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^{31}P NMR Studies of the Structure of Cation–Nucleotide Complexes Bound to Porcine Muscle Adenylate Kinase†

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ABSTRACT: The paramagnetic effects on the spin-relaxation rates of ^{31}P nuclei in complexes of porcine muscle adenylate kinase with ATP, GTP, GDP, and AMP were measured in the presence of two dissimilar activating paramagnetic cations, Mn(II) and Co(II), to examine the structures of the enzyme-bound complexes. Experiments were performed exclusively on enzyme-bound complexes to limit contributions to observed relaxation rates to two exchanging complexes (with and without cation). Measurements were made at three frequencies, 81, 121.5, and 190.2 MHz, and as a function of temperature in the range 5–30 °C to determine the effect of exchange on the observed relaxation rates. Relaxation rates in the E·MnATP, E·MnGTP, and E·MnGDP complexes were shown to be exchange-limited and therefore without structural information. Relaxation rates for the complexes E·CoATP, E·CoGTP, and E·CoGDP were shown to depend on Co(II)–^{31}P distances. Inability to precisely estimate spectral densities arising from electronic relaxation of Co(II) restricts calculations of Co(II)–^{31}P distances in these complexes to upper and lower limits. At the center of these limits, the Co(II)–^{31}P distances of β-P and γ-P in E·CoATP and E·CoGTP, and of β-P (E·CoGDP), are in the range 3.1–3.5 Å appropriate for the first coordination sphere. For all these complexes, the corresponding distance for α-P is appreciably larger in the range 3.9–4.5 Å. In the quaternary complex, E·MnGDP·AMP, while the ^{31}P relaxation rates of α-P and β-P (GDP) were exchange-limited, that for ^{31}P in AMP was only partially exchange limited at 121.5 MHz [because of the longer Mn(II)–^{31}P distance] as evidenced by its frequency dependence and an activation energy of 4 kcal/mol. The ability to measure structure-dependent ^{31}P relaxation rates for AMP in the quaternary complex with both Mn(II) and Co(II) at all the frequencies allowed approximate estimates of the lifetime and correlation time for the Mn(II) complex. On this basis a value of 5.9 Å appears appropriate for the Mn(II)–P(AMP) distance in the complexes E·MnGDP·AMP and E·CoGDP·AMP.

Adenylate kinase (EC 2.7.4.3) catalyzes the reversible reaction[1]

$$\text{MgATP} + \text{AMP} \rightleftharpoons \text{MgADP} + \text{ADP} \qquad (1)$$

The enzyme is most abundant in tissues, such as muscle, in which the energy turnover is considerable (Noda, 1973) and is essential for production of adenine nucleotides beyond the monophosphate. Kinetic and NMR experiments have detected two distinct substrate binding sites, one of which binds MgATP and MgADP while the other is specific for uncomplexed AMP and ADP (Noda, 1958; Rhoads & Lowenstein, 1968; Hamada & Kuby, 1978; Nageswara Rao et al., 1978). GTP and MgGTP are known to bind at the MgATP site with reasonable affinity (Price et al., 1973). In contrast, however, the AMP site is highly specific for adenine nucleotides.

There is continuing debate about the positions of the two substrate binding sites on adenylate kinase despite the fact that

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; E·M·S, enzyme–metal–substrate; E·S, enzyme–substrate; NTP, nucleoside 5′-triphosphate; NDP, nucleoside 5′-diphosphate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; CD, circular dichroism.

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both the amino acid sequence (Heil et al., 1974) and the X-ray structure (Sachsenheimer & Schulz, 1977; Dreusicke et al., 1988) have been determined. The "glycine-rich loop", residues 15–22 in the sequence, appears to be an anion hole in a substrate binding domain (Dreusicke & Schulz, 1986) which was interpreted from X-ray data as part of the AMP binding site (Pai et al., 1977). However, part of the loop, Lys-21, is a candidate for part of the ATP binding site (Tagaya et al., 1987). Also, His-36 is claimed as part of the MgATP binding domain (Schirmer et al., 1970; McDonald et al., 1975). ^{1}H NMR relaxation evidence (Smith & Mildvan, 1982; Fry et al., 1985, 1987) concerning the placement of the two nucleotide binding domains within the X-ray structure was used to suggest that the substrate positions should be the reverse of those given by the X-ray data (Pai et al., 1977). Recent X-ray work with the P^1,P^5-bis(5′-adenosyl) pentaphosphate complex of yeast adenylate kinase (Egner et al., 1987) (which shows a fair degree of similarity with the porcine muscle enzyme in the presumed binding domains (Tomasselli et al., 1986; Schulz et al., 1986; Dreusicke & Schulz, 1986), appears to agree with the NMR results regarding the AMP binding site while leaving the position of the ATP binding site undetermined. The identity and designation of the binding sites is thus not unequivocally established.

Structural measurements on enzyme–nucleotide complexes involving the cation are motivated by the general question of the role of the cation in catalyzing these enzymatic reactions. In the case of adenylate kinase, there is an additional feature unique to this enzyme in that the reverse reaction of adenylate kinase may be viewed as a phosphoryl transfer from ADP as the donor to another ADP as an acceptor. There is compelling evidence (Rhoads & Lowenstein, 1968; Nageswara Rao et al., 1978) that the cation is associated only with the acceptor ADP (similar to all kinases) and not to the donor ADP. It has been suggested that metal-chelated ATP is bound to an enzyme either in β,γ-bidentate coordination with the resulting metal-chelated ADP being β-monodentate or as a α,β,γ-tridentate coordinate with a α,β-bidentate metal-chelated ADP as the product (Mildvan, 1974; Mildvan & Fry, 1987). Recent studies of creatine kinase (Leyh et al., 1985; Jarori et al., 1985), 3-P-glycerate kinase (Ray & Nageswara Rao, 1988a,b), and arginine kinase[2] have all concluded upon direct coordination to ATP as the α,β,γ-tridentate metal chelate and to ADP as the α,β-bidentate chelate. Adenylate kinase, however, appears to be different from these other enzymes. Adenylate kinase is specific for the Δ screw-sense β,γ-bidentate, exchange-inert CrATP complex whereas 3-P-glycerate kinase (Jaffe et al., 1982), creatine kinase, and arginine kinase are virtually inactive with all exchange-inert CrATP complexes (Dunaway-Mariano & Cleland, 1980). Furthermore, the results of both Mn(II) EPR (Kalbitzer et al., 1983) and metal effects on nucleotide phosphorothioate stereoisomer specificity (Tomasselli & Noda, 1983; Tomasselli et al., 1984) agree that α-P (ATP or GDP) is not coordinated to metal in enzyme-bound complexes. Indeed, the results of Mn(II) EPR (Kalbitzer et al., 1983) suggested the possibility of β-monodentate coordination for enzyme-bound MnATP, which is not incompatible with the results of the phosphorothioate work but does present some problems with understanding the results on the exchange-inert CrATP complexes. Thus, the nature of the chelation of the obligatory divalent cation to enzyme-bound ATP (or acceptor ADP) is not clearly resolved.

[2] G. K. Jarori, B. D. Ray, and B. D. Nageswara Rao, unpublished results.

Paramagnetic effects on nuclear spin-relaxation rates is one of the few methods available for determination of distances between divalent cations and substrate nuclei (Mildvan et al., 1980; Villafranca, 1984; Jarori et al., 1985; Mildvan & Fry, 1987; Ray & Nageswara Rao, 1988b). However, extreme caution needs to be exerted in interpreting the data obtained by this method to avoid the many and various pitfalls inherent in the method (Mildvan & Gupta, 1978; Burton et al., 1979; Mildvan et al., 1980; Jarori et al., 1985; Ray & Nageswara Rao, 1988b). In particular, it is important to determine that the relaxation rate data obtained are not dominated by the exchange rate (Jarori et al., 1985; Ray & Nageswara Rao, 1988b). Some of the early attempts to use this method for complexes with pyruvate kinase (Mildvan et al., 1976) and phosphoribosylpyrophosphate synthetase (Granot et al., 1980) led to results that disagreed with those of other methods such as ^{17}O superhyperfine structure effects in Mn(II) EPR spectra (Lodato & Reed, 1987) and metal ion effects on phosphorotioate analogues of nucleotides (Gibson & Switzer, 1980). These discrepancies appear to be a result of incorrect assessment of the contribution of exchange to the observed relaxation rate.

This paper presents ^{31}P spin-relaxation measurements on enzyme-bound substrate complexes of porcine muscle adenylate kinase with Mn(II) and Co(II). These experiments were designed to determine cation-^{31}P distances in different enzyme complexes with ATP, GTP, GDP, and AMP and were performed by adapting the strategy previously used for creatine kinase (Jarori et al., 1985) and 3-P-glycerate kinase (Ray & Nageswara Rao, 1988b). Because adenylate kinases are active in the presence of ADP, the inactive and more weakly bound GDP was substituted for ADP in those cases where an ADP-like complex was desired. Results with the E·MnGDP·AMP complex provide an example in which the distance-dependent paramagnetic relaxation rate and the lifetime of the paramagnetic complex contribute at a comparable level to the observed relaxation rate. The analysis of such data is of relevance to the use of the paramagnetic relaxation method for the determination of distances in the range of about 5–7 Å.

Experimental Procedures

Materials. ATP, AMP, DTT, 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. $CoCl_2$ (Puratronic) was from AESAR, and GTP and GDP were from Boehringer. All other chemicals were of analytical reagent grade. ATP, AMP, GTP, GDP, and buffer solutions were passed through a Chelex 100 column before use in the NMR experiments.

Enzyme Preparation. Porcine skeletal muscle adenylate kinase was purified from a mitochondria-free supernatant (Crane et al., 1956) by a procedure originally used to prepare beef heart cytosolic adenylate kinase (Tomasselli & Noda, 1980). Purified enzyme was concentrated by lyophilization from a buffered solution containing 5 mM DTT.

Lyophilized enzyme was resuspended in 200 mM K-Hepes and 5 mM DTT buffer (pH 8.2) and dialyzed extensively against 200 mM K-Hepes buffer containing preequilibrated Chelex 100. Enzyme concentrations obtained in this way were usually between 5.3 and 6.5 mM. Typically, the specific activity at 25 °C of resuspended enzyme treated in this manner was 1500 units/mg in a coupled assay system with AMP, ATP, 2 mg/mL BSA, pyruvate kinase, and lactate dehydrogenase (Price et al., 1973). Protein and nucleotide concentrations were determined spectrophotometrically with $\epsilon_{280}^{mg/mL} = 0.538$ cm^{-1}

and a molecular weight of 21 700 (Noda, 1973) 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 NMR measurements at 121.5 MHz were made on an NT-300 wide-bore NMR spectrometer equipped with a 12-mm multinuclear probe, a 293C pulse programmer, a Nicolet 1280 computer, and a variable-temperature controller. A typical 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 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, 1973; 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, 1988b). The relevant equations for analysis of the data in this paper are given below: Given a sample that contains two exchanging complexes, one 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

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

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, τ_1 and ω_I are respectively the gyromagnetic ratio and the resonance frequency of the relaxaing 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).

The measurements presented 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 $(T_{1P})^{-1}$. Since structural information is not available from measurements in which $\tau_M \gg T_{1M}$ and can be computed from measurements in which $\tau_M \sim T_{1M}$ only if τ_M is known, the contribution of τ_M to T_{1P} must be determined (see eq 2 and 3). This was done by making T_{1P} measurements as a function of temperature in the range 5–30 °C and at three ^{31}P frequencies (81, 121, and 190 MHz). Note that the 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 $(pT_{1P})^{-1}$ obtained for the ^{31}P nuclei in the phosphate groups of E·MnATP, E·MnGTP, and E·MnGDP at 5 °C are given in Table I along with values of $(pT_{1P})^{-1}$ obtained for the MnGDP and MnGTP complexes and previously published data for MnATP (Jarori et al., 1985). Data presented are based on measurements made at 121.5 and 190.2 MHz. For the enzyme complexes with ATP, on the basis of previously published dissociation constants (Price et al., 1973), the fractional concentration of paramagnetic complexes free in solution ([M·S]/[E·M·S]) never exceeds ~3%. Although the published dissociation constants for GTP and GDP complexes (Price et al., 1973) suggest that substantial fractions of free GTP and GDP may be present for the concentrations chosen here (see Table I), the measured relaxation rates and their temperature dependences in the range 5–30 °C do not indicate substantial free Mn(II)–nucleotide in either case. It appears that, under the sample conditions of the NMR measurements, MnGTP and MnGDP bind with greater affinity than that suggested by the data of Price et al. (1973).

The relaxation rates for α-P and β-P of GDP in E·MnGDP and for α-P, β-P, and γ-P of ATP and GTP in E·MnATP and E·MnGTP are unchanged within experimental error between 121.5 and 190.2 MHz. Furthermore, Arrhenius plots of $(pT_{1P})^{-1}$ yield activation energies (ΔE)in the range 5–8 kcal/mol for the ^{31}P nuclei in these enzyme-bound complexes. The values of ΔE are in the range appropriate for τ_M and are much larger than would be expected for τ_R or τ_V. Thus, by these criteria (Jarori et al., 1985), the ^{31}P relaxation data in the Mn(II)–nucleotide complexes bound to adenylate kinase appear to be determined by the lifetimes of these complexes.

Co(II)–Nucleotide Complexes. Co(II) is known to be a good activator of the adenylate kinase reaction (Noda, 1973; Tomaselli et al., 1980). However, dissociation constants involving the cation are not usually known for Co(II) complexes with an accuracy comparable to those for Mn(II) complexes (Price et al., 1973). It is, therefore, implicitly assumed that these dissociation constants for the Co(II) complexes are generally in the same ranges as those for the corresponding Mn(II) complexes (Jarori et al., 1985). Since the experimental protocols use a sufficient excess of enzyme and low concentrations of paramagnetic cations, the variability in the concentrations resulting from this assumption does not alter the analysis of the data for the Co(II) complexes.

Values of $(pT_{1P})^{-1}$ obtained for the ^{31}P nuclei in the phosphate groups of E·CoATP, E·CoGTP, and E·CoGDP at 5 °C are given in Table II along with values of $(pT_{1P})^{-1}$ measured on CoGDP and CoGTP complexes and previously published data for CoATP (Jarori et al., 1985). Data for the enzyme-

Table I: Paramagnetic Effect $(pT_{1P})^{-1}$ of Mn(II) on ^{31}P Relaxation Rates and Corresponding Activation Energies ΔE for Various GDP, GTP, and ATP Complexes Free in Solution and Bound to Adenylate 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/P(AMP) $(pT_{1P})^{-1}$ (s^{-1})	γ-P/P(AMP) ΔE (kcal/mol)
MnATP[b] (ATP, 4 mM; $MnCl_2$, 2.5–15 μM)	121.5	5570 ± 200		7270 ± 200		6930 ± 200	
MnGTP[c] (GTP, 5 mM; $MnCl_2$, 1.5–6.9 μM)	121.5	7400 ± 300		9600 ± 300		8200 ± 200	
MnGDP[c] (GDP, 5 mM; $MnCl_2$, 2.5–10.3 μM)	121.5	11300 ± 400		11900 ± 400			
E·MnGDP[c] (enzyme, 6.5 mM; GDP, 2.1 mM;	121.5	2040 ± 200	5.5 ± 0.4	2100 ± 200	5.3 ± 0.4		
$MnCl_2$, 4.5–21 μM)	190.2	1830 ± 200		1960 ± 200			
E·MnATP[c] (enzyme, 6.2 mM; ATP 2.5 mM;	121.5	660 ± 40	6.4 ± 0.4	860 ± 90	7.8 ± 0.5	1030 ± 150	8.4 ± 0.4
$MnCl_2$, 2.7–29.3 μM)	190.2	630 ± 40		990 ± 90		1200 ± 150	
E·MnGTP[c] (enzyme, 5.5 mM; GTP 2.5 mM;	121.5	4125 ± 350	6.0 ± 0.4	5800 ± 500	9.2 ± 1.0	5270 ± 500	6.0 ± 0.4
$MnCl_2$, 1.7–76 μM)	190.2	3810 ± 350		6120 ± 500		6020 ± 500	
E·MnDGP·AMP[d] [enzyme, 5.6 mM; GDP, 2.7 mM;	81.0					305 ± 20	7.0 ± 0.5
AMP, 2.7–5.2 mM (121.5 MHz); $MnCl_2$, 21.9–43	121.5					215 ± 20	4.0 ± 0.3
μM (81 MHz), 12–72 μM (121.5 MHz), and	190.2					145 ± 10	1.0 ± 0.4
38–106 μM (190.2 MHz)]							

[a] Samples for this work were in 200 mM K-Hepes, pH 8.2 The $(pT_{1P})^{-1}$ values given were measured at 5 °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 MnATP were taken from Jarori et al. (1985) and are presented for comparison with the enzyme–nucleotide complex data in this work. [c] Measurements were made with four to six values of p = [Mn(II)]/[nucleotide]. [d] Measurements were made with four to six values of p = [Mn(II)]/[AMP]. $(pT_{1P})^{-1}$ for P(AMP) at 121.5 MHz was found unchanged within experimental error, for the range of concentrations used for [AMP]. The paramagnetic complexes are thus almost entirely quaternary.

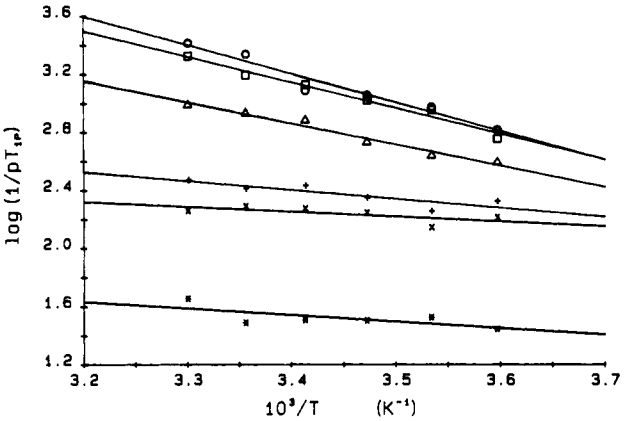

FIGURE 1: log $(pT_{1P})^{-1}$ vs $10^3/T$ for α-P, β-P, and γ-P of E·MnATP (△, □, ○), and E·CoATP (*, ×, +). Typical sample conditions and activation energies (ΔE) obtained are given in Tables I and II.

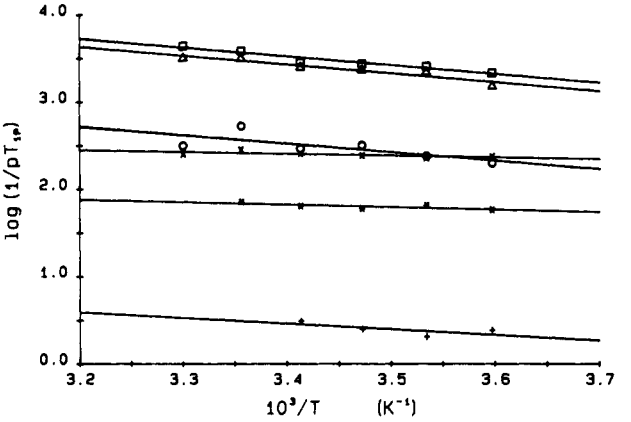

FIGURE 2: log $(pT_{1P})^{-1}$ vs $10^3/T$ for α-P and β-P in E·MnGDP (△, □) and E·CoGDP (*, ×) and for P(AMP) in E·MnGDP·AMP (○) and E·CoGDP·AMP (+). Typical sample conditions and activation energies obtained are given in Tables I and II.

bound complexes were obtained at three frequencies, viz., 80, 121.5, and 190.2 MHz. As was previously seen with CoADP (Jarori et al., 1985), $(pT_{1P})^{-1}$ for CoGDP showed a dependence on GDP concentration, and therefore, the values at high (5 mM) and low (0.5 mM) concentrations along with those obtained by extrapolation to infinite dilution are given. The data for CoGTP do not show concentration dependence in the same range. For the enzyme-bound complexes, temperature dependence of $(pT_{1P})^{-1}$ at 121.5 MHz was measured in the range 5–30 °C. Figures 1 and 2 present Arrhenius plots for E·CoATP and E·CoGDP, respectively. Values for ΔE are included in Table II. By analogy with the results for E·MnGTP and E·MnGDP, the contribution of free CoGTP and CoGDP to the relaxation rates observed for E·CoGTP and E·CoGDP are considered negligible under the sample conditions chosen. For all the three E·Co–nucleotide complexes, the ΔE values are in the range 1–3 kcal/mol and are significantly lower than for the correspondng Mn(II)–nucleotide complexes. Also, the $(pT_{1P})^{-1}$ values for these complexes show a reproducible, although small, frequency dependence. Relaxation rates in these Co(II) complexes are, therefore, primarily determined by T_{1M} with negligible contribution from τ_M.

The theoretical problems involved in estimating a correct value of $f(\tau_C)$ for Co(II) complexes have been discussed earlier in some detail (Benetis et al., 1983; Jarori et al., 1985). 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 creatine kinase (Jarori et al., 1985) and 3-P-glycerate kinase (Ray & Nageswara Rao, 1988b). 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, the same range as above again appears appropriate. Since $f(\tau_C)$ 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), it is not expected that the range of values for $f(\tau_C)$ would vary much, for similar complexes, from protein to protein. Therefore, the 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 expected 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, 1988). Calculated distances are given in Table III. These distances signify direct coordination of Co(II) with the β-P and γ-P of ATP and GTP, and with the β-P of GDP, and lack of direct coordination with the α-P in all three Co(II)–nucleotide

Table II: Paramagnetic Effect $(pT_{1P})^{-1}$ of Co(II) on ^{31}P Relaxation Rates and Corresponding Activation Energies ΔE for Various GDP, GTP, and ATP Complexes Free in Solution and Bound to Adenylate 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/P(AMP) $(pT_{1P})^{-1}$ (s^{-1})	γ-P/P(AMP) ΔE (kcal/mol)
CoATP[b] (ATP, 2.7 mM; $CoCl_2$, 15–25 μM)	121.5	120 ± 20		150 ± 20		210 ± 20	
CoGTP (GTP, 4.95 mM; $CoCl_2$, 15–103 μM)	121.5	120 ± 15		340 ± 20		260 ± 15	
CoGDP (GDP, 5 mM; $CoCl_2$, 25–145 μM)	121.5	145 ± 10		580 ± 40			
CoGDP (GDP, 0.5 mM; $CoCl_2$, 3.6–14 μM)	121.5	120 ± 10		310 ± 20			
CoGDP (extrapolated values)[c]	121.5	115 ± 10		287 ± 20			
E·CoGDP (enzyme, 5.4 mM; ADP, 2.4 mM;	81.0	69 ± 10		220 ± 20			
$CoCl_2$, 45–240 μM)	121.5	58 ± 10	0.9 ± 0.1	230 ± 20	0.9 ± 0.1	230 ± 20	
	190.2	86 ± 10		290 ± 25			
E·CoATP (enzyme, 6 mM; ATP 2.5 mM; $CoCl_2$,	81.0	54 ± 5		220 ± 20		350 ± 25	
36–254 μM)	121.5	27 ± 3	2.0 ± 0.1	160 ± 10	2.0 ± 0.1	230 ± 20	2.2 ± 0.1
	190.2	24 ± 2		135 ± 10		160 ± 15	
E·CoGTP (enzyme, 6 mM; GTP 5.5 mM;	81.0	72 ± 10		170 ± 15		170 ± 15	
$CoCl_2$, 21–212 μM)	121.5	44 ± 5	1.0 ± 0.1	130 ± 10	2.2 ± 0.5	120 ± 10	1.4 ± 0.3
	190.2	67 ± 10		200 ± 20		200 ± 20	
E·CoGDP·AMP[d] (enzyme, 6.1 mM; GDP, 2.5 mM;	81.0					4.5 ± 1	
AMP, 2.5 mM; $CoCl_2$, 150–1200 μM)	121.5					5.1 ± 1	2.0 ± 0.3
	190.2					7.6 ± 1	

[a] Samples for this work were in 200 mM K-Hepes, pH 8.2. The $(pT_{1P})^{-1}$ values were measured at 5 °C with at least four different values of p = [Co(II)]/[nucleotide]. Measurements were made at 81, 121.5, and 190.2 MHz. The ΔE values were obtained at 121.5 MHz from Arrhenius plots (see Figures 1 and 2) of $(pT_{1P})^{-1}$ in the temperature range 5–25 °C. The errors were estimated on the basis of computer fits of the T_1 data and of the appropriate functions involved in obtaining the final values. [b] CoATP data were taken from Jarori et al. (1985) and are presented for comparison with the enzyme–nucleotide complex data in this work. [c] At infinite dilution (see text). [d] Measurments were made with at least five different values of p = [Co(II)]/[AMP].

Table III: Distances of ^{31}P Nuclei from the Cation [Mn(II) or Co(II)] in Various E·M·S Complexes with Adenylate Kinase

complex	cation–^{31}P distance (Å) α-P	β-P	γ-P	P(AMP)
CoATP[a]	3.0–4.0	2.9–3.8	2.7–3.6	
CoGDP[b,c]	3.1–4.0	2.6–3.4		
CoGTP[c]	3.0–4.0	2.6–3.3	2.7–3.5	
MnGDP[d]	3.3	3.3		
MnGTP[d]	3.5	3.4	3.5	
E·CoGDP[c]	3.4–4.5	2.7–3.6		
E·CoGTP[c]	3.6–4.7	3.0–3.9	3.0–4.0	
E·CoATP[c]	3.9–5.1	2.9–3.8	2.7–3.6	
E·CoGDP·AMP[c]				5.1–6.8
E·MnGDP·AMP[e]				5.9

[a] Data for CoATP taken from Jarori et al. (1985). [b] Based on extrapolated values of $(pT_{1P})^{-1}$. [c] Calculations are based on $(pT_{1P})^{-1}$ data at 121.5 MHz (see Table II) by using eq 4 with C = 675 Å $s^{-1/3}$ and 10^{-12} s $\leq f(\tau_C) \leq 5 \times 10^{-12}$ 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 $f(\tau_C)$. [d] Calculations are based on eq 4 and 6 with C = 601 Å $s^{-1/3}$ and $\tau_C = 10^{-10}$ s^{-1}. [e] Calculations are based on analysdis of frequency dependence of T_{1M} with correction for the partial exchange limitation (see text).

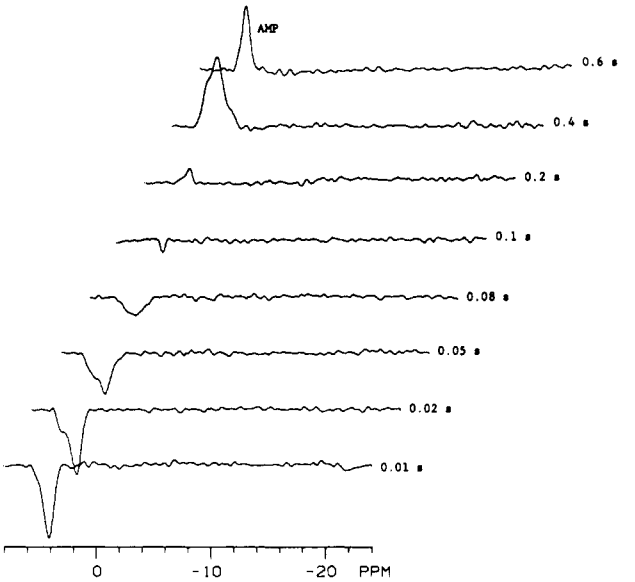

FIGURE 3: T_{1P} measurement at 121.5 MHz and high [Mn(II)] for ^{31}P nuclei in E·MnGDP·AMP (p = 0.026, T = 5 °C). A standard inversion–recovery sequence with a composite π pulse was used. NMR parameters: π/2 pulse width, 18 μs; sweep width, ±2400 Hz; data size, 2048; line broadening, 60 Hz; number of scans, 256; and recycle delay, 0.8 s. Computer fit gives 0.181 s as T_{1P} for ^{31}P of AMP. Signals for α-P and β-P of GDP are too broad to be observed.

complexes. Molecular models indicate that a cation chelated to β-P and γ-P of ATP is approximately at a distance of 4.9 ± 0.4 Å from the ^{31}P nucleus in α-P (Mildvan, 1974). The ranges of Co(II)–^{31}P distances for α-P in the enzyme complexes, given in Table III, are not appropriate either for a first or for a second coordination sphere.

The Quaternary Complexes E·MnGDP·AMP and E·CoGDP·AMP. In adenylate kinase, as was the case for 3-P-glycerate kinase, the recipient substrate, AMP, contains a ^{31}P nucleus. Thus, it should be possible to measure structure-dependent relaxation rates for AMP in dead-end complexes with the metal-chelated substrate, GDP. Figure 3 shows a typical T_1 measurement for P(AMP) in the E·MnGDP·AMP complex at Mn:AMP = 0.026. At this high concentration of Mn(II), the ^{31}P resonances of α-P and β-P (GDP) in the complex are too broad to be observed. In calculating $(pT_{1P})^{-1}$ for P(AMP) in the complex E·MnGDP·AMP, it should be noted that the value of p is strictly given by [E·MnGDP·AMP]/$[AMP]_{total}$, which is difficult to determine accurately. However, if the paramagnetic complexes are almost entirely quaternary (E·MnGDP·AMP), p may be set equal to $[Mn]_{total}/[AMP]_{total}$. In order to verify if this condition is valid, T_{1P} measurements on Mn(II) complexes were made at various concentrations of AMP from [AMP] = [GDP] up to [AMP] = [E]. T_{1P} was found to be unchanged within experimental error (see footnote *d* of Table I).

Values of $(pT_{1P})^{-1}$ measured at 81, 121.5, and 190.2 MHz for P(AMP) in E·MnGDP·AMP and E·CoGDP·AMP complexes at 5 °C are presented in Tables I and II, respectively. The relaxation rates for P(AMP) in both the Co(II) and

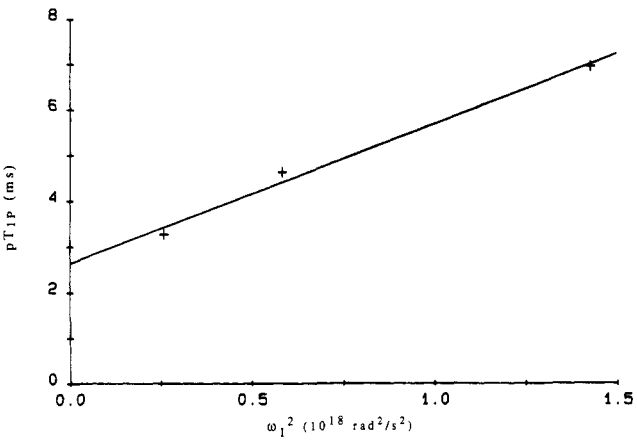

FIGURE 4: pT_{1P} (ms) vs ω_1^2 (10^{18} rad^2 s^{-2}) for the ^{31}P nucleus of AMP in E·MnGDP·AMP. Data points shown are for 81, 121.5, and 190 MHz. Solid line is based on a least-squares fit with an intercept of 2.63 ms and a slope of 3.047×10^{-21} s^3.

Mn(II) complexes depend on frequency. Temperature dependence of this relaxation rate in E·CoGDP·AMP shows an activation energy of 2 kcal/mol. Thus, the relaxation rate for P(AMP) in the E·CoGDP·AMP complex is primarily determined by T_{1M} and not by τ_M. The range of distances calculated from Co(II) to P(AMP) in E·CoGDP·AMP (see Table III) is 5.1–6.8 Å. On the other hand, temperature dependence of the ^{31}P relaxation rate of P(AMP) in E·MnGDP·AMP shows activation energies of 7.0, 4.0, and 2.0 kcal/mol at 81, 121.5, and 190.2 MHz, respectively (see Table I).[3] This result clearly indicates that τ_M is significantly larger than T_{1M} at 81 MHz, significantly smaller than T_{1M} at 190.2 MHz, and comparable to T_{1M} at 121.5 MHz. The frequency dependence of pT_{1P} for P(AMP) in E·MnGDP·AMP is plotted as a function of ω_I^2 in Figure 4. On the basis of eq 3, 4, and 6, the data should be linear with slope = $(r/C)^6\tau_{C1}/3$ and intercept = $\tau_M + (r/C)^6/(3\tau_{C1})$ if τ_{C1} is independent of frequency. It may be seen from eq 8 that τ_{C1} will be independent of frequency either if τ_{S1} is much longer than τ_R or if τ_{S1} is comparable to τ_R and its value is also independent of frequency in the range studied. The plot shown in Figure 4 does not show appreciable departure from linearity to cause one to suspect frequency dependence of τ_{C1}. The question, then, is regarding the contribution of τ_M to the intercept in Figure 4. The slope of the frequency dependence is independent of τ_M. Thus, given the least-squares slope, the limits for r from the Co(II) data (5.1 Å $< r <$ 6.8 Å) can be used to compute limits for τ_{C1}. These limis can then be back-substituted into the formula for the intercept, and limits for the value of τ_M can be calculated. The limits calculated in this manner were 4.36 ns $< \tau_{C1} <$ 24.5 ns and 2.467 ms $< \tau_M <$ 2.623 ms. Note that a 6% increase in τ_M leads to over a 5-fold increase in τ_{C1} and about 30% change in the value of r. In this situation, pinpointing the value of τ_M in this range with precision carries no meaning. Based on the accuracy of these data alone, it is not possible to narrow down the range for r. If the value of 5.9 Å at the center of the range obtained for the Co(II) complex were used for the Mn(II) complex, one obtains the values τ_{C1} = 10.4 ns and τ_M = 2.6 ms. This would mean that, at 121.5 MHz, about 56% of the value of pT_{1P} can be attributed to τ_M and such a contribution is compatible with both the frequency dependence and the activation energy for P(AMP) in E·MnGDP·AMP. Thus, r = 5.9 Å and τ_{C1} = 10.4 ns appear appropriate for the E·MnGDP·AMP complex. These values may be improved if a more accurate estimate of τ_{C1} becomes possible in the future, perhaps from relaxation data for other nuclei in this complex.

Discussion

Structure-dependent ^{31}P relaxation rates of CoATP, CoGTP, and CoGDP bound to adenylate kinase show that the cation is directly coordinated only to β-P (GDP) or β-P and γ-P (ATP or GTP), and not to α-P in these complexes.[4] β,γ-Bidentate coordination for NTP and β-monodentate coordination for NDP is in agreement with the results of studies with stable CrATP complexes (Dunaway-Mariano & Cleland, 1980). However, the conclusion of β,γ-bidentate coordination reached in the present work for cation–ATP or cation-GTP complexes of adenylate kinase is in contrast with the Mn(II) EPR results, which show evidence for Mn(II) coordination to only with β-P (Kalbitzer et al., 1983). Work with nucleotide phosphorothioates (Tomasselli et al., 1984) agrees with the conclusion of β-monodentate coordination for NDP complexes but did not address the question of coordination for NTP complexes.

The conclusion of direct coordination of the cation to only the β-P of E·GDP and to only the β-P and γ-P of E·ATP or E·GTP for the adenylate kinase complexes is in contrast with results obtained for the corresponding complexes of 3-P-glycerate kinase (Ray & Nageswara Rao, 1988a,b), creatine kinase (Leyh et al., 1985; Jarori et al., 1985), and arginine kinase.[2] It is interesting to note that these results parallel those for substrate activity of β,γ-bidentate CrATP complexes for different kinases on the basis of CD assay. 3-P-glycerate kinase (Jaffe et al., 1982), creatine kinase, and arginine kinase show no activity in the CD assay with the bidentate complexes whereas adenylate kinase does (Dunaway-Mariano & Cleland, 1980). While the range of Co(II)–^{31}P distances obtained for α-P in E·CoATP, E·CoGTP, and E·CoGDP (Table III) are too long for direct coordination, they are too short for the second coordination sphere; i.e., there is not enough room for an intervening water molecule. How this particular disposition of the α-P with respect to the cation is fashioned on this enzyme in contrast with the other enzymes may well be related to the amino acid residues in the vicinity of the phosphate groups of the nucleotides bound to the active site. Reliable characterization of these binding sites may throw further light on the issue.

Experiments with the quaternary complex E·MnGDP·AMP demonstrated a feature that is expected to recur when this method is used on other complexes with observed nuclei at different distances from the cation, viz., simultaneous occurrence of exchange limitation for some relaxation rates and partial exchange limitation with some structure dependence for the others. Careful examination of both the frequency dependence and the activation energy[3] is needed in such cases along with the use of a second, weaker paramagnetic cation to avoid mistakenly considering such relaxation rates to be totally structure dependent and free of contribution from τ_M.

[3] The temperature dependence of T_{1P} arises from that of both T_{1M} and τ_M. Assuming each of these shows an Arrhenius behavior, the composite does not strictly show a similar variation. However, because of the limited temperature range used in these experiments, the variation of T_{1P} with temperature can be approximated by an Arrhenius plot and an effective activation energy may be obtained. The frequency dependence of such an effective activation energy reflects the magnitude of T_{1M} at different frequencies relative to τ_M, which is frequency independent.

[4] There is some debate regarding ATP binding also at the AMP site on adenylate kinase (Shyy et al., 1987). It is however, generally accepted that such an auxiliary binding is substantially weaker in affinity than to the ATP site and occurs exclusively with ATP uncomplexed with the cation. Thus, regardless of how the quantitative aspects of this question are resolved, it does not affect the results presented here.

Thus, these results shed further light on factors to be recognized in using this method for measuring cation to nucleus distances in the range of ~5–7 Å either on the substrate to which the cation is chelated or on substrates (or inhibitors) present at the active site that do not bind the cation to a significant extent (either on or off the enzyme). Mn(II)-induced relaxation rates for distances in the intermediate range of ~5–7 Å are likely to include contributions from both τ_M and T_{1M}. The protocol of making measurements exclusively on substrates in enzyme–bound form, and of measuring activation energies and frequency dependencies for all complexes, appears to be essential for the delineation of the role of exchange and for the determination of structure from the measured relaxation rates. In spite of making all the aforementioned measurements, it was not possible to precisely evaluate the correlation time τ_{C1} and determine the Mn(II)–P(AMP) distance in E·MnGDP·AMP due to the fact that, for the ^{31}P data, the pT_{1P} vs ω_I^2 plot has an intercept of which over 90% is contributed by τ_M. Thus slight changes in τ_M well within experimental uncertainties cause large changes in τ_{C1}. It may be possible to overcome this difficulty by obtaining data on nuclei in the same complex with longer relaxation times, in which case values of τ_M, τ_C, and r[Mn(II)–P(AMP)] may all be evaluated with required precision. Such experiments are being planned with 2-^{13}C-labeled nucleotides. If successful, the Mn(II) data from these measurements can then be used to "calibrate" the spectral density for the Co(II) complexes. For the time being the data in Mn(II) and Co(II) complexes were used to choose a τ_{C1} of 10.4 ns and r[(Mn(II)–P(AMP)] of 5.9 Å. This distance corresponds to the center of the range suggested by the Co(II) data. The distance *appears* to be in very good agreement with the result of 5.9 ± 0.3 Å obtained by Fry et al. (1987) for the inhibitor complex E·CrAMPPCP·AMP. However, the agreement must be regarded as a coincidence; the equality of the distances may actually imply an inconsistency in the structure of the two complexes. The cation Cr(III) used in the work of Fry et al. (1987) is present in a bidentate complex of an ATP analogue whereas Mn(II) or Co(II) in the present work is in a monodentate complex of an ADP analogue. The distances of P(AMP) from the cation in the two cases are not expected to be the same.

The methodology for interpreting paramagnetic effects on nuclear spin relaxation is not yet sufficiently well developed to permit proper analysis of relaxation data from enzyme-bound reaction complexes. Therefore, since adenylate kinase catalyzes a phosphoryl transfer from ADP to ADP, it was not possible to measure distances in E·ADP complexes. This would require accounting for the additional exchange effects that arise from the interconversion of enzyme-bound reactants and products as well as from the interchange of donor and acceptor ADP molecules on this enzyme (Vasavada et al., 1984). Attempts to develop methodology to interpret such data are in progress.

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Hydration of CO_2 by Carbonic Anhydrase: Intramolecular Proton Transfer between Zn^{2+}-Bound H_2O and Histidine 64 in Human Carbonic Anhydrase II†

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ABSTRACT: The energy barrier for the intramolecular proton transfer between zinc-bound water and His 64 in the active site of human carbonic anhydrase II (HCA II) has been studied at the partial retention of diatomic differential overlap (PRDDO) level. The most important stabilizing factor for the intramolecular proton transfer is the zinc ion, which lowers the pK_a of zinc-bound water and electrostatically repels the proton. The energy barrier of 127.5 kcal/mol for proton transfer between a water dimer is completely removed in the presence of the zinc ion. The zinc ligands, which donate electrons to the zinc ion, raise the barrier slightly to 34 kcal/mol for a 4-coordinated zinc complex including three imidazole ligands from His 94, His 96, and His 119 and to 54 kcal/mol for the 5-coordinated zinc complex including the fifth water ligand. A few model calculations indicate that these energy barriers are expected to be reduced to within experimental range (~10 kcal/mol) when large basis set, correlation energies, and molecular dynamics are considered. The proton-transfer group, which functions as proton receiver in the intramolecular proton transfer, helps to attract the proton; and the partially ordered active site water molecules are important for proton relay function.

Carbonic anhydrase is a zinc metalloenzyme that catalyzes the reversible hydration of CO_2 to bicarbonate ion and a proton. In human carbonic anhydrase II (HCA II) the maximal turnover number is 10^6 s^{-1} at 25 °C. It is now widely accepted that the initial nucleophilic attack occurs by a Zn^{2+}-bound hydroxide ion and that the subsequent proton transfer are catalyzed by a non-Zn^{2+}-liganded histidine and by buffer in HCA II (Lindskog, 1983; Lindskog et al., 1984; Lipscomb, 1983; Pocker & Sarkanen, 1978; Prince, 1979; Coleman, 1980). A plausible sequence (Figure 1) for the hydration reaction consists of (1) binding of CO_2 near Zn^{2+}; (2) conversion of CO_2 to HCO_3^- by nucleophilic attack of Zn^{2+}-bound OH^- on C of CO_2; (3) internal proton transfer within Zn^{2+}-bound HCO_3^-; (4) binding of H_2O to Zn^{2+} and ionization of this Zn^{2+}-bound H_2O (probably assisted by the negatively charged Glu 106-Thr 199 proton network) to facilitate release of HCO_3^-; and (5) the coordinated transfer of H^+ from Zn^{2+}-bound H_2O to a proton-transfer group (His 64 in HCA II), then to buffer, and finally to solvent. In this mechanism, the mechanistic role of a 5-coordinated Zn^{2+} species is unclear, although such intermediates could be accommodated in several steps depicted in Figure 1. In step 2, for instance, one oxygen of CO_2 could bind to Zn^{2+} at the fifth coordination site. Also, in step 4, both an OH and terminal O of HCO_3^- could bind to Zn^{2+} before the product release.

It is known that the active site of human carbonic anhydrase II is a conical cavity, which is about 15 Å wide at the entrance and 16 Å deep reaching toward the center of the enzyme (Eriksson et al., 1986). The zinc ion is located near the apex, and His 64, the proton-transfer group in HCA II, is about 7 Å away from Zn^{2+}. Active site water molecules between the zinc ion and His 64 (Figure 2) are partially ordered (Eriksson et al., 1986). In the X-ray structure, these water molecules are not in contact with solvent further out in the cavity. His 64 bridges these two solvent areas (Eriksson et al., 1986). Thus, for the deprotonation step (step 5, Figure 1) of the enzyme-catalyzed CO_2 hydration, two independent proton transfers are required: (1) an intramolecular proton transfer from the zinc-bound H_2O to His 64 and (2) a proton transfer from the protonated His 64 to buffer and then to solvent. The buffer-assisted proton transfer is the rate-limiting step at low buffer concentrations (Jönsson et al., 1976), while the intramolecular proton transfer is rate limiting at high buffer concentrations (Steiner et al., 1975; Pocker & Bjorkquist, 1977). It is possible that the intramolecular proton transfer may involve more than two protons: the deuterium isotope effects

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