $\varepsilon << |R_A\text{-}R_B|/2$, A and B recoveries will be essentially uncoupled, with the rate constants incremented by the interconversion rate (ε), as given by

$$R_D + p(R_A + \varepsilon), \text{ and } R_D + p(R_B + \varepsilon). \tag{7}$$

(3) If the resonances of A(A′) and B(B′) superpose, the recovery of the total magnetization is given by:

$$\begin{aligned}&(m_A + m_{A'} + m_B + m_{B'}) = \\ &-2M_0[e^{-\Omega_+ t}(1 - \sin 2\varphi) + e^{-\Omega_- t}(1 + \sin 2\varphi)].\end{aligned} \tag{8}$$

(4) If the A(A′) and B(B′) resonances are separated, each of the recoveries is biexponential. Note further that $\varphi \leq \pi/4$ (see Equation 4) so that $\cos\varphi \geq \sin\varphi$. Thus, the amplitudes of the two exponentials are of the same sign for A, and of opposite sign for B (A and B are distinguished by the choice $R_A > R_B$ in Equation 4).

By analyzing the observed recoveries in terms of Equations 3–8, or by using the more general version given in Nageswara Rao (1995), R_A and R_B may be evaluated. If R_A and R_B are dominated by dipolar interactions, the ratio of r_A and r_B, the cation nucleus distances in the two complexes, is given by

$$(r_A/r_B) = (R_B/R_A)^{1/6}. \tag{9}$$

Thus the structural changes occurring due to enzyme turnover may be determined.

Experimental procedures

Materials

ADP, ATP, 0.1 mM $MnCl_2$ solution in 0.15M NaCl, arginine, P-arginine, lactate dehydrogenase, and pyruvate kinase were obtained from Sigma, Puratronic $CoCl_2$ from Alfa/Aesar Chemicals, Hepes from Research Organics, and Chelex-100 from Bio-Rad. All other chemicals used were of analytical reagent grade.

Sample preparation

Arginine kinase was purified from the lobster muscles by method of Blethen and Kaplan (1967), except that the hemocyanin was removed from the purified enzyme by chromatography on Sephadex-G75, and adenylate kinase was removed by chromatography over DEAE cellulose (acetate) (Jarori et al., 1989). The specific activity of the enzyme was determined spectrophotometrically by a coupled assay with pyruvate kinase and lactate dehydrogenase. The enzyme was finally dialyzed against 0.1 M Hepes-K^+, pH 8.1 containing pre-equilibrated Chelex-100 resin to eliminate trace metal ions from the sample, and concentrated to ∼240–300 mg/ml in an Amicon ultra-filtration cell (Model-8010). All buffers and ligand solutions were also passed through a Chelex-100 column. Protein and nucleotide concentrations were determined spectrophotometrically, with $\varepsilon_{280}^{mg/ml} = 0.670\ cm^{-1}$ and a molecular weight of 40 000 (Morrison, 1973) for the enzyme, and $\varepsilon_{280}^{mg/ml} = 15.4\ cm^{-1}$ for ATP and ADP.

NMR measurements

^{31}P NMR measurements at 202 MHz, were made on an Inova-500 spectrometer with a high-stability variable temperature controller, 10 mm multinuclear probe from Cryomagnet Systems, and SUN Microsystems SPARCstation 5/85 host computer. T_1 measurements were made using a standard inversion recovery sequence with a composite π pulse. The relaxation rates in the paramagnetic complexes were determined by averaging the measurements for 4–6 values of cation concentration. For these measurements, a typical sample contained ∼1.9 ml of buffered enzyme solution with about 35% D_2O in a 10-mm o.d. NMR sample tube. Temperature dependence of $(pT_{1P})^{-1}$ was measured for fixed cation concentrations over the range of 5–25 °C and the relaxation data for all of the signals were fitted by nonlinear least squares to a multiple biexponential model.

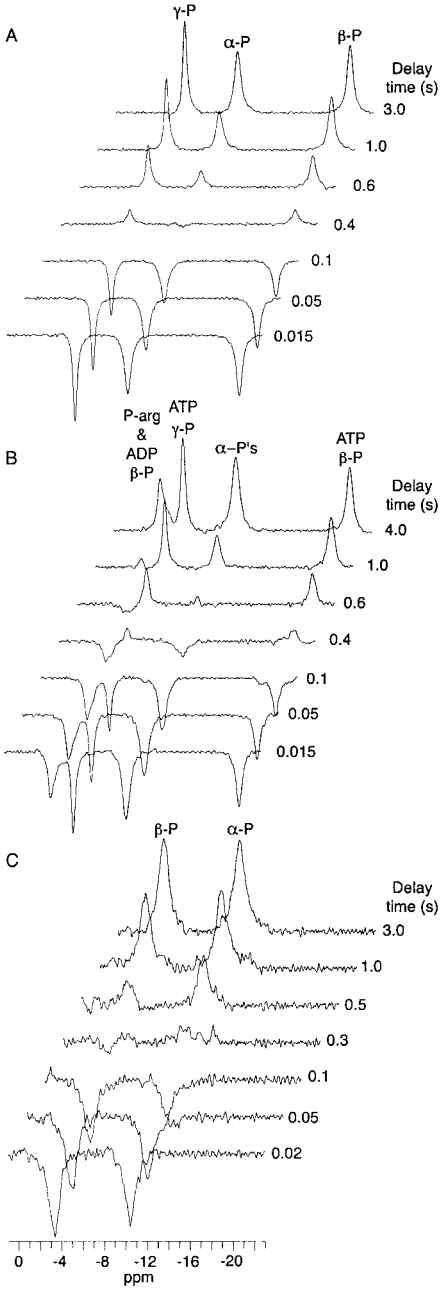

Figure 1. Typical T_1 measurements for ^{31}P at 202 MHz and 5 °C in arginine kinase complexes: (A) ECo(II)ATP, (B) enzyme-bound equilibrium mixture, and (C) ECo(II)ADP. [Co(II)]/[nucleotide] = 0.034 for all three cases. Resonance assignments are labeled. Sample conditions: (A) 5.0 mM enzyme sites, 3.8 mM ATP, and 27–195 μM $CoCl_2$; (B) 4.7 mM enzyme sites, reaction initiated with 3.6 mM ATP, 3.72 mM arginine, and 25–175 μM $CoCl_2$; and (C) 5.0 mM enzyme sites, 2.9 mM ADP, and 27–190 μM $CoCl_2$. All the solutions were buffered in 0.1 M Hepes, pH 8.1. NMR parameters: For all three cases π/2 pulse width, 24.4 μs; sweep width, 8500 Hz; data size, 8K; and line broadening, 30 Hz. Recycle delay, (A), (C) 3 s, and (B) 4.5 s; transients (A), (B) 64, and (C) 32.

Results and analysis

^{31}P measurements with Co(II)

Shown in Figures 1A and 1C are stack plots of T_1 measurements on ^{31}P nuclei in E•ATP and E•ADP complexes of arginine kinase, respectively, in the presence of a fractional Co(II) given by [Co(II)]/[nucleotide]= 0.034. The assignments for the ^{31}P signals are marked. Sandwiched between them in Figure 1B is the T_1 stack plot for an independent sample of the enzyme-bound equilibrium mixture with the same value of fractional Co(II). In all three samples, the enzyme concentrations are in sufficient excess such that the concentration of free nucleotides does not exceed 8% of the total. The concentrations of the various enzyme-bound complexes and free substrates were computed on the basis of known dissociation constants (Buttlaire and Cohn, 1974) by means of a computer program. In making these computations, estimates of some of the dissociation constants, and synergistic effects associated with these were included. This information is based on previous enzyme-bound diamagnetic equilibrium-mixture experiments in which the stoichiometric equilibrium constant was estimated to be ∼1.0 (Nageswara Rao et al., 1976; Vasavada et al., 1980) under conditions analogous to those employed in the current experiments. The computations show the following distribution of complexes: Under the conditions used for obtaining Figure 1B, at least 92% of the nucleotides are bound to the enzyme, and over 50% of these are present in enzyme-bound reaction complexes (with and without the cation); E•ADP•arginine complexes are about 25–30% of the reaction complexes; and the rest of the substrates (nucleotides, arginine and P-arginine) are present either in binary complexes with the enzyme or free in solution. The concentrations of the three complexes containing P-arginine and Co(II), viz., E•Co(II)ADP•P-arginine, E•Co(II)•P-arginine and Co(II)•P-arginine are approximately in the ratio 99.6:0.3:0.1. Thus, P-arginine is exposed to the cation primarily in the nucleotide complexes because the affinity of the nucleotide for the cation is significantly larger than that of P-arginine. Since the relaxation rate of P-arginine due to the presence of Co(II) in the enzyme-bound equilibrium mixture is the quantity of interest, and the observed ^{31}P signal arises from all the complexes, the contribution of the unproductive complexes to the experimentally measured relaxation rate was estimated. For this purpose,

the ^{31}P relaxation rates of P-arginine and E•P-arginine have been independently measured in the presence of Co(II) (see Table 1). The data show that 95% of the observed relaxation rate in the presence of Co(II) is due to E•Co(II)ADP•P-arginine and that E•Co(II)•P-arginine and Co(II)•P-arginine together contribute no more than 5% of the rate.

A comparison of the three stack plots in Figure 1 readily reveals that the ^{31}P signal of bound P-arginine relaxes markedly more slowly than all the signals from ATP and ADP (some of which are superposed in Figure 1B). In particular, the ^{31}P relaxation time of P-arginine in the equilibrium mixture (Figure 1B) is considerably longer than that for γ-ATP in both Figures 1A and 1B. Since the relaxation rate due to dipolar interaction between the cation (Co(II)) and a nucleus (^{31}P) is inversely proportional to the sixth power of the cation-nucleus distance, this difference indicates qualitatively that the Co(II)-^{31}P (P-arginine) distance in E•Co(II)ADP•P-arginine is longer than the Co(II)-^{31}P (γ-P) distance in E•Co(II)ATP, and E•Co(II)ATP•arginine. The elongation in this distance is a measure of the excursion of the itinerant phosphoryl moiety on the surface of the enzyme in the arginine kinase reaction.

The ^{31}P relaxation rates obtained for the ternary complexes E•Co(II)ATP and E•Co(II)ADP are given in Table 1. These rates were previously shown not to be limited by exchange due to association and dissociation (Jarori et al., 1989). It was also demonstrated recently that scalar (contact and pseudo-contact) interactions between Co(II) and ^{31}P make a negligible contribution (a factor of 10^{-4} weaker) to ^{31}P relaxation compared to the dipole-dipole interaction between them (Ray et al., 1999). The ^{31}P relaxation rates of bound ATP and ADP interconverting in the equilibrium mixture, given in Table 1, are generally similar to those in the individual bound complexes suggesting that they are not exchange limited in the equilibrium mixture also. However, in order to verify that such is the case, the temperature dependence of the relaxation rates was measured in the range 5–25 °C. The measurements confirm that the observed rates are not limited by exchange due to association and dissociation. Analysis of the data in the equilibrium mixture by the use of Equations 3–8, however, requires an estimate of the interconversion rate ε. This was obtained by first noting that the apparent relaxation rate of the P-arginine signal in the equilibrium mixture is about 20 s^{-1}. Since this ^{31}P nucleus is interchanging with that of γ-P(ATP) during the reaction, and the latter has a relaxation rate of ∼76 s^{-1}, it can be concluded that the interchange of this pair belongs to case 2 (see Theory) viz., $\varepsilon < |R_A - R_B|/2$ so that $(R_B + \varepsilon) \approx 20\ s^{-1}$. On the other hand, assay of the enzyme with Mg(II), Mn(II), and Co(II) at 22 °C yielded turnover numbers for the *overall* reaction of 100, 90 and 4 s^{-1}, respectively. It was shown previously that at pH 8.0 the interconversion rate (∼90 s^{-1}) of the arginine kinase reaction is not rate-limiting for the overall reaction with Mg(II) as the activator (Nageswara Rao and Cohn, 1977; Vasavada et al., 1980). Assuming that this condition is also valid for the paramagnetic cations suggests that the value of ε in the presence of Co(II) is larger than 4 s^{-1}, i.e., $4\ s^{-1} \leq \varepsilon \leq 20 s^{-1}$. Attempts to fit the recovery curves with several different values in this range lead to the choice of 7 s^{-1} for ε. Analysis of the temperature dependence of $(pT_{1P})^{-1}$ of P-Arg and γ-P(ATP) signals yielded results in agreement with this choice of 7 s^{-1} for ε.

The relaxation rates obtained for the ^{31}P nuclei in the equilibrium mixture are given in Table 1. Experimental and calculated recoveries for the four different ^{31}P signals (see Figure 1B) are shown in Figure 2. It may be noted that the superposed α-P resonances of ADP and ATP belong to case 3, and the separated β-P resonances of ADP and ATP belong to case 4, as described in the Theory section. Furthermore, in the case of the superposed resonances of β-P(ATP) and P-arginine, the recoveries of the individual nuclei need to be separately calculated and summed to obtain the theoretical curve. In the computations, the stoichiometric equilibrium constant was assumed to be unity, which was the case for arginine kinase in the presence of Mg(II) (Nageswara Rao and Cohn, 1977; Vasavada et al., 1980). The values of p for ATP, for ADP, and for P-arginine were computed on the basis of known dissociation constants. They were found to be unequal and this was incorporated into the simulations. These factors, along with the uncertainty in the estimate of ε discussed above, contribute to the overall uncertainty in the relaxation rates (R_A and R_B) deduced for the interchanging nuclei. However, since the cation-nucleus distances are proportional to the reciprocal sixth power of the relaxation rates, the accuracy of the distances calculated is not significantly affected. Thus, even in an extreme case where the estimated relaxation rate of P-arginine deviates by a factor of two, the Co(II)-^{31}P(P-arginine) distance will have an error of 12% (see below).

From the point of view of the structural changes accompanying phosphoryl transfer, the two relaxation

Table 1. ^{31}P spin relaxation rates in the presence of Co(II) for enzyme-bound nucleotide and equilibrium-mixture complexes of arginine kinase

Complex	^{31}P relaxation rate (s^{-1})			
	α-P	β-P	γ-P	P-arginine
Free substrates:				
Co(II)ATP[d]	135	160	225	
Co(II)ADP[d]	260	492		
Enzyme-bound complexes:				
E•Co(II)ATP[c]	42	64	81	
E•Co(II)ADP[c]	101	65		
Equilibrium mixture[a]	36 (ATP & ADP)	[b]	76	
		72 (ATP)		20

[a]For details of the sample conditions, see text and figure legends. The experimental errors in these measurements, based on data fitting and the deviations between measurements with independent samples, are about ±10% of the values given.
[b]β-P(ADP) not resolvable.
[c]Sample conditions: 1. P-arginine + Co(II): 5 mM P-arginine and 50–400 μM $CoCl_2$; and 2. E•P-arginine + Co(II): 5.6 mM enzyme sites, 4.95 mM P-arginine, and 139–788 μM $CoCl_2$. Both samples were buffered with 100 mM Hepes buffer, pH 8.1.
[d]From Jarori et al. (1985).

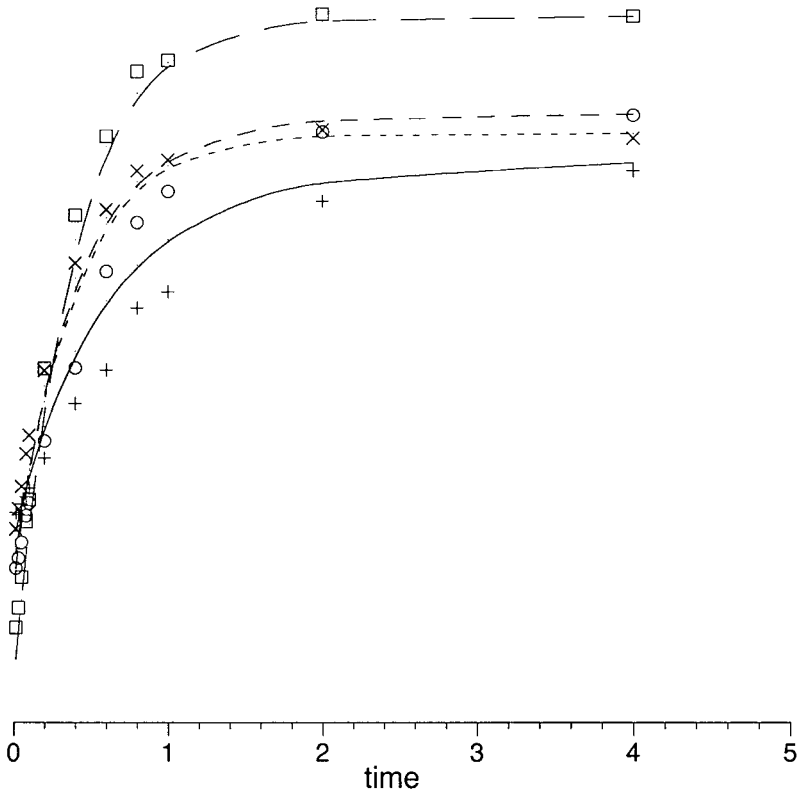

Figure 2. Experimental and theoretical T_1-recoveries for the ^{31}P signals in the enzyme-bound equilibrium mixture. The points represent experimental values of magnetization in arbitrary units: the superposed resonances of P-arginine and β-P(ADP) (+), γ-P(ATP) signal (□), the superposed resonances of α-P(ATP) and α-P(ADP) (○), and the resonance of β-P(ATP) (×). Theoretical recoveries are calculated with the relaxation rates of 20 s^{-1} (P-arginine), 81 s^{-1} (γ-P(ATP)), 64 s^{-1} (β-P(ATP)), 42 s^{-1} (α-P(ATP)), 101 s^{-1} (α-P(ADP)), and 65 s^{-1} (β-P(ATP)), value of p for each species taken from theoretical computations (see text), $\varepsilon = 7\ s^{-1}$, and [Co(II)]/[nucleotide] = 0.034.

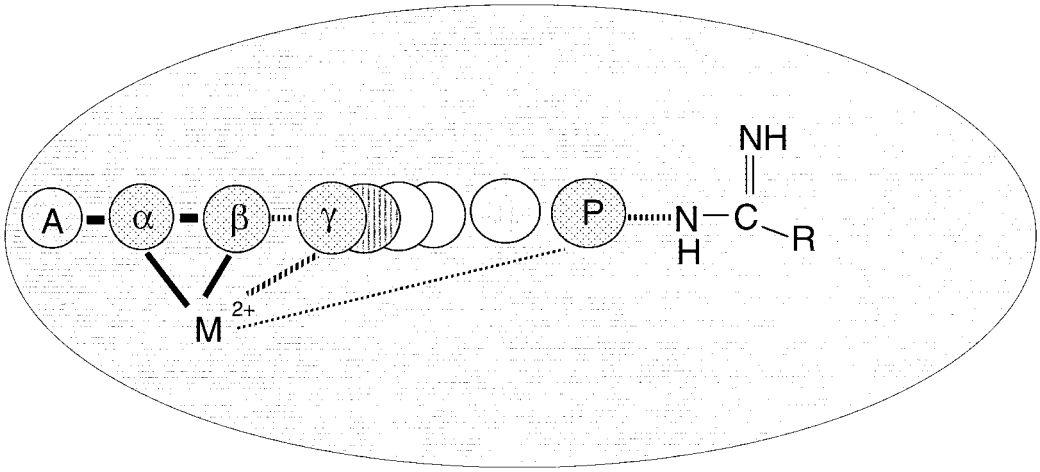

Figure 3. Depiction of the movement of the itinerant phosphate with respect to the divalent metal ion.

rates of 76 ± 5 s^{-1} for γ-P(ATP) and of 20 ± 2 s^{-1} for the P(P-arginine) allow an estimate of the change in this Co(II)-^{31}P distance, on the basis of Equation 9, leading to

$$(r(P - \text{arginine})/r(\gamma - P(ATP)) = 1.32 \pm 0.05. \quad (10)$$

It may be noted, however, that unlike the relaxation data for Mn(II) complexes, precise evaluation of distances from Co(II) data on the basis of frequency-dependent measurements is beset with the unavailability of an exact theory. Previous estimates of Co(II)-^{31}P distances in ternary complexes (Jarori et al., 1985, 1989; Ray and Nageswara Rao, 1988; Ray et al., 1988) were based on an acceptable range of $10^{-12}s \leq f(\tau_c) \leq 5 \times 10^{-12}$ s for the spectral density function, $f(\tau_c)$, that enters the calculations. However, setting r(γ-P(ATP)) to be 3.0 ± 0.2 Å appropriate for direct coordination of the cation with the oxygen on the γ-P(ATP) (Jarori et al., 1985, 1989), leads to r(P-arginine) = 4.0 ± 0.4 Å. (This range adequately covers for a maximum uncertainty of a factor of two for the P-arginine relaxation rate.) The two distances r(γ-P(ATP)) and r(P-arginine) represent two sides of a triangle with the cation at the apex. The third side of this triangle is the displacement of the phosphoryl group. *Therefore, in a coordinate frame with the cation at the origin, the phosphoryl group moves at least 1.0 Å as the enzyme turns over.* This movement is depicted in Figure 3.

Discussion

An attractive feature of high resolution NMR investigations of biological macromolecular structure is not only that structural information is obtained when the macromolecule is present in an active state in solution, but also that the technique offers considerable scope for monitoring dynamic processes taking place in the macromolecular complexes. For example, the lineshapes of interconverting enzyme-bound reaction complexes were used to isolate and determine the interconversion rate on the surface of the enzyme (Nageswara Rao and Cohn, 1977; Vasavada et al., 1980). The work presented in this paper represents the next step of acquiring structurally relevant information in such complexes. The results obtained illustrate that nuclear relaxation measurements on interconverting substrates and products bound to enzymes activated by paramagnetic cations are interpretable in terms of structural changes accompanying enzyme turnover. Such measurements should be attempted on enzyme complexes for which the dissociation constants and spectral features are conducive, and adequate information already exists regarding paramagnetic cationic effects on individual enzyme-substrate complexes. Sample conditions for the enzyme-bound equilibrium mixture should be chosen such that unproductive complexes are minimized, and it is necessary to make sure that the relaxation rates of interest are not exchange limited. Analysis of the relaxation data requires knowledge of ε, the interconversion rate. While a reasonable value of ε may be obtained from the NMR lineshapes of enzyme-bound equilibrium mix-

tures with Mg(II), there is no reliable way to do the same with paramagnetic cations. It may, therefore, be necessary to deduce this information by iterative fitting of the observed recoveries themselves. Thus, the analysis of the relaxation data, although not simple, is not excessively complicated if the different parameters entering the theoretical expressions are carefully examined.

Structural changes in the reaction complexes are caused by appropriate movements of the protein enveloping the substrates at the active site. Considerable evidence is available from different structural methods that domain movements occur in the protein structure when the substrates and cofactors bind the enzyme, leading to a closing up of the active site. However, information regarding the resultant conformational changes in the reaction complex is not readily obtained from standard structural methodologies. The results of this paper represent an attempt to acquire such information for the arginine kinase reaction. That it was possible to do so is due to a unique feature of the NMR method in that experiments may be deliberately devised such that the data gathered for structural measurements contain dynamic information as well.

Distance changes in the enzyme-bound reaction complex representing the excursion of an itinerant moiety, such as those deduced for the terminal phosphoryl group of ATP from ^{31}P measurements, indicate a topography of the active site that accommodates such a movement. However, concomitant structural adjustments take place in other regions of the reaction complex, such as the reorientation of the phosphate chain in the nucleotide along with a displacement of the cation. Interpretation of these structural changes must be made with explicit cognizance of the reference frames with respect to which they are specified. The change in the cation-P distance is specified with reference to the cation at the origin, and the simultaneous displacement of the cation is specified with respect to a fixed adenosine moiety. As data of this kind are accumulated for other nuclei in the reaction complex, a picture of the internal movement of the entire active complex is likely to emerge.

The structural changes at the active site reveal that the amino-acid environment not only engineers the productive conformations of the bound substrates to facilitate the reaction, but also orchestrates a set of coherent structural movements in them as the reaction proceeds on the surface of the enzyme. Microscopic reversibility of the reaction requires that these movements trace a reversible path as the reaction occurs in the forward and reverse directions with equal facility. The specific structural alterations accompanying the enzyme turnover are likely to be unique for the enzyme. Interpretation of these changes, along with the corresponding structure of the protein environment, with the help of computer-based iterative molecular modeling methods has the potential of yielding fresh insights into the functional roles of amino-acid residues spanning the active site.

The method used here, and the theoretical basis for it (Nageswara Rao, 1995), is specific to nuclear spin relaxation effects due to paramagnetic cations, and is, therefore, restricted to enzymes activated by these cations such as the ATP-utilizing enzymes. However, there are a large number of these enzymes, and moreover there are various categories among them, viz., phosphoryl transfer, adenyl transfer, and pyrophosphoryl transfer, in which the cleavage occurs at different points on the phosphate chain (Cohn, 1959). All these enzymes are typically activated by Mn(II) and Co(II). There is thus a range of questions pertinent to these enzymes that could be examined by the method used here. For example, it was suggested that there are induced-fit movements in adenylate kinase as the reaction complex is formed (Schulz et al., 1990). Similarly, it was postulated that hinge-bending movements occur in 3-P-glycerate kinase (Bernstein et al., 1997) when all the components of the reaction complex are present on the enzyme. Measurements of the kind presented in this paper can be used to determine the structural changes that occur in the reaction complex as a consequence of, and in concert with, such protein domain movements.

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