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^{31}P and ^{1}H NMR Studies of the Structure of Enzyme-Bound Substrate Complexes of Lobster Muscle Arginine Kinase: Relaxation Measurements with Mn(II) and Co(II)†

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ABSTRACT: The paramagnetic effects of Mn(II) and Co(II) on the spin–lattice relaxation rates of ^{31}P nuclei of ATP and ADP and of Mn(II) on the spin–lattice relaxation rate of the δ protons of arginine bound to arginine kinase from lobster tail muscle have been measured. Temperature variation of ^{31}P relaxation rates in E·MnADP and E·MnATP yields activation energies (ΔE) in the range 6–10 kcal/mol. Thus, the ^{31}P relaxation rates in these complexes are exchange limited and cannot provide structural information. However, the relaxation rates in E·CoADP and E·CoATP exhibit frequency dependence and ΔE values in the range 1–2 kcal/mol; i.e., these rates depend upon ^{31}P–Co(II) distances. These distances were calculated to be in the range 3.2–4.5 Å, appropriate for direct coordination between Co(II) and the phosphoryl groups. The paramagnetic effect of Mn(II) on the ^{1}H spin–lattice relaxation rate of the δ protons of arginine in the E·MnADP·Arg complex was also measured at three frequencies (viz., 200, 300, and 470 MHz). These ^{1}H experiments were performed in the presence of sufficient excess of arginine to be observable over the protein background but with MnADP exclusively in the enzyme-bound form so that the enhancement in the relaxation rates of the δ protons of arginine arises entirely from the enzyme-bound complex. Both the observed frequency dependence of these rates and the $\Delta E \leq 1.0 \pm 0.3$ kcal/mol indicate that this rate depends on the ^{1}H–Mn(II) distances. From the frequency dependence of the relaxation rate an effective τ_C of 0.6 ns has also been calculated, which is most likely to be the electron spin relaxation rate (τ_{S1}) for Mn(II) in this complex. The distance estimated on the basis of the reciprocal sixth root of the average relaxation rate of the δ protons was 10.9 ± 0.3 Å.

Knowledge of the structures of substrates and their relative dispositions at the active sites of enzyme-bound complexes is essential to the understanding of the mechanism of catalysis of multisubstrate enzymes. Paramagnetic enhancement of spin–lattice relaxation rates provides a useful method of determination of distances of various ligand nuclei from the paramagnetic probe in an enzyme–substrate complex (Mildvan et al., 1980; Villafranca, 1984; Jarori et al., 1985; Ray & Nageswara Rao, 1988; Ray et al., 1988). These distances can then be used to characterize the substrate conformation at the active site. This method is particularly attractive for ATP[1]-utilizing enzymes that require a divalent cation, Mg(II), as an obligatory component in vivo but can accept paramagnetic cations such as Mn(II) and Co(II) in most cases. Recently, ^{31}P relaxation measurements in the presence of Mn(II) and Co(II) were used to characterize the enzyme–substrate complexes of creatine kinase (Jarori et al., 1985), 3-P-glycerate kinase (Ray & Nageswara Rao, 1988), and adenylate kinase (Ray et al., 1988). These studies reveal that considerable care

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; E·M·S, enzyme–metal–substrate; E·S, enzyme–substrate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

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needs to be exercised in assessing the contribution of exchange to the observed relaxation rates in the enzyme complexes. The use of two dissimilar cations, such as Mn(II) and Co(II), proved beneficial in overcoming exchange-limitation problems, and the necessity of arranging the experimental protocol so that the paramagnetic probe is present only in the macromolecular complex of interest has been demonstrated repeatedly. Some of the anomalies in the structural information deduced by the paramagnetic relaxation enhancement method in the past [compare, for example, the NMR results of Mildvan and Sloan (1976) with the Mn(II) EPR results of Lodato and Reed (1987) on pyruvate kinase complexes] appear to be due to incorrect assessment of the role of exchange in the NMR relaxation data.

Structural data derived from ^{31}P and ^{1}H relaxation measurements on enzyme–substrate complexes of arginine kinase in the presence of Mn(II) and Co(II) are presented in the paper. Arginine kinase (EC 2.7.3.3) catalyzes the reversible phosphorylation of L-arginine

$$\text{ATP} + \text{L-arginine} \xrightleftharpoons{\text{M(II)}} \text{ADP} + \text{L-P-arginine} \quad (1)$$

where M(II) is a divalent cation. Mn(II) and Co(II) activate this reaction (Virden et al., 1965). The enzyme from *Homarus americanus* is a monomer of molecular weight of 40 000 and is stable in solution in the presence of 2-mercaptoethanol (Blethen & Kaplan, 1967). The reaction is analogous to that of creatine kinase in that the phosphorylation of a guanidino nitrogen is catalyzed. Thus, arginine kinase is a guanidino kinase that appears to serve the same function in muscle contraction in invertebrates as creatine kinase does in vertebrates (Morrison, 1973). Comparison of amino acid compositions of several arginine and creatine kinases further indicates the similarities of these two enzymes (Blethen & Kaplan, 1967; Ratto & Christen, 1988). Indeed, the sea cucumber variant of arginine kinase is even reported to form a hybrid with creatine kinase in vitro (Seals & Grossman, 1988). Similarities between arginine kinase and creatin kinase were evident in the results of magnetic resonance experiments such as Mn(II) EPR (Buttlaire & Cohn, 1974b; Reed & Cohn, 1972), proton relaxation enhancement (Buttlaire & Cohn, 1974a; Reed et al., 1972), and ^{31}P NMR of enzyme-bound substrate complexes in the presence of diamagnetic Mg(II) (Nageswara Rao & Cohn, 1977, 1981). There are, however, some differences in the pH dependence of the ^{31}P chemical shift of β-P(E·MgADP). Furthermore, it may be noted that creatine kinase has two subunits, whereas lobster arginine kinase consists of a single polypeptide chain. The active-site structure data presented below allow another basis for comparison of the complexes of these two enzymes.

The ^{31}P relaxation measurements are useful for locating the phosphorus nuclei in the phosphate chains of enzyme-bound nucleotides with reference to the cation in the complexes. The ^{1}H NMR measurements, on the other hand, are aimed at determining the distances of the δ protons of arginine from the cation in the E·MnADP·Arg complex. A number of considerations relevant for experiments directed at deducing structural parameters characteristic of the second substrate (which is not directly chelated to cation) arise in the design and analysis of the ^{1}H NMR measurements.

Experimental Procedures

Materials. ATP, ADP, 2-mercaptoethanol, 0.1 M $MnCl_2$ solution in 0.15 M NaCl, and pyruvate kinase and lactate dehydrogenase from rabbit muscle were purchased from Sigma. Hepes was from Research Organics, and $CoCl_2$ (Puratronic) was from AESAR. D_2O (99.8%) and deutero glycine (98%) for ^{1}H NMR experiments were from Cambridge Isotope Laboratories. All other chemicals were of analytical reagent grade. ATP, ADP, L-arginine, KNO_3, and buffer solutions were passed through a Chelex 100 column before use in NMR experiments.

Enzyme Preparation. Arginine kinase was purified from lobster (*H. americanus*) tail muscle by the method of Blethen and Kaplan (1967) except that hemocyanin was removed from the purified enzyme by chromatography on Sephadex G-75. The specific activity of the enzyme determined by means of a coupled assay with pyruvate kinase and lactate dehydrogenase was 160 units/mg at 22 °C and pH 8.0. Purified enzyme was stored at 5 °C in 0.2 M K-Hepes, pH 8.0, with 5 mM 2-mercaptoethanol and 25% ammonium sulfate.

Arginine kinase for ^{1}H NMR was extensively dialyzed against 20 mM perdeuterated potassium glycine containing 5 mM 2-mercaptoethanol, pH 8.0. Final dialysis buffer included preequilibrated Chelex 100 to remove adventitious metal ions. The enzyme solution was then concentrated in a 3-mL Amicon ultrafiltration cell to 160–180 mg/mL. Concentrated enzyme was then quick-frozen and lyophilized (two cycles to remove exchangeable protons) and finally dissolved in 99.8% D_2O containing 5 mM 2-mercaptoethanol. This was centrifuged in a Beckman microfuge, and the supernatant was used for ^{1}H NMR experiments. There was at most a 10% loss in specific activity upon lyophilization of the enzyme.

Arginine kinase for ^{31}P NMR was extensively dialyzed against 0.2 M K-Hepes, pH 8.0, containing 5.0 mM 2-mercaptoethanol with preequilibrated Chelex 100 suspended in the dialysis buffer. The enzyme was then concentrated in a 3-mL Amicon ultrafiltration cell to 4.8–6.5 mM. Protein and nucleotide concentrations were determined spectrophotometrically with $\epsilon_{280}^{mg/mL} = 0.67\ cm^{-1}$ and a molecular weight of 40 000 (Blethen & Kaplan, 1967) for the enzyme and $\epsilon_{259}^{mM} = 15.4\ cm^{-1}$ for ATP and ADP. A Beckman Altex Model 3500 digital pH meter was used for pH measurements.

NMR Measurements. ^{31}P and ^{1}H NMR measurements at 121.5 and 300 MHz, respectively, were made on a NT-300 wide-bore NMR spectrometer equipped with a 12-mm multinuclear probe, a 5-mm ^{1}H probe, a 293C pulse programmer, a Nicolet 1280 computer, and a variable-temperature controller. A typical ^{1}H sample contained 450 μL of solution in D_2O in a 5-mm NMR tube. A typical ^{31}P sample contained ~0.8 mL of the enzyme in an 8-mm-o.d. NMR sample tube placed inside a 12-mm NMR tube. D_2O for field-frequency lock was added between the two tubes. Measurements at 81 and 190.2 MHz for ^{31}P and at 200 and 470 MHz for ^{1}H were made on NTC-200 and NTC-470 spectrometers at the Purdue University Biochemical Magnetic Resonance Laboratory. T_1 measurements were made by using a standard inversion-recovery sequence with a composite π pulse for inversion. The errors quoted for relaxation rates and activation energies are based on standard deviations given by computer fits and deviations between measurements made with independent samples.

Theoretical Details. The theory of nuclear spin relaxation in the presence of paramagnetic cations has been extensively reviewed (Dwek, 1973; James, 1975; Mildvan & Gupta 1978; Burton et al., 1979; Jardetzky & Roberts, 1981). A summary of this theory and the experimental strategy arising from it has previously been published (Jarori et al., 1985; Ray & Nageswara Rao, 1988; Ray et al., 1988). The relevant equations for analysis of the data in this paper are given below: Given a sample that contains two exchanging complexes, one

Table I: Paramagnetic Effect $(pT_{1P})^{-1}$ (s^{-1}) of Mn(II) on ^{31}P and ^{1}H Relaxation Rates and Corresponding Activation Energies (ΔE) (kcal/mol) for Various ADP and ATP Complexes Free in Solution and Bound to Arginine Kinase[a]

complex (sample composition)	NMR frequency (MHz)	α–P $(pT_{1P})^{-1}$ (s^{-1})	α–P ΔE (kcal/mol)	β–P $(pT_{1P})^{-1}$ (s^{-1})	β–P ΔE (kcal/mol)	γ–P $(pT_{1P})^{-1}$ (s^{-1})	γ–P ΔE (kcal/mol)	δ–^{1}H(arginine) $(pT_{1P})^{-1}$ (s^{-1})	δ–^{1}H(arginine) ΔE (kcal)/mol)
MnADP[b] (ADP, 5 mM; $MnCl_2$, 2.5–15 μM)	121.5	5400 ± 300		7200 ± 500					
MnATP[b] (ATP, 4 mM; $MnCl_2$, 2.5–15 μM)	121.5	5570 ± 200		7270 ± 200		6930 ± 200			
E·MnADP[c] (enzyme, 7.1 mM; ADP, 3.6 mM; $MnCl_2$, 16.2–104.2 μM)	121.5 190.2	190 ± 20 201 ± 10	10.9 ± 1	174 ± 20 161 ± 10	10.4 ± 1				
E·MnATP[c] (enzyme, 6.8 mM; ATP, 3.9 mM; $MnCl_2$, 9.8–55.9 μM)	121.5	240 ± 20	6.2 ± 0.5	430 ± 20	6.8 ± 0.6	350 ± 30	6.6 ± 0.6		
E·MnADP·Arg[d] (enzyme, 2.5 mM; ADP, 2 mM; L-Arg 26 mM; $MnCl_2$ 0.16–1.5 mM)	200 300 470							191 ± 15 101 ± 4 71 ± 4	2.2 ± 0.4[e]

[a] Samples for ^{31}P measurements were in 50 mM K-Hepes, pH 8.0, containing 5 mM 2-mercaptoethanol. The $(pT_{1P})^{-1}$ values given were measured at 15 °C. The ΔE values were obtained from Arrhenius plots (see Figures 1 and 2) of $(pT_{1P})^{-1}$ in the temperature range 5–30 °C. The errors were estimated on the basis of computer fits of the T_1 data and of the appropriate functions involved in obtaining final values. [b] Data for MnADP and MnATP were taken from Jarori et al. (1985). [c] Measurements were made for five values of p = [Mn(II)]/[nucleotide]. [d] Samples for ^{1}H measurements were in 20 mM deuterated potassium glycine, pH 8.0, containing 5 mM 2-mercaptoethanol. The $(pT_{1P})^{-1}$ values given were measured at 19 °C, and measurements were made for five values of p = [Mn(II)]/[arginine]. [e] This ΔE value is obtained as the *positive* slope of log $(pT_{1P})^{-1}$ vs T^{-1}.

paramagnetic and the other diamagnetic, with fractional concentrations p and $(1 - p)$ and nuclear relaxation rates $(T_{1M})^{-1}$ and $(T_{1D})^{-1}$, respectively, such that $(T_{1M})^{-1} \gg (T_{1D})^{-1}$, the observed relaxation rate is given by

$$(T_{1,obs})^{-1} = \frac{(1-p)}{T_{1D}} \frac{T_{1M} + \tau_M}{T_{1M} + (1-p)\tau_M} + \frac{p}{T_{1M} + (1-p)\tau_M} \quad (2)$$

where τ_M is the lifetime of the paramagnetic complex. If $p \ll 1$, eq 2 reduces to the commonly used form

$$(T_{1P})^{-1} = p/(T_{1M} + \tau_M) \quad (3)$$

Neglecting the contribution of the scalar hyperfine interaction, T_{1M} is related to the cation–nucleus distance by

$$(T_{1M})^{-1} = (C/r)^6 f(\tau_C) \quad (4)$$

where

$$C = [(2/15)S(S+1)g^2\gamma_I^2\beta^2]^{1/6} \quad (5)$$

and

$$f(\tau_C) = 3\tau_{C1}/(1 + \omega_I^2\tau_{C1}^2) \quad (6)$$

for Mn(II) complexes (with $\omega_S\tau_{C2} \gg 1$), but

$$f(\tau_C) = 3\tau_{S1} + 7\tau_{S2}/(1 + \omega_S^2\tau_{S2}^2) \quad (7)$$

for Co(II) complexes (with $\tau_{Ci} = \tau_{Si}$). Also

$$\tau_{Ci}^{-1} = \tau_R^{-1} + \tau_{Si}^{-1} \qquad i = 1, 2 \quad (8)$$

In eq 5–8, S, g, and ω_S are, respectively, the spin, the g factor, and the Larmor frequency for the cation, γ_I and ω_I are, respectively, the gyromagnetic ratio and the resonance frequency of the relaxing nucleus, β is the Bohr magneton, τ_R is the isotropic rotational correlation time of the complex, and τ_{S1} and τ_{S2} are the electronic longitudinal and transverse relaxation times of the paramagnetic cation. These equations assume an isotropic g factor and that the zero-field splitting is smaller than the Zeeman interaction of the cation, both of which are acceptable for complexes with Mn(II) but not for those with Co(II). For Mn(II) complexes τ_{S1} is given by

$$(\tau_{S1})^{-1} = B[\tau_V/(1 + \omega_S^2\tau_V^2) + 4\tau_V/(1 + 4\omega_S^2\tau_V^2)] \quad (9)$$

where B is a constant related to the crystal field and τ_V is the correlation time for its fluctuations.

^{31}P measurements presented here were made exclusively on enzyme-bound substrate complexes, so that the exchange is limited to two complexes, E·S and E·M·S, as implied in eq 2, and maximizes the contribution of E·M·S to $(T_{1P})^{-1}$. In the case of the ^{1}H measurements, however, a new tactic was used. Because the binding of Mn(II) to L-arginine is much weaker than binding of Mn(II) to ADP in the E·M·S complex, L-arginine in excess of the enzyme concentration can be used as long as [E] > [ADP] > [Mn] such that ADP and Mn(II) are present predominantly in enzyme-bound complexes (see below). The exchanging species then becomes S_2, E·S_2, E·S_1·S_2, and E·M·S_1·S_2, where S_1 is ADP and S_2 is L-arginine. For the purposes of eq 2, S_2, E·S_2, and E·S_1·S_2 can be taken together as being the diamagnetic species in exchange with the single paramagnetic species E·M·S_1·S_2. Note that this only works if S_2 does not have any appreciable affinity for M compared to both E·S_1 and S_1.

It must be stressed that for either the ^{31}P or the ^{1}H measurements structural information is not available from T_{1P} measurements if $\tau_M \gg T_{1M}$ and can be deduced from measurements in which τ_M is comparable to T_{1M} only if τ_M is known. Therefore, the contribution of τ_M to T_{1P} must be determined (see eq 2 and 3). This was done in the present work by making T_{1P} measurements for both ^{1}H and ^{31}P samples as a function of temperature in the range 5–30 °C and by making measurements at three frequencies (viz., 81, 121.5, and 190.2 MHz for ^{31}P and 200, 300, and 470 MHz for ^{1}H). Note that activation energies of T_{1M} are usually 1–3 kcal/mol, while those for τ_M are 5–20 kcal/mol. Furthermore, T_{1M} depends on frequency and τ_M does not.

Results and Analysis

Mn(II)–Nucleotide Complexes. Values of $(pT_{1P})^{-1}$ and ΔE obtained at 121.5 MHz for the ^{31}P nuclei in the phosphate groups of E·MnADP and E·MnATP at 15 °C and at 190.2 MHz and 15 °C for E·MnADP are given in Table I along with previously published data for MnATP and MnADP (Jarori et al., 1985). For the enzyme complexes, on the basis of known dissociation constants (Buttlaire & Cohn, 1974a), the fractional concentration of paramagnetic complexes free in solution ([M·S]/[E·M·S]) did not exceed 0.5%.

Table II: Paramagnetic Effect $(pT_{1P})^{-1}$ of Co(II) on ^{31}P Relaxation Rates and Corresponding Activation Energies (ΔE) for Various ADP and ATP Complexes Free in Solution and Bound to Arginine Kinase[a]

complex (sample composition)	^{31}P NMR frequency (MHz)	α–P $(pT_{1P})^{-1}$ (s^{-1})	α–P ΔE (kcal/mol)	β–P $(pT_{1P})^{-1}$ (s^{-1})	β–P ΔE (kcal/mol)	γ–P $(pT_{1P})^{-1}$ (s^{-1})	γ–P ΔE (kcal/mol)
CoADP[b]	121.5	280 ± 20		560 ± 50			
CoATP[b] (ATP, 2.7 mM; $CoCl_2$, 15–25 μM)	121.5	120 ± 20		150 ± 20		210 ± 20	
E·CoADP (enzyme, 5.3 mM; ADP, 3.5 mM;	81.0	160 ± 15		140 ± 10			
$CoCl_2$, 347–774 μM)	121.5	91 ± 6	2.2 ± 0.2	58 ± 5	1.8 ± 0.2		
	190.2	185 ± 15		106 ± 10			
E·CoATP (enzyme, 6.8 mM; ATP, 3.9 mM;	81.0	91 ± 10		180 ± 10		73 ± 10	
$CoCl_2$ 108–470 μM)	121.5	51 ± 5	1.3 ± 0.1	85 ± 5	1.4 ± 0.10	84 ± 9	1.8 ± 0.1
	190.2	71 ± 10		108 ± 10		169 ± 10	
E·CoADP·Arg (enzyme, 4.8 mM; ADP, 3.2 mM; $CoCl_2$, 726 μM; Arg, 6.2 mM)	121.5	34 ± 3	8.5 ± 1.6	32 ± 3	7.6 ± 1.4		
E·CoADP·NO_3^-·Arg (enzyme, 4.8 mM; ADP, 3.2 mM; $CoCl_2$, 726 μM; Arg, 6.2 mM; KNO_3, 21 mM)	121.5	13 ± 1	20.0 ± 3.0	24 ± 2	13.0 ± 2.0		

[a] All samples for this work were in 50 mM K-Hepes, pH 8.0, containing 5 mM 2-mercaptoethanol. The $(pT_{1P})^{-1}$ values given were measured at 15 °C with five different values of p = [Co(II)]/[nucleotide]. The ΔE values were obtained at 121.5 MHz from Arrhenius plots (see Figure 2) of $(pT_{1P})^{-1}$ in the temperature range 5–25 °C. [b] CoADP and CoATP data were taken from Jarori et al. (1985).

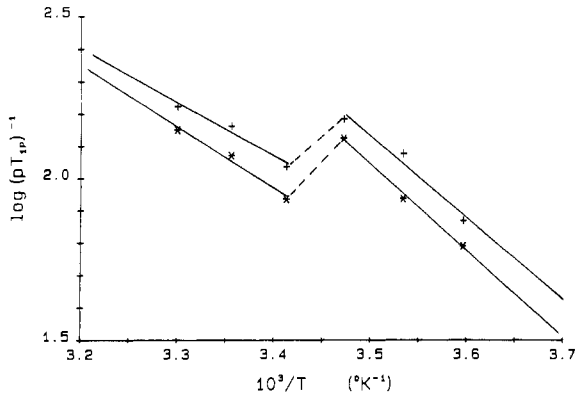

FIGURE 1: log $(pT_{1P})^{-1}$ vs $10^3/T$ for α–P and β–P of E·MnADP (*, +). Typical sample conditions and activation energies (ΔE) obtained are given in Table I.

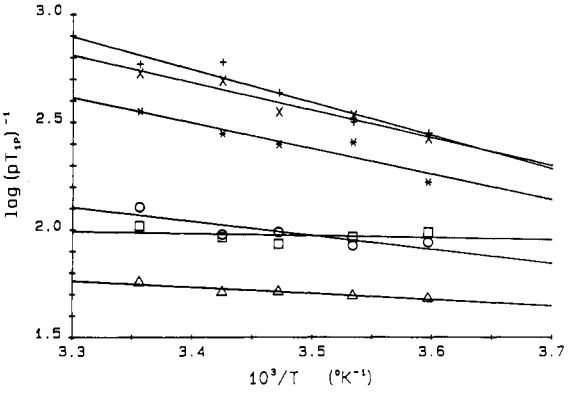

FIGURE 2: log $(pT_{1P})^{-1}$ vs $10^3/T$ for α–P, β–P, and γ–P of E·MnATP (*, +, ×) and E·CoATP (△, □, ○). Typical sample conditions and activation energies (ΔE) obtained are given in Tables I and II.

The temperature dependence of $(pT_{1P})^{-1}$ for α–P and β–P E·MnADP, shown in Figure 1, displays a marked discontinuity between 15 and 20 °C. However, the variation follows an Arrhenius behavior with nearly the same activation energy of 10–11 kcal/mol on either site of this discontinuity. The ^{31}P line shapes of MgADP complexes of arginine kinase exhibit features below 15 °C that are interpretable in terms of conformational heterogeneity (Nageswara Rao & Cohn, 1977). The discontinuity in Figure 1 may be related to such a heterogeneity and the attendant conformational transitions as the temperature is elevated. Evidence for a similar conformational heterogeneity was indicated for creatine kinase in ^{31}P NMR spectra of E·MgADP (Nageswara Rao & Cohn, 1981), in ^{31}P relaxation rates of E·CoADP (Jarori et al., 1985), and in some Mn(II) EPR measurements[2] (McLaughlin et al., 1976). As far as the temperature dependence of $(pT_{1P})^{-1}$ shown in Figure 1 is concerned, the discontinuity takes place without a discernible change in energy activation energy of 10–11 kcal/mol, which is a value normally associated with τ_M. The discontinuity in $(pT_{1P})^{-1}$ in Figure 1 is due to an abrupt change in the lifetime of the E·MnADP complex rather than to a change in the ^{31}P relaxation time. This exchange limitation was confirmed for the E·MnADP complex by the frequency independence of the relaxation rate. The temperature dependence of $(pT_{1P})^{-1}$ of E·MnATP shown in Figure 2 yields ΔE values of 6–7 kcal/mol for the three phosphate groups (see Table I). In contrast with ADP complexes, the ATP complexes of either arginine kinase or creatine kinase do not show features of conformational heterogeneity in the various types of ^{31}P NMR and Mn(II) EPR measurements discussed above. Nevertheless, the ^{31}P relaxation rates of Mn–nucleotide complexes are bereft of structural information and are governed by the lifetimes of these complexes. It may be recalled that exchange limitation was also prevalent for similar complexes of creatine kinase (Jarori et al., 1985), 3-P-glycerate kinase (Ray & Nageswara Rao, 1988), and adenylate kinase (Ray et al., 1988).

Co(II) Complexes. Table II gives the relaxation rates observed for the ^{31}P nuclei in the complexes E·CoATP, E·CoADP, E·CoADP·Arg, and E·CoADP·NO_3–Arg along with relaxation rates previously measured for CoADP and CoATP (Jarori et al., 1985). A typical stack plot for a ^{31}P T_1 measurement for E·CoATP is shown in Figure 3. Relaxation rates for all complexes were measured as a function of temperature in the range 5–25 °C. In addition, relaxation rates for E·CoADP and E·CoATP were measured at three different frequencies, viz., 81, 121.5, and 190.2 MHz.[3] The observed

[2] G. D. Markham, G. H. Reed, and M. Cohn, unpublished experiments; see McLaughlin et al. (1976).

[3] In free solution Co(II) undergoes a reaction with 2-mercaptoethanol, yielding a colored precipitate. However, in the experiments with the enzyme complexes with Co(II) no evidence for such reaction was detectable in spite of the presence of 5 mM 2-mercaptoethanol in the samples. It appears the Co(II) is entirely sequestered by the enzyme-bound nucleotides. The effect on the measured relaxation rates due to any reduction in the Co(II) concentration is not significant (<5%).

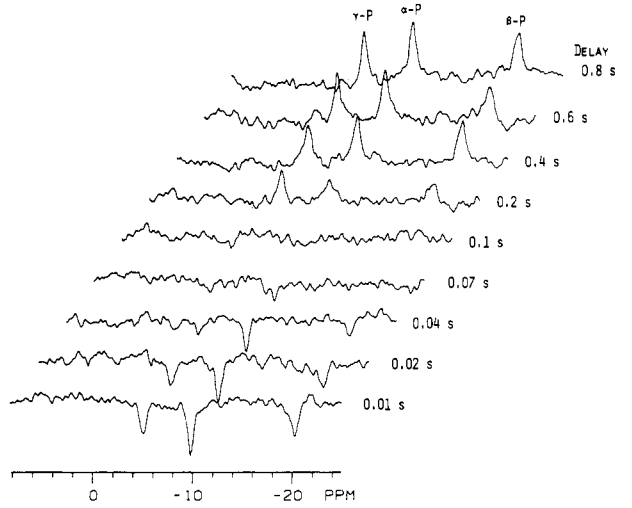

FIGURE 3: Typical T_{1P} measurement at 121.5 MHz and 15 °C for ^{31}P nuclei in ATP bound to arginine kinase in the presence of Co(II) ($p = 0.097$). A standard inversion-recovery sequence with composite π pulse was used. NMR parameters: $\pi/2$ pulse width, 19.5 μs; sweep width, ±2000 Hz; data size, 2048; line broadening, 40 Hz; number of scans, 160; recycle delay, 0.8 s. Computer fit gives T_{1P} values of 0.15, 0.09, and 0.08 s (standard deviation ~0.01 s) for α–P, β–P, and γ–P, respectively.

frequency dependence and low activation energies (ΔE) indicate that the relaxation rates for these two complexes are governed by Co(II)–^{31}P distances. However, the activation energies for the quaternary complex E·CoADP·Arg and for the transition-state analogue E·CoADP·NO_3^-·Arg are ~8 and 20 kcal/mol, respectively, which indicates that the $(pT_{1P})^{-1}$ values for the ^{31}P nuclei in these complexes are severely exchange limited.

To calculate the Co(II)–^{31}P distances from $(pT_{1P})^{-1}$ values for E·CoADP and E·CoATP, estimates of $f(\tau_C)$ in these complexes are required (see eq 4). The theoretical problems involved in estimating a correct value of $f(\tau_C)$ for Co(II) complexes have been discussed earlier in some detail (Jarori et al., 1985; Benetis et al., 1983). On the basis of the frequency dependence exhibited by the relaxation rates as well as previously published data for various enzyme complexes with Co(II) (Mildvan et al., 1980; Villafranca, 1984), the range 10^{-12} s $< f(\tau_C) < 5 \times 10^{-12}$ s was used to analyze 121.5-MHz relaxation data for Co(II) complexes of enzymes previously studied (Jarori et al., 1985; Ray & Nageswara Rao, 1988; Ray et al., 1988). The relaxation data in Table II also show frequency sensitivity in the range 80–190 MHz for ^{31}P, i.e., $\omega_S = 1.7$–4.2×10^{12} rad s^{-1} for Co(II) (with $g = 4.33$). Thus, it is reasonable to consider the same range as above for $f(\tau_C)$. For Co(II) complexes $f(\tau_C)$ is determined exclusively by the electron relaxation times (eq 7), which are in the neighborhood of 10^{-12} s, and is unaffected by τ_R ($\tau_R > 10^{-9}$ s). The *range* of values for $f(\tau_C)$ is, therefore, not expected to vary to a considerable degree for similar complexes of different proteins. Therefore, distances are calculated by using the range 10^{-12} s $< f(\tau_C) < 5 \times 10^{-12}$ s and $C = 675$ Å s$^{-1/3}$. This range of values is likely to absorb any other corrections (Sternlicht, 1965; Rubinstein et al., 1971) such as that due to the anisotropic g tensor of Co(II) (Vasavada & Nageswara Rao, 1989). The distances signify direct coordination of Co(II) with all the phosphate groups in both ADP and ATP (see Table III).

^1H Relaxation Rate Measurements of δ Protons of Arginine in E·MnADP·Arg. As described under Experimental Procedures, these ^1H NMR measurements were performed in the presence of a significant excess of arginine concentration over that of the enzyme and with [Mn(II)] and [ADP] chosen so that MnADP is predominantly in the enzyme-bound form. Note that these measurements require significantly larger Mn(II) concentrations than those used for the measurements with nucleotides (see Table I). Nevertheless, at the concentrations used in the experiments the ratio [MnADP]/[E·MnADP)] never exceeded 1.5%.

Table III: Distances of ^1H and ^{31}P Nuclei from the Cation [Mn(II) or Co(II)] in Various Complexes of Arginine Kinase and in CoADP and CoATP

complex	cation–^{31}P distance (Å) α–P	β–P	γ–P	cation–^1H distance (Å) δ–H
CoADP[a]	2.6–3.5	2.4–3.1		
CoATP[a]	3.0–4.0	2.9–3.8	2.7–3.6	
E·CoADP[b]	3.2–4.2	3.4–4.5		
E·CoATP[b]	3.5–4.6	3.2–4.2	3.2–4.2	
E·MnADP·Arg[c]				10.9 ± 0.3

[a] Data for CoADP and CoATP taken from Jarori et al. (1985). [b] Calculations are based on $(pT_{1P})^{-1}$ data at 121.5 MHz from eq 4 with $C = 6.75$ Å s$^{-1/3}$ and $10^{-12} \le f(\tau_C) \le 5 \times 10^{-12}$ s. Distances in Co(II) complexes are given as a range corresponding to the range chosen for $f(\tau_C)$. Errors arising from $(pT_{1P})^{-1}$ are ~2–4%. Most of the uncertainty in the distances is due to the estimation of $f(\tau_C)$. [c] Distance was calculated on the basis of the frequency dependence of the relaxation times, pT_{1P}, in the range 200–470 MHz (see Table I) with $C = 812$ Å s$^{-1/3}$ (see text).

The ^1H signal of the δ protons (at ~3.2 ppm) of arginine is the only resonance that could be clearly identified in the presence of the proton spectrum of the protein. This signal might overlap the δ proton signals from the 16 arginines in the protein (Blethen & Kaplan, 1967). It is assumed that the enhancement in the relaxation rate observed is exclusively due to the interaction of the substrate δ protons with Mn(II) in the enzyme complex. Control measurements with Mn(II) and free arginine show that this assumption is valid.

There are two δ protons that give rise to an unresolved NMR signal for this sample, and hence the composite relaxation rate of this resonance is a biexponential with rates arising from the two cation–proton distances. Denoting these distances by r_1 and r_2, if r_1^{-6} and r_2^{-6} differ by a factor of 3 or more, the biexponential character of relaxation is expected to be detectable. If not, the superposition yields an average rate so that the distance calculated, r, will be $[^1/_2(r_1^{-6} + r_2^{-6})]^{-1/6}$. The latter is the case in the current measurements. It is important to recognize this point in interpreting the distance calculated from the relaxation data. It is evident that because of the reciprocal sixth power the shorter of the two distances r_1 and r_2 will be close to the calculated value r.

The $(pT_{1P})^{-1}$ values for the δ protons of arginine exhibit strong frequency dependences in the range 200–470 MHz (see Table I). The plot of pT_{1P} vs ω_I^2, shown in Figure 4, is approximately linear. However, since there are only three experimental points, each associated with some uncertainty, possible nonlinearities cannot be ruled out on the basis of the data alone. On the other hand, the temperature dependence of $(pT_{1P})^{-1}$ showed a *positive* slope for log $(pT_{1P})^{-1}$ vs T^{-1} with $\Delta E = 2.4 \pm 0.3$ kcal/mol (see Figure 5). Such a temperature dependence (opposite to an Arrhenius type of variation) obtains if $\omega_I \tau_{C1} \ge 1$, $\tau_{C1} \approx \tau_{S1}$, $\tau_{S1}^{-1} = B\tau_V$ (see eq 5 and 9), and τ_V has an Arrhenius type of temperature dependence. Furthermore, if $\tau_{S1} \propto \tau_V^{-1}$, $\omega_S \tau_V \ll 1$ (see eq 9), and $\tau_{C1} \approx \tau_{S1}$, τ_C is frequency independent and, therefore, pT_{1P} vs ω_I^2 will be linear (see eq 5). Thus, a linear fit of pT_{1P} vs ω_I^2 shown in Figure 4 is consistent with the temperature dependence in Figure 5.

It may also be seen from eq 2 and 5 that a plot of pT_{1P} vs ω_I^2 has a slope given by $(r/C)^6(\tau_{C1}/3)$ and a y intercept equal to $[(r/C)^6(3\tau_{C1})^{-1} + \tau_M]$. Thus, if τ_M is known, the values

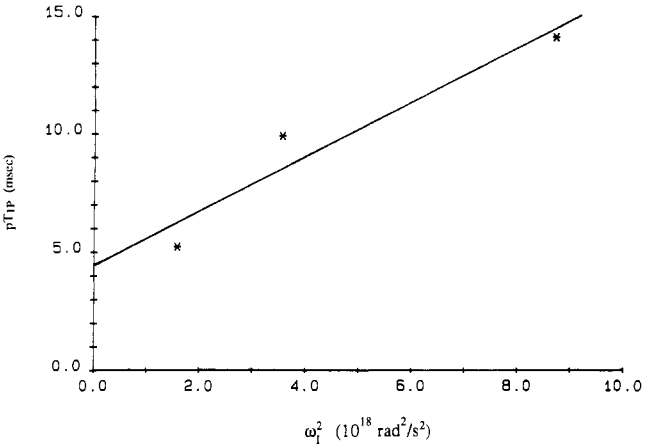

FIGURE 4: pT_{1P} (ms) vs ω_I^2 for δ-^{1}H of L-arginine in E·MnADP·Arg. Experimental data points are shown for 200, 300, and 470 MHz together with the least-squares fit. Computed values from least-squares fit with C = 812 Å $s^{-1/3}$, τ_C = 0.6 ± 0.02 ns, and r = 10.9 ± 0.3 Å.

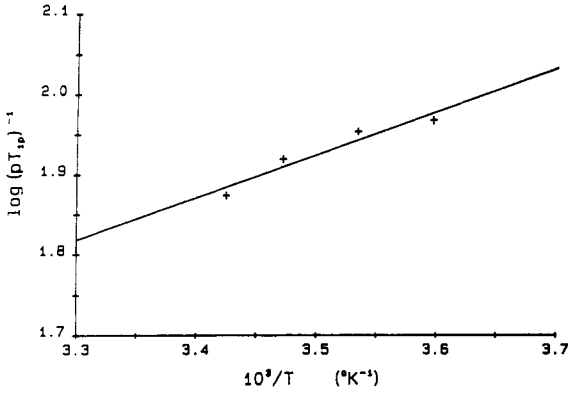

FIGURE 5: log $(pT_{1P})^{-1}$ vs $10^3/T$ for δ protons of arginine in E·MnADP·Arg complex. Typical sample conditions and results are given in Table I. Note that the sign of the slope is opposite to that for a simple Arrhenius behavior for $(pT_{1P})^{-1}$ (see text).

of r and τ_{C1} can be obtained from the slope and y intercept of Figure 4. For the relaxation of nuclei in arginine τ_M is determined by exchanges between E·MnADP·Arg and all those diamagnetic complexes in the sample containing arginine. The frequency dependence of $(pT_{1P})^{-1}$ and the ΔE value of 2.4 kcal/mol suggests that τ_M is not a major part of pT_{1P}. The y intercept in Figure 4 is ~5 ms. The ^{31}P relaxation rate of α-P and β-P of E·MnADP is exchange limited with a τ_M value of ~5 ms and ΔE ~ 10 kcal/mol. This value of τ_M is clearly not appropriate for arginine on the basis of either its magnitude or its activation energy. The τ_M value for arginine thus appears to be primarily governed by the rate of dissociation of arginine from the E·MnADP·Arg complex. This dissociation rate is expected to be larger than that for MnADP since MnADP binds to the enzyme an order of magnitude stronger than does arginine (Buttlaire & Cohn, 1974a). All the evidence, therefore, suggests that τ_M makes a minor contribution (≤30%) to the y intercept of Figure 4. On this basis, the values of slope and y intercept in Figure 4 yield τ_{C1} = τ_{S1} = 0.6 ± 0.2 ns and r = 10.9 ± 0.3 Å.[4] To verify if the value of τ_{C1} is reasonable, ^{1}H relaxation rates of solvent H_2O were also measured at all three frequencies for the same complexes in which the relaxation rates of δ protons were measured. The frequency dependences of the solvent H_2O proton relaxation rates also yield a value of τ_C = τ_{S1} = 0.5 ± 0.12 ns, in agreement with that obtained above from the δ proton relaxation data.

The value of 0.6 ± 0.2 ns deduced for τ_{S1} on the basis of the frequency dependence of relaxation rates for δ protons and for solvent H_2O in the range 200–470 MHz is similar to the τ_{S1} values obtained from the frequency dependence of solvent H_2O relaxation in Mn(II) complexes or arginine kinase (Buttlaire & Cohn, 1974b) in the range 8–60 MHz. If there is a single τ_{S1} with a frequency dependence governed by eq 9 valid for both the frequency ranges, the value of τ_{S1} in the higher frequency range should have exceeded the rotational correlation time τ_R and the observed correlation time would then have been τ_R. However, the observed temperature dependence, as well as the value of 0.6 ± 0.2 ns being too short to be a rotational correlation time for an enzyme of molecular mass of 40000, suggests that the deduced correlation time τ_{C1} is primarily contributed by the electron spin relaxation time. A similar paradox was observed in the value of τ_{S1} deduced for the ^{31}P relaxation rates of 3-P-glycerate in the E·MnADP·3-P-glycerate complex (Ray & Nageswara Rao, 1988).

Discussion

The structure–dependent ^{31}P relaxation rates of E·CoATP and E·CoADP show that the cation is directly coordinated to oxygens of all the phosphate groups in the nucleotide on each of these complexes. This pattern of direct coordination of the cation is similar to the results obtained for creatine kinase (Leyh et al., 1985; Jarori et al., 1985) and 3-P-glycerate kinase (Ray & Nageswara Rao, 1988). Thus, among the enzymes studied thus far by this method, adenylate kinase is the only enzyme for which a departure from direct coordination of the cation to all the phosphate groups of bound ATP and ADP was detected. In the adenylate kinase complexes the cation–^{31}P (α-P) distances were found to be intermediate between those for direct coordination and a second coordination sphere (Ray et al., 1988). For all the enzymes ^{31}P relaxation rates of enzyme-bound Mn–nucleotide complexes were clearly exchange limited. In this assessment the choice of experimental protocols such that cation-binding substrates are exclusively in enzyme-bound form, along with measurement of temperature and frequency dependence of the relaxation rate, played a crucial role.

The ^{31}P NMR results presented in this paper on arginine kinase complexes generally parallel those obtained previously for creatine kinase (Jarori et al., 1985). Since both these enzymes form long-lived transition-state analogue complexes with nitrate in the presence of MnADP and the second substrate (arginine or creatine as the case may be), it was possible to monitor the increase in lifetime with the progressive addition of the different components of the quinary complex. Thus, while the ^{31}P relaxation rates of E·CoATP were structure dependent for both enzymes, addition of creatine did not make a significant difference for the creatine kinase complexes, whereas addition of arginine made these rates become exchange limited for the arginine kinase complexes. This behavior is consistent with the fact that the binding of the substrates to arginine kinase is appreciably tighter than to creatine kinase (Buttlaire & Cohn, 1974a; Reed et al., 1972). Addition of nitrate to form the transition-state analogue complexes produced analogous effects for both the enzymes. The lifetimes became long, and the activation energies are elevated to the 15–20 kcal/mol range.

[4] On the basis of eq 3, 4, and 6, it may be readily seen that from the graph of pT_{1P} vs ω_I^2 in Figure 4, $r = C\,[9 \times \text{slope} \times (\text{intercept} - \tau_M)]^{1/12}$. Thus, the uncertainty in the value of τ_M has a negligible effect on the value of r calculated; for example, if τ_M is 30% of the value of the intercept, the value of r is reduced by just 3%. However, the corresponding τ_C increases by 20%.

The temperature dependence of the exchange-limited ^{31}P relaxation rates in the E·MnADP complexes displayed an unmistakable discontinuity around 15–20 °C. A qualitatively similar behavior, although much less pronounced, was noticed for MnADP bound to creatine kinase (Jarori et al., 1985) about 10–15 °C. In the case of creatine kinase, the ^{31}P relaxation rate for β–P(E·CoADP) showed an unusual temperature dependence in contrast with that of α–P (Jarori et al., 1985). All these results taken along with previously published results on the ^{31}P NMR line shapes of β–P(E·MgADP) (Nageswara Rao & Cohn, 1977, 1981) and Mn(II) EPR measurements (McLaughlin et al., 1976) suggest the presence of conformational heterogeneity in the cation–ADP complexes of both the enzymes. It is difficult to determine the nature of this conformational heterogeneity from the magnetic resonance measurements alone, but the fact that both these enzymes exhibit parallel results is indicative of a similarity in their mechanism.

Distance determinations in biological macromolecules by NMR, either by paramagnetic relaxation or by NOE measurements, are subject to uncertainties arising from such assumptions as an isotropic rotational correlation time or an isotropic g tensor. These assumptions are usually required to make the theoretical analysis tractable; nevertheless, they place a limitation on the methodology. To offset the effect of such unavoidable factors, it is useful to acquire significantly more than the minimum amount of distance data and determine structures on the basis of agreement with maximal data. It is with such a view that the ^1H NMR measurements on E·MnADP·Arg complexes presented in this paper were attempted. The superposed ^1H resonances of the δ protons of arginine yield the only clearly identifiable peak in the spectrum in spite of the large excess of arginine, due to overlap with other signals from the protein and J coupling induced dispersion of signals. Thus, it must be recognized that the relaxation rate being measured is proportional to the average of the reciprocal sixth power of the distances of the two protons from the cation. Nevertheless, this is the only distance involving arginine that ^1H NMR will provide. The experiments were performed by keeping the nucleotide and Mn(II) entirely in the enzyme complex so that enhancement in the relaxation of the δ protons arises from the bound complex alone. This was verified by ^1H NMR measurements on arginine δ protons as well as on solvent H_2O. The results indicate that the procedure yields reliable relaxation rates.

The distance of 10.9 ± 0.3 Å between Mn(II) and the δ protons of arginine in E·MnAPD·Arg suggests, on the basis of a Dreiding molecular model, that the enzyme active site is in a partially closed conformation with a gap between where the oxygens of the γ–P(ATP) would be and the recipient guanidino nitrogen. Formation of the reaction complex, i.e., the presence of MnATP rather than MnADP, may be required for complete closure. A complete structural characterization of arginine on the enzyme will, of course, require measurements using L-arginine labeled by ^{13}C or ^{15}N at specific positions.

The correlation time of 0.6 ± 0.2 ns deduced from the ^1H relaxation data is too short to be rotational correlation time of a protein of the size of arginine kinase, and hence it was concluded that it is the electronic relaxation time. Similar values of correlation time attributed to electron relaxation were deduced at smaller magnetic fields (Buttlaire & Cohn, 1974a). If there is a single τ_{s1} for the cation, the field dependence of the electron relaxation time does not appear to follow the standard form of the Bloembergen–Morgan (1961) theory (see eq 9) over the whole range. Alternatively, there could be multiple values of τ_{s1}, each dominating in a different frequency range. Either way, the nuclear spin relaxation process arising from interaction with a rapidly relaxing paramagnetic cation may be considerably more complicated than the prescription of the commonly used form of the theory for the electron relaxation.

Paramagnetic effects on the relaxation rates of the ^{31}P nuclei of ATP served the purpose of locating the cation with reference to phosphate chain. To complete the conformational picture of bound ATP, distance information pertaining to the adenine base and to the ribose with reference to the cation is required. Paramagnetic measurements with ^{13}C and ^{15}N labeled at specific position in the adenosine moiety offer the best hope of acquiring these data. Such experiments are in progress.

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^{1}H NMR Structural Characterization of a Recombinant Kringle 2 Domain from Human Tissue-Type Plasminogen Activator†

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ABSTRACT: The kringle 2 domain of human tissue-type plasminogen activator (t-PA) has been characterized via ^{1}H NMR spectroscopy at 300 and 620 MHz. The experiments were performed on the isolated domain obtained by expression of the 174-263 portion of t-PA in *Escherichia coli* [Cleary et al. (1989) *Biochemistry 28*, 1884-1891]. The spectrum of t-PA kringle 2 is characteristic of a globular structure and shows overall similarity to that of the plasminogen (PGN) kringle 4. Spectral comparison with human and bovine PGN kringle 4 identifies side-chain resonances from Leu^{46}, which afford a fingerprint of kringle folding, and from most of the aromatic ring spin systems. Assignment of signals arising from the His^{13}, His^{48a}, and His^{64} side chains, which are unique to t-PA kringle 2, was assisted by the availability of a His^{64} → Tyr mutant. Ligand-binding studies confirm that t-PA kringle 2 binds L-lysine with an association constant K_a ~11.9 mM^{-1}. The data indicate that homologous or conserved residues relative to those that compose the lysine-binding sites of PGN kringles 1 and 4 are involved in the binding of L-lysine to t-PA kringle 2. These include Tyr^{36} and, within the kringle inner loop, Trp^{62}, His^{64}, Trp^{72}, and Tyr^{74}. Acid/base titration of aromatic singlets in the presence of L-lysine yields pK_a* ~6.25 and ~4.41 for His^{13} and His^{64}, respectively, and shows that the His^{48a} imidazole group does not protonate down to pH* ~4.3. Thus, the His^{48a} and His^{64} side chains are in solvent-shielded locations. As observed for the PGN kringles, the Trp^{62} indole group titrates with pK_a* ~4.60, which indicates proximity of the side chain to a titratable carboxyl group, most likely that of Asp^{57} at the binding site. Several labile NH protons of t-PA kringle 2 exhibit retarded H-exchange kinetics, requiring more than a week in ^2H_2O for full deuteration in the presence of L-lysine at 37 °C. This reveals that kringle 2 is endowed with a compact, dynamically stable conformation. Proton Overhauser experiments in ^1H_2O, centered on well-resolved NH resonances between 9.8 and 12 ppm, identify signals arising from the His^{48a} imidazole NH3 proton and the three Trp indole NH1 protons. A strong dipolar interaction was observed among the Trp^{25} indole NH1, the Tyr^{50} amide NH, and the His^{48a} imidazole CH2 protons, which affords evidence for an aromatic cluster in t-PA kringle 2 similar to that found at the hydrophobic kernel of PGN kringles. Overall, the data indicate a highly structured conformation for the recombinant t-PA kringle 2 that is closely related to that of the previously investigated PGN kringles 1, 4, and 5.

Tissue-type plasminogen activator (t-PA)[1] is a serine protease that converts the proenzyme plasminogen (PGN) into plasmin, which, in turn, efficiently degrades the fibrin network of blood clots (Collen, 1980). It consists of several distinct structural domains (Pennica et al., 1983): a finger domain homologous to fibronectin type 1 structures (Bányai et al., 1983), a growth factor domain resembling the mammalian epidermal growth factor (Doolittle et al., 1984), two kringle domains somewhat homologous to those in PGN, and a C-terminal proteolytic domain similar to the trypsin-like serine proteases (Patthy, 1985). The activation of PGN by t-PA is greatly enhanced by fibrin or its fragments (Hoylaerts et al., 1982; Rånby, 1982; Rijken et al., 1982). The acceleration seems to be due to the high affinity of these proteases for the fibrin matrix, ensuring that the activation occurs efficiently on the surface of a fibrin clot.

Systematic studies using domain deletion mutants suggest that the t-PA kringle 2 domain is involved in fibrin binding and, therefore, mediates the fibrin-dependent activation of PGN by t-PA (van Zonneveld et al., 1986a,b; Verheijen et al., 1986; Gething et al., 1988). These studies have also shown

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[1] Abbreviations: bPGN, bovine plasminogen; CD, circular dichroism; CIDNP, chemically induced dynamic nuclear polarization; COSY, two-dimensional chemical-shift-correlated spectroscopy; FID, free induction decay; hPGN, human plasminogen; K_a, ligand-kringle equilibrium association constant; K2, kringle 2; K4, kringle 4; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; PGN, plasminogen; pH*, glass electrode pH reading uncorrected for deuterium isotope effects; pK_a*, pK_a determined from acid/base titration in ^2H_2O, uncorrected for deuterium isotope effects; t-PA, human tissue-type plasminogen activator; u-PA, urokinase plasminogen activator; 2D, two dimensional.

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