Analysis of ^{31}P NMR Spectra of Enzyme-Bound Reactants and Products of Adenylate Kinase Using Density Matrix Theory of Chemical Exchange†

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ABSTRACT: ^{31}P NMR spectra of equilibrium mixtures of enzyme-bound reactants and products of the adenylate kinase reaction

$$ATP + AMP \overset{Mg^{2+}}{\rightleftharpoons} 2ADP$$

were analyzed by using computer simulations based on density matrix theory of chemical exchange. Since adenylate kinase has the unique feature that the reactants in the reverse direction are both ADP molecules, which are indistinguishable off the enzyme, the density matrix equations are formulated for the ABC + D ⇄ A′B′ + A″B″ exchange appropriate for the reaction, in which the interchange of A′B′ and A″B″ is explicitly introduced. It is shown that the consideration of this interchange is essential to explain the experimentally observed line shapes. By comparison of the computer-simulated spectra with various values for the rates of the exchange with the experimental spectra for porcine adenylate kinase at pH 7.0 and $T = 4$ °C, the following characteristic rates were determined: interconversion rates, 375 ± 30 s^{-1} (ATP formation) and 600 ± 50 s^{-1} (ADP formation); interchange rates of donor and acceptor ADP's, 100 ± 30 s^{-1} (in the presence of optimal Mg^{2+} concentration), 1500 ± 100 s^{-1} (in the absence of Mg^{2+}). It is shown that under the conditions of the experiments the interchange rate is the lower limit of the dissociation rate of ADP (or MgADP from the acceptor site if Mg^{2+} was present) from the enzyme complexes. The significance of these interchange rates and their values relative to the interconversion rates is discussed with special reference to the role of the Mg^{2+} ion in the differentiation of the two nucleotide binding sites on adenylate kinase.

Phosphorus-31 NMR[1] is a convenient method for the investigation of enzyme–substrate interactions of phosphoryl transfer enzymes since the spectrum consists exclusively of signals that may be readily assigned to the phosphate groups in the substrates and products of the enzymatic reaction. A number of enzymes of the kinase classification have recently been studied, by ^{31}P NMR, with the use of enzyme concentrations in sufficient excess of those of the substrates so that the substrates are observed in their enzyme-bound form (Nageswara Rao, 1979; Cohn & Nageswara Rao, 1979; Nageswara Rao & Cohn, 1977a,b, 1981; Nageswara Rao et al., 1976, 1978a,b, 1979). By designing the experiments such that the reactants and products are all in the enzyme-bound form and all components of the reaction are present in the sample, it is possible to monitor exclusively the interconversion step of the reaction. The ^{31}P NMR spectrum of such an equilibrium mixture (enzyme bound) exhibits a variety of line-shape features arising from chemical exchange due to the fact that as the reactants and products interconvert the NMR spectral parameters (chemical shifts and spin–spin coupling constants) of the different ^{31}P nuclei change accordingly. The line shapes thus contain information about the kinetics associated with the interconversion step. Accurate analysis of these line shapes is, however, complicated by the multiplet structure of the ^{31}P resonances arising from indirect spin–spin coupling between neighboring ^{31}P nuclei in the phosphate chain. Furthermore, in the presence of spin–spin coupling the density matrix theory of chemical exchange in NMR (Kaplan & Fraenkel, 1980) should be used for a proper analysis. Recently, Vasavada et al. (1980) gave a computational basis for the calculation of the ^{31}P line shapes in an ABC ⇄ A′B′ + C′ type of chemical exchange on the basis of the density matrix theory, which is valid for arbitrary values of the strength of spin–spin coupling and of the rates of exchange. This procedure is directly applicable to phosphoryl transfer enzyme reactions in which the second substrate, which reacts with ATP and gets phosphorylated, does not contain a ^{31}P nucleus. In this paper, a density matrix analysis of the ^{31}P NMR line shapes in the enzyme-bound equilibrium mixtures of the porcine adenylate kinase reaction

$$ATP + AMP \overset{Mg^{2+}}{\rightleftharpoons} 2ADP \qquad (1)$$

is presented. Mg^{2+} is an obligatory component of the reaction. The enzyme is ubiquitous and plentiful in tissues turning over energy from adenine nucleotides and is essential for the synthesis of adenine nucleotides beyond the monophosphate level (Noda, 1962, 1973). Kinetic studies indicate a random Bi-Bi mechanism for the catalysis by this enzyme (Rhoads & Lowenstein, 1968). Among the kinases, adenylate kinase is unique in that it catalyzes, in the reverse reaction, phosphoryl transfer from one ADP as a donor to another ADP as an acceptor. The formulation of the basic equations for the density matrix theory not only requires them to be extended to the case where all four substrates contain ^{31}P nuclei (this extension is straightforward) but must also take explicit cognizance of the fact that the two substrate molecules in the reverse reaction are identical with each other, which injects an interesting new feature in the analysis.

^{31}P NMR spectra of the reactants and products and of the equilibrium mixture of the adenylate kinase reaction, all in the enzyme-bound form, have been studied in some detail (Nageswara Rao et al., 1978a). These experiments reveal an unmistakable differentiation of the two nucleotide binding sites on the enzyme; the ^{31}P chemical shifts of donor and acceptor ADP molecules are significantly different in the enzyme-bound complexes. Furthermore, the experiments provide evidence

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NMR, nuclear magnetic resonance.

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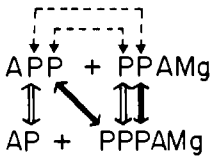


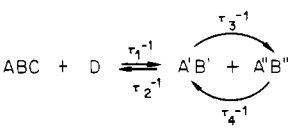
FIGURE 1: ^{31}P exchanges in an equilibrium mixture of reactants and products bound to adenylate kinase. The solid and open arrows represent fast and slow (NMR) exchanges, respectively. The dashed lines represent the interchange of the ADP's between the donor and acceptor sites.

that the obligatory cation, Mg^{2+}, plays a distinct role in affecting such a differentiation in that while both Mg-bound and free nucleotides bind at the acceptor ADP (or ATP) site, only free nucleotides bind at the donor ADP (or AMP) site. In the interpretation of the ^{31}P NMR spectra the qualitative features of the line shapes exhibited by the resonances were used to reach the conclusions summarized above, but no quantitative analysis could be performed because the requisite theoretical procedures were not readily available. In addition, since the interconversion rates of enzyme-bound reactants and products are rather large (400–600 s^{-1}), the ^{31}P spectra of the equilibrium mixtures were too complex (or deceptively simple) even for a simple-minded quantitative analysis. The interconversion rates were, therefore, estimated from spectra in which less than optimal Mg^{2+} concentration was used, thereby slowing down the apparent rates of interconversion. Lastly, the reaction complexes for the reverse reaction of adenylate kinase are affected by one more chemical exchange process arising from the fact that the acceptor and donor ADP molecules are indistinguishable off the enzyme and can, therefore, interchange their sites (through the intermediary of the solution). Throughout this paper, this particular exchange is referred to as the "interchange process" to avoid confusion with the other chemical exchanges in the sample. The theoretical analysis presented below consists of (i) formulating the density matrix equations appropriate for the adenylate kinase reaction in which the interchange of donor and acceptor ADP molecules is explicitly considered and (ii) computer calculation of the line shapes with different exchange parameters and comparison with experimental line shapes obtained under different conditions to determine the exchange parameters. The determination of the various exchange rates provides a quantitative basis for the scrutiny of qualitative interpretations (made earlier) regarding the differentiation of the nucleotide binding sites and the role of Mg^{2+} ion therein. Of particular interest is the effect of the possible interchange of the donor and acceptor ADP molecules (in the reverse reaction) on the interconversion step of the adenylate kinase reaction.

The experimental spectra included in this paper for the purpose of comparison with the computer calculations are from previously published work (Nageswara Rao et al., 1978a). While some of the sample conditions and NMR parameters used are given in the figure legends here, the original paper should be consulted for further details about the experiments.

Computations of Line Shapes

The ^{31}P exchanges involved in the adenylate kinase reaction are depicted in Figure 1. These exchanges may be formally represented as



(2)

Table I: ^{31}P Chemical Shifts (δ) in Parts per Million (ppm) from 85% H_3PO_4 and Spin-Spin Coupling Constants (J) in Hertz and Line Widths $(\pi T_2)^{-1}$ in Hertz of Reactants and Products Bound to Porcine Adenylate Kinase (at pH 7.0 and $T = 4$ °C) Used in the Calculation of Spectra[a]

substrate	δ (ppm) α-P	β-P	γ-P	J (Hz)[b] $J_{\alpha\beta}$	$J_{\beta\gamma}$
	No Added Mg^{2+} (Figure 6A)				
ATP	11.1 (10)	21.6 (15)	7.0 (10)	17.3	21.0
ADP (donor)	11.0 (10)	7.5 (10)		22.0	
ADP (acceptor)	9.4 (10)	3.5 (10)		22.0	
AMP	-3.9 (12)				
	Optimal Mg^{2+} (Figure 2B)				
ATP	11.1 (10)	17.7 (15)	6.4 (10)	15.1	14.7
ADP (donor)	11.0 (10)	7.0 (10)		22.0	
ADP (acceptor)	9.6 (10)	3.5 (10)		18.2	

[a] Lind widths are given in parentheses. Upfield shifts are positive. [b] $J_{\alpha\beta}$ for ADP cannot be measured in the enzyme-bound complexes due to interchange so values in free ADP and MgADP were used.

where A, B, and C represent respectively the ^{31}P nuclei in α-P, β-P, and γ-P of ATP, D represents that in AMP, A′B′ represents those in α-P and β-P, respectively, of acceptor ADP, and A″B″ represents the corresponding nuclei in donor ADP. τ_1^{-1} and τ_2^{-1} are the interconversion rates of the reaction (on the surface of the enzyme), and τ_3^{-1} and τ_4^{-1} are the rates of interchange of the donor and acceptor ADP molecules between their respective binding sites on the enzyme. The basic equations required for the calculation of line shapes under the exchanges represented by eq 2 are summarized in the Appendix. The procedure requires a point by point calculation of spectral intensities covering the width of the observed spectrum for a set of chosen values of τ_1, τ_2, τ_3, and τ_4. τ_1 and τ_2 are related by the equilibrium constant of enzyme-bound reactants and products by

$$\tau_1^{-1}/\tau_2^{-1} = [\text{E·MgADP·ADP}]/[\text{E·MgATP·AMP}] = K'_{eq} \quad (3)$$

K'_{eq} was estimated to be 1.6 for the porcine adenylate kinase (Nageswara Rao et al., 1978a). The interchange rates τ_3^{-1} and τ_4^{-1} are set equal to each other; this may be seen to be reasonable on the basis of the interpretation of these rates (see later). Thus there are two independent rates, τ_1^{-1} and τ_3^{-1}, that need to be chosen for the calculation of the line shape. The remaining parameters required for the computation are the NMR spectral parameters (chemical shifts, spin–spin coupling constant, and line widths) of the various enzyme-bound nucleotide complexes (in the absence of the exchange). The values of these parameters used in the calculation are summarized in Table I.[2]

Figure 2 shows the experimental ^{31}P NMR spectrum of an equilibrium mixture of enzyme-bound substrates and products of porcine and carp adenylate kinase at pH 7.0 and 4 °C along with the control spectrum obtained for a catalytic concentration of the enzyme [from Nageswara Rao et al. (1978a)]. [The spectrum for the carp enzyme (Figure 2C) is shown for

[2] A few of the chemical shifts given in Table I differ slightly (<0.3 ppm) from the chemical shifts measured for individual substrates bound to the enzyme (Nageswara Rao et al., 1978a). (Note that the spectral parameters of the reaction complex itself cannot be experimentally measured in the *absence* of exchange.) These changes were made for obtaining the best fit with experiment. In a sense, this is a reasonable method to *determine* the spectral parameters of the reaction complex, which are rendered difficult to measure by the line-shape features arising from the exchanges (Vasavada et al., 1980).

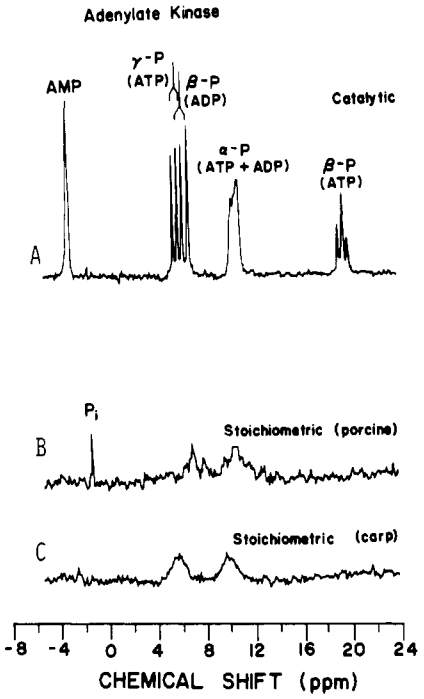


FIGURE 2: ^{31}P NMR spectra (at 40.3 MHz) of equilibrium mixtures of porcine and carp adenylate kinase at pH 7.0 and T = 4 °C. (A) Equilibrium mixture with catalytic enzyme concentration. Intial concentrations were as follows: ATP, 15 mM; $MgCl_2$, 15 mM; AMP, 17.3 mM; porcine enzyme, ~5 μM; K-Hepes, 100 mM. (B) Equilibrium mixture with enzyme concentration > substrate. Initial concentrations were as follows: ATP, 2.9 mM; AMP, 2.6 mM; $MgCl_2$, 2.9 mM; porcine enzyme, 3.5 mM; K-Hepes, 145 mM. The P_i signal arises from hydrolysis of ATP during the accumulation of the NMR data. (C) Same as (B) except reaction initiated from reverse direction. Concentrations were as follows: ADP, 2.1 mM; $Mg(CH_3COO)_2$, 1.15 mM; carp enzyme, 1.2 mM; K-Hepes, 100 mM. NMR parameters were as follows: band width 2 kHz; (A) 650 scans, memory size 16K, line broadenine 0.8 Hz, and pulse repetition rate 90.0 s; (B) 4000 scans, memory size 16K, line broadening 3.8 Hz, and pulse repetition rate 4.5 s; (C) 5200 scans, memory size 8K, line broadening 1.5 Hz, and pulse repetition rate 6.0 s.

the purpose of general line-shape comparison and is not used for actual analysis since the spectral parameters are not all determined for this enzyme.] The P_i resonance at −2 ppm in Figure 2B arises from hydrolysis of ATP. The spectrum is deceptively simple in that there are only two rather broad features at ~7 and ~10 ppm while the chemical shifts in the absence of the reaction span −4 to 20 ppm (see Figure 1A and Table I). Although the signal to noise ratio of the spectrum is not very good, this spectrum will have to be used for the present analysis since accumulation of the data for longer periods of time to improve the spectrum leads to rather dramatic changes in the general features of the spectrum arising from the coupling of the irreversible hydrolysis with the adenylate kinase reaction. [These changes have enabled the differentiation of the β-P resonances of the donor and acceptor ADP molecules bound to the enzyme (Nageswara Rao et al., 1978a).] The spectrum for the carp enzyme shows the line-shape features at ~7 and ~10 ppm in the equilibrium mixture. The hydrolysis of ATP does not occur for the carp enzyme to the same extent as for the porcine enzyme, hence, the better signal to noise ratio of Figure 2C.

The first step in computer calculation of the line shapes is to determine if the interchange process of the donor and acceptor ADP's needs to be considered for the analysis. Theoretical line shapes for the spectrum of the equilibrium mixture are thus simulated by setting $\tau_3^{-1} = 0$ and varying τ_1^{-1} from 0 (no reaction) to 700 s^{-1} in steps of 100 s^{-1} and are shown

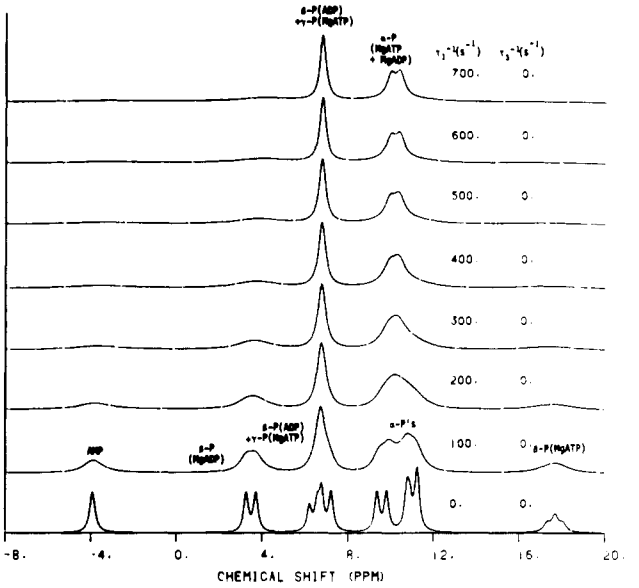


FIGURE 3: Computer-simulated ^{31}P NMR spectra for the enzyme-bound equilibrium mixture of the adenylate kinase reaction for different values of τ_1^{-1} and $\tau_3^{-1} = 0$. The other parameters used are listed in Table I.

in Figure 3. The calculated spectra for $\tau_1^{-1} > 400$ s^{-1} provide the clue that the absence of observable resonance intensities in the regions other than ~7 and ~10 ppm requires interconversion rates to be rather large. For these rates, the resonances participating in the exchanges AMP ⇄ α-P(ADP) and β-P(MgADP) ⇄ β-P(MgATP) are still in slow exchange; i.e., the exchange rate is small compared to the chemical shift difference, and thus these four resonances acquire line broadening comparable to the exchange rate (without appreciable shift in their line positions) and are therefore too broad to be observed. On the other hand, for the same exchange rate, the resonances participating in the two remaining exchanges α-P(MgADP) ⇄ α-P(MgATP) and β-P(ADP) ⇄ γ-P(MgATP) are in intermediate or fast exchange, where the exchange rate is comparable or large compared to the corresponding chemical shift difference, hence, leading to partial or full coalescence of the resonances involved in each exchange and thus leading to the observed signals at ~7 and ~10 ppm. However, none of the simulated spectra in Figure 2 agree with the observed spectrum in the details of the line shapes at ~7 and ~10 ppm.

The fact that spectra calculated by setting $\tau_3^{-1} = 0$, i.e., by ignoring the possible interchange of donor and acceptor ADP's, do not agree with the experiment raises the question whether the introduction of the interchange in the computer simulations might produce agreement with the observed spectrum. Figure 4 presents computer-simulated spectra in which τ_1^{-1} was kept fixed at 600 s^{-1} and τ_3^{-1} was varied from 0 to 300 s^{-1} in steps of 50 s^{-1}. The introduction of τ_3^{-1} produces appropriate changes in the line shape and a good agreement with experiment for $\tau_3^{-1} = 100$ s^{-1} (and $\tau_1^{-1} = 600$ s^{-1}). This result was the first clear evidence that the rate of interchange of donor and acceptor molecules influences the observed spectrum.

In order to obtain the values of τ_1^{-1} and τ_3^{-1} that agree best with the experiment and determine the extent of interplay between these exchanges as depicted in the calculated line shapes, the spectra were also computed keeping τ_3^{-1} fixed at 100 s^{-1} and varying τ_1^{-1} from 200 to 1000 s^{-1}. These results are shown in Figure 5. These and several other simulations using different choices of the values of τ_1^{-1} and τ_3^{-1} clearly show that the best agreement with the experimental spectrum obtains for $\tau_1^{-1} = 600$ s^{-1} and $\tau_3^{-1} = 100$ s^{-1}. While it is

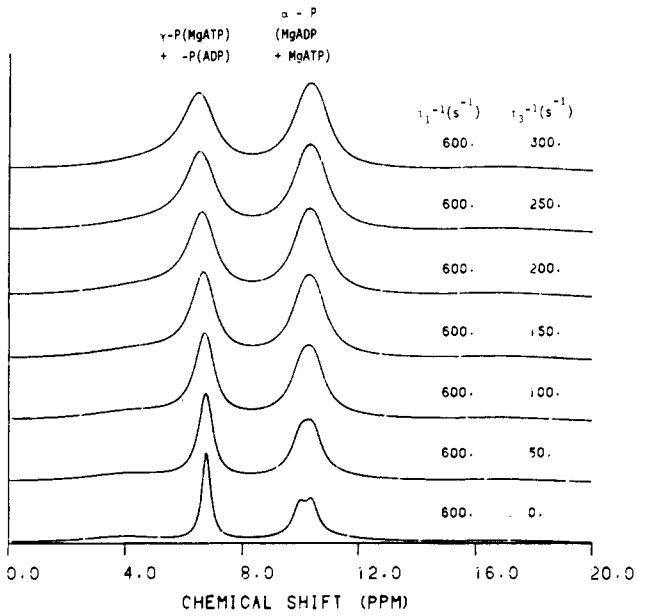


FIGURE 4: Computer-simulated ^{31}P NMR spectra for the enzyme-bound equilibrium mixture of the adenylate kinase reaction for τ_1^{-1} = 600 s^{-1} and for different values of τ_3^{-1}. The other parameters are listed in Table I.

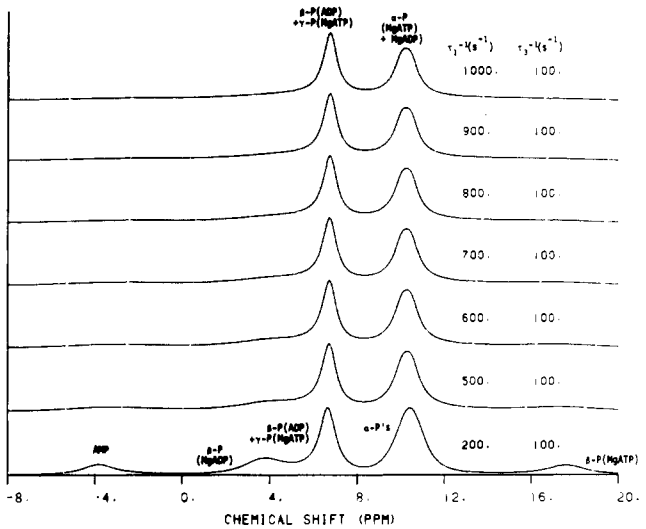


FIGURE 5: Computer-simulated ^{31}P NMR spectra for the enzyme-bound equilibrium mixture of the adenylate kinase reaction for τ_3^{-1} = 100 s^{-1} and for different values of τ_1^{-1}. The other parameters are listed in Table I.

difficult to determine a unique measure of the errors in these quantities, on the basis of discernible deviations between the calculated and experimental spectra, a range of ±50 s^{-1} for τ_1^{-1} and ±30 s^{-1} for τ_3^{-1} were estimated.

In light of the above result that the line shapes involving the enzyme-bound ADP resonances exhibit a clear dependence on the rate of interchange of ADP molecules between the donor and the acceptor sites, it will be useful to determine the interchange rate (τ_3^{-1}) in the absence of Mg^{2+} in order to examine the role of Mg^{2+} in affecting a differentiation of the two bound ADP's [suggested earlier by Nageswara Rao et al. (1978a)]. The experimental ^{31}P NMR spectrum of enzyme-bound reactants and products of porcine adenylate kinase in the absence of externally added Mg^{2+} is shown in Figure 6A [from Nageswara Rao et al. (1978a)]. The equilibrium of the reaction was established due to adventitious activating metal ions present in the sample. It is clear that the ADP resonance in Figure 6A acquired a line shape arising from the interchange

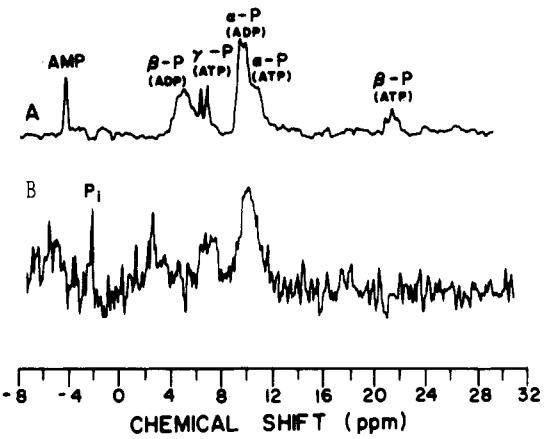


FIGURE 6: ^{31}P NMR spectra (at 40.3 MHz) of equilibrium mixtures of porcine adenylate kinase at T = 4 °C obtained at different Mg^{2+} concentrations: (A) pH 7.0; (B) pH 8.0. (A) No added Mg^{2+}. Equilibrium was established because of minute quantities of activating divalent cations present in the sample as contaminants. Concentrations were as follows: enzyme, 3.2 mM; ADP, 5.8 mM. (B) [Mg]/[ADP] = 2.19. Concentrations were as follows: enzyme, 3.9 mM; ADP, 6.9 mM; $Mg(CH_3COO)_2$, 15.1 mM. NMR parameters were as follows: (A) 5000 scans, band width 2 kHz, memory size 8K, line broadening 3.8 Hz, and pulse repetition rate 2.5 s; (B) same as (A) except number of scans 2000.

of the two ADP molecules. This spectrum may be calculated on the basis of the chemical shifts of the various nuclei in the enzyme-bound species in the absence of Mg^{2+} by setting τ_1^{-1} = 0 and simply varying τ_3^{-1}. (The feeble interconversion rate that was responsible for the establishment of the equilibrium in Figure 6A is <5 s^{-1} and is essentially included in the line widths of the various resonances in the spectrum.) The results of computer simulations obtained by varying τ_3^{-1} (with τ_1^{-1} = 0) are shown in Figure 7. Comparison of the spectra in Figure 7 with the observed spectrum in Figure 6A shows that the appropriate value of τ_3^{-1} = 1500 ± 100 s^{-1}. Thus in the absence of Mg^{2+}, the interchange of the ADP's bound at the donor and acceptor sites of adenylate kinase proceeds at a rate that is more than one order of magnitude faster than that in the presence of Mg^{2+}.

Discussion

The density matrix theory of chemical exchange of coupled spin systems provides a convenient framework to analyze the manner in which the simultaneous presence of two different categories of exchange such as the interconversion of enzyme-bound reactants and products and the interchange of the donor and acceptor ADP molecules bound to adenylate kinase influence the observed spectrum.[3] Computer simulation of the ^{31}P NMR spectra on the basis of the theory, given in the Appendix, allowed the examination of the two processes prevalent in the adenylate kinase reaction. The simulated spectra clearly show that in order to account for the experimentally observed line shapes, the rate of interchange of the two ADP's must be explicitly included in the calculation. The characteristic exchange rates for the porcine adenylate kinase reaction at pH 7.0 and T = 4 °C, determined on the criterion of the best agreement between calculated and experimental

[3] In the presence of catalytic enzyme concentrations at which the exchanges do not dramatically alter the NMR line shapes, the exchange processes could be elegantly studied by two-dimensional NMR techniques (Balaban & Ferretti, 1983). Such experiments, however, do not provide information on the exchanges involving the enzyme-bound complexes. Two-dimensional NMR experiments on enzyme-bound equilibrium mixtures are rather difficult to perform on enzyme systems like adenylate kinase.

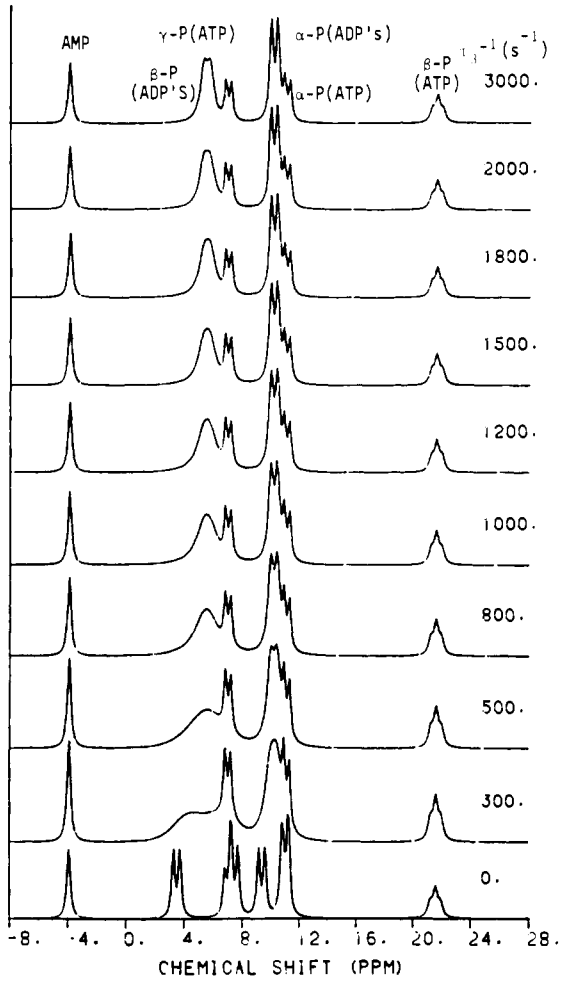


FIGURE 7: Computer-simulated ^{31}P NMR spectra of enzyme-bound reactants and products of adenylate kinase in the absence of Mg^{2+}. (Compare with Figure 6A for different value of τ_3^{-1} with $\tau_1^{-1} = 0$.) The other parameters are listed in Table I.

spectra, are as follows.[4] For interconversion rates (at equilibrium), τ_2^{-1}(ATP formation) = 375 ± 30 s^{-1} and τ_1^{-1}(ADP formation) = 600 ± 50 s^{-1}. For interchange rates (τ_3^{-1}) of donor and acceptor ADP molecules, the values are 100 ± 30 s^{-1} (in the presence of optimal Mg^{2+} concentration) and 1500 ± 100 s^{-1} (in the absence of Mg^{2+}). The interconversion rates estimated previously (420 s^{-1} and 690 s^{-1} for ATP and ADP formation, respectively) on the basis of line widths of ^{31}P resonances in the presence of suboptimal Mg^{2+} concentrations agree reasonably well with the values given above. Considering the approximations involved in such a simplified estimate, the agreement may well be fortuitous. However, it may be noted that computer-simulation procedures yield exchange rates smaller than those obtained by linewidth measurements—and the same trend occurred also in the case of arginine kinase (Vasavada et al., 1980). The consistently lower exchange rates obtained by computer simulation relative to those obtained on the basis of line-width changes may be ascribed to the fact that the latter method implicitly ignores the contribution to the observed line width arising from the residual spin–spin splitting and, therefore, overestimates the exchange rate. The interconversion rates determined here are still much larger than the overall rates of the porcine adenylate kinase reaction, 80 s^{-1} and 43 s^{-1} (Nageswara Rao et al., 1978a), for ATP and ADP formation, respectively. Thus the conclusion that the interconversion step is not rate limiting for the overall reaction, previously made, remains unaltered.

Interchange Rates. Clearly an important consequence of the computer-simulated line-shape analysis based on the density matrix theory is the determination of the rates of interchange of ADP molecules between the donor and acceptor sites on the enzyme through the intermediary of the solution. This type of interchange occurs, in general, whenever there are multiple sites available on the enzyme for a particular molecule. However, in the case of ADP binding adenylate kinase, these are the two sites of the reacting substrates for the reverse reaction. An interchange of these two molecules influences the kinetics of the catalytic processes in the sense that it introduces additional (catalytically unproductive) steps in the reverse reaction. Thus the drastic reduction of the interchange rate from 1500 s^{-1} in the absence of Mg^{2+} to 100 s^{-1} in the presence of Mg^{2+}, i.e., in the reaction complex, appears appropriate in view of the fact that the rate of reaction between the two ADP's on the enzyme (i.e., rate of ATP formation) is 375 s^{-1}. If the rapid rate of interchange (1500 s^{-1}) prevalent in the absence of Mg^{2+} were to persist in the reaction complex, the catalysis in the reverse direction must be hampered, and as a consequence, the equilibrium of the reaction $E{\cdot}S_1{\cdot}S_2 \rightleftarrows E{\cdot}P_1{\cdot}P_2$ would be affected. However, by the attenuation of the interchange in the presence of Mg^{2+} down to 100 s^{-1}, a value sufficiently smaller than the reaction rate of the enzyme-bound ADP's (375 s^{-1}), the adverse influence of the interchange on the rate of ATP formation is made negligible.

The fact that the rate of interchange of the donor and acceptor ADP's is attenuated in the presence of Mg^{2+} compared to that in the absence of Mg^{2+} was earlier inferred qualitatively on the basis of the general features of the ^{31}P NMR spectra (Nageswara Rao et al., 1978a) of the enzyme-bound equilibrium mixtures of porcine adenylate kinase. A similar inference was also made from the ^{31}P NMR spectra of diadenosine pentaphosphate (Ap_5A), a potent inhibitor of the enzyme, bound to adenylate kinase (Nageswara Rao & Cohn, 1977b). These results, coupled with the previous knowledge that the optimum Mg^{2+} concentration corresponds to one Mg^{2+} ion per reaction complex, were used to conclude that the metal–nucleotides bind adenylate kinase at the acceptor ADP (or ATP) site with significantly higher affinity than at the other nucleotide site. The computer simulations are consistent with these interpretations. Furthermore, the role of Mg^{2+} in the differentiation of the two nucleotide binding sites is brought into sharp focus in that the rate of interchange of the two ADP's attenuated from a value much larger (in the absence of Mg^{2+}) to a value sufficiently smaller (in the presence of Mg^{2+}) than the (eventual) interconversion rate in the reverse direction such that the catalytic rate is not severely affected by the interchange.

Although the interchange rate, as it is defined thus far, appears to be the parameter to be chosen naturally for the description of the effect of the interchange on NMR line shapes, it should be noted that this rate per se does not enter explicitly in a kinetic scheme for an enzymatic reaction between two substrates that are indistinguishable off the enzyme. The interchange process is itself a composite of several kinetic steps involving the dissociation of the ADP molecules bound at the donor and acceptor sites on the enzyme from their enzyme complexes and their subsequent reassociation with the enzyme in a manner that their binding sites are switched.

[4] The accuracy in the determination of these rates could be further improved by obtaining data at more than one value of the spectrometer operating frequency (i.e., for different values of chemical shifts between exchanging species).

Therefore, in order to appreciate the significance of the dynamical rate determined by analyzing the NMR line shapes, it is useful to consider its relationship to the rates of the kinetic steps involved in the interchange. To obtain such a relationship, on the basis of kinetic equations, should be a formidable task.[5] However, for the particular kind of experiments considered here, in which the interchanging molecules are observed in their enzyme-bound complexes in the presence of enzyme concentrations in sufficient excess over those of the substrates, the following simple relationship appears reasonable although it may be somewhat qualitative. Since the interchange process in the absence of Mg^{2+} involves dissociation of the two substrates from their enzyme complexes and their subsequent reassociation, it is clear that in the presence of excess enzyme concentrations used in the experiments such that at least 90% of ADP is in the enzyme-bound species, the two rates of reassociation are going to be very fast compared to the dissociation rates. Considering first the interchange in the absence of Mg^{2+}, the rate-determining steps in the interchange are then the dissociation steps of the two substrates. The interchange rate is thus a lower limit for the slower of the dissociation rates of the two interchanging molecules from their enzyme complexes. According to this interpretation, the interchange rate is governed by the slowest dissociation rate in the process, and this should be nearly the same in both directions, thus lending justification for setting $\tau_3^{-1} = \tau_4^{-1}$ in the computer calculations (see earlier). The measured interchange rate of 1500 s^{-1} (in the absence of Mg^{2+}) for the donor and acceptor ADP's bound to porcine adenylate kinase, therefore, implies that the rate of dissociation of ADP from either site is 1500 s^{-1} or larger.

On the other hand, in the presence of Mg^{2+}, i.e., in the reaction complex, the interchange rate is reduced to 100 s^{-1}. Since the donor ADP site is specific to metal-free nucleotides, the interchange in the presence of adequate concentrations of Mg^{2+} (such that acceptor ADP is chelated by Mg^{2+} and donor ADP is not) involves dissociation of MgADP and ADP, respectively, from the acceptor and donor sites and reassociation with the sites reversed before which the Mg^{2+} ion must be exchanged between the two ADP molecules. For the adenylate kinase reaction, the optimum Mg^{2+} concentration is known to be equal to [ATP] + (1/2)[ADP] (Noda, 1973; Rhoads & Lowenstein, 1968). Under these conditions the exchange of Mg^{2+} between MgADP and ADP (free in solution) will be governed by the rate of dissociation of Mg^{2+} from MgADP. This rate was determined to be 2500 s^{-1} at 25 °C and pH 6.0 in the presence of 0.1 M KNO_3 (Eigen & Hammes, 1961; Frey & Steuhr, 1974). Even at the conditions of the ^{31}P NMR experiments (see legend of Figure 2), which were performed at 4 °C, this rate is expected to be at least 1000 s^{-1}. [At 5 °C and pH 8.0 in the presence of 100 mM Hepes, the dissociation rate for Mg^{2+} from MgATP, which is known to be slower than that from MgADP, is determined to be 1000 s^{-1} from NMR line-shape analysis (K. V. Vasavada, B. D. Ray, and B. D. Nageswara Rao, unpublished experiments).] Thus the interchange rate of 100 s^{-1} in the presence of Mg^{2+} may be understood in two alternative ways. (1) Addition of Mg^{2+} alters the dissociation rates of ADP and MgADP so that the interchange is governed by the slower of the two dissociation rates. If the addition is assumed to primarily alter the kinetic constants of the site that binds metal–nucleotides (acceptor ADP site), it leads to the conclusion that the rate of dissociation of MgADP from porcine adenylate kinase is about 100 s^{-1} under the conditions of the experiment. (2) Alternatively,[6] if it is assumed that the dissociation rates of the two ADP's are not much altered by the addition of Mg^{2+}, these rates ($\sim$1500 s^{-1}) remain fast compared to the interchange rate. Under these conditions the observed interchange rate will be a weighted average of the rates on the enzyme (which may be considered to be zero) and in solution. Thus the observed interchange rate may be explained by postulating that (a) the dissociation rates of donor and acceptor ADP's from the enzyme are much faster than the interchange rate (b) about 10% of ADP is free in solution at any time. [Estimates of the free ADP in the sample (used in Figure 2) yield 5–10%.] Although the fact that the binding of ATP and ADP to kinases becomes stronger in the presence of Mg^{2+} suggests that the first of the two alternatives above is the more likely explanation, on the basis of the quantitative information available, it is not possible to distinguish between them. Experimental data with much larger excess of enzyme concentrations (thus reducing the amount of ADP free in solution) might provide a discernment between the two explanations.

Figure 6B shows a ^{31}P NMR spectrum of enzyme-bound equilibrium mixture obtained with Mg^{2+} concentration 4-fold in excess of the optimum value given above. This spectrum reveals two features that can be attributed to the excess Mg^{2+} concentrations. First, the signals of AMP and β-P(MgADP) begin to appear at intensities larger than may be expected on the basis of the ATPase activity indicated by the P_i signal. The appearance of AMP and β-P(MgADP) signals was explained (Nageswara Rao et al., 1978a) on the basis of a reduced availability of metal-free ADP for binding at the donor sites, leading to the formation of inert complexes of the type E·MgADP·AMP. Consequently, a reduced line width is observed for these resonances. Second, it may be noted that the strong resonances at $\sim$7 and $\sim$10 ppm in Figure 6B are narrower than the corresponding resonances in Figure 2B, which were obtained with optimum Mg^{2+} concentration. This reduction in the line width can be understood on the basis of the simulated spectra of the equilibrium mixture in Figure 4. The depletion of metal-free ADP caused by the presence of excess of Mg^{2+} must also make the interchange of the two ADP's less frequent in view of the specificity of the donor site to metal-free nucleotides. Thus in the presence of excess Mg^{2+}, the interchange rate must be reduced below 100 s^{-1}, and it may be seen from Figure 4 that lower values of τ_3^{-1} lead to narrower resonances at $\sim$7 and $\sim$10 ppm. Thus a consistent picture is obtained regarding the influence of the interchange on the observed spectra and the relationship of this rate to the kinetic parameters associated with the specific experimental conditions.

The quantitative analysis of the ^{31}P NMR spectra using computer simulations based on density matrix theory presented here yields results that substantiate the qualitative interpretations of these spectra made earlier (Nageswara Rao et al., 1978a). Furthermore, the analysis allows the determination of the dynamical parameters characteristic of the reaction, viz.,

[5] A general formulation of the problem should involve rate equations governing the concentrations of all the species present in the sample, e.g., $E{\cdot}S_1$, $E{\cdot}S_2$, $E{\cdot}S_1{\cdot}S_2$, $E{\cdot}P_1$, $E{\cdot}P_2$, $E{\cdot}P_1{\cdot}P_2$, MS_1, S_1, S_2, MP_1, P_1 and P_2. A preliminary attempt at the formulation indicated that such an approach is too complicated and cumbersome for the purpose of the present analysis in which certain simplifications are possible in view of the special conditions chosen in the experiments. Nevertheless, it will be of considerable interest to investigate the general problem and obtain quantitative relationships that will allow a critical evaluation of the validity of simplified approaches that may be used for specific experiments. Such studies are contemplated.

[6] We thank the referee for pointing out this alternative explanation to us.

the interconversion rates and the interchange rate of the donor and acceptor ADP molecules between their binding sites on the enzyme, which is a rather unique feature of the adenylate kinase reaction. The determination of the interchange rate not only enhances the information previously available from the ^{31}P NMR spectra but also provides an additional parameter to examine the role of Mg^{2+} in this reaction. In addition to facilitating the catalysis of the reaction as for all kinases, Mg^{2+} is shown to play an unmistakable role in differentiating the two nucleotide binding sites of this enzyme through the specificity of the donor ADP to binding metal-free nucleotides. The interchange rates of the ADP's determined here, in the absence and in the presence of Mg^{2+}, and their values relative to the interconversion rates lend incisive supporting evidence to the role of the divalent cation in affecting the differentiation of the donor and acceptor ADP's bound to adenylate kinase.

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Appendix

The signal intensity in the NMR spectrum at the angular frequence ω is given by

$$\mathcal{J}(\omega) \propto [ABC] \sum_{ABC} [\mathrm{Im}(\chi_{ABC}(\omega))_{kk'} + [D]\mathrm{Im}(\chi_D(\omega))_{kk'}] + [A'B'] \sum_{A'B'} \mathrm{Im}(\chi_{A'B'}(\omega))_{kk'} + [A''B''] \sum_{A''B''} \mathrm{Im}(\chi_{A''B''}(\omega))_{kk'} \quad (A1)$$

where e.g., in the first term [ABC] is the concentration, $\mathrm{Im}(\chi_{ABC}(\omega))_{kk'}$ is the imaginary part of the k,k' matrix element of χ_{ABC} (as a function of ω), which is the deviation in the spin density matrix element of the ABC spin system from its equilibrium value, and the sum $\sum_{ABC}$ extends over all the allowed transitions k,k' of this spin system. The four terms in eq A1 represent the four spin systems ABC, D, A'B', and A''B'' in eq 2. The matrix elements of χ's are determined by the solution of the coupled simultaneous equations

$$[i(\omega_{kk'}(ABC) - \omega) + (T_2^{-1})_{kk'} + \tau_1^{-1}](\chi_{ABC})_{kk'} - \tau_1^{-1}(\rho^{col}_{ABC})_{kk'} = (1/8)K \sum_{i=A,B,C} (I_y(i))_{kk'} \quad (A2)$$

$$[i(\omega_{kk'}(D) - \omega) + (T_2^{-1})_{kk'} + \tau_1^{-1}](\chi_D)_{kk'} - \tau_1^{-1}(\rho^{col}_D)_{kk'} = (1/2)K(I_y)_{kk'} \quad (A3)$$

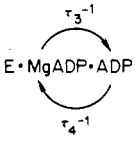
$$[i(\omega_{kk'}(A'B') - \omega) + (T_2^{-1})_{kk'} + \tau_2^{-1} + \tau_3^{-1}](\chi_{A'B'})_{kk'} - \tau_2^{-1}(\rho^{col}_{A'B'})_{kk'} - \tau_3^{-1}(\tilde{\rho}^{col}_{A'B'})_{kk'} = (1/4)K \sum_{i=A'B'} (I_y(i))_{kk'} \quad (A4)$$

$$[i(\omega_{kk'}(A''B'') - \omega) + (T_2^{-1})_{kk'} + \tau_2^{-1} + \tau_4^{-1}](\chi_{A''B''})_{kk'} - \tau_2^{-1}(\rho^{col}_{A''B''})_{kk'} - \tau_4^{-1}(\tilde{\rho}^{col}_{A''B''})_{kk'} = (1/4)K \sum_{i=A''B''} (I_y(i))_{kk'} \quad (A5)$$

In the above, $\omega_{kk'}$ and $(T_2^{-1})_{kk'}$ are the frequency and the half line width (at half-height), respectively, of the $k \leftrightarrow k'$ transition in the absence of the exchange (in rad/s) and τ_1, τ_2, τ_3, and τ_4 are the lifetimes associated with the reactions

$$\text{E·MgATP·AMP} \underset{\tau_2^{-1}}{\overset{\tau_1^{-1}}{\rightleftharpoons}} \text{E·MgADP·ADP} \quad (A6)$$

and



(A7)

K is an overall constant, which may be ignored since relative rather than absolute intensities are to be calculated. Numerical factors $^1/_8$, $^1/_2$, and $^1/_4$ on the right-hand side of eq A2–A5 equal the reciprocal of the number of spin states of the respective spin systems. $I_y(i)$ is the y component of the spin-angular momentum operator. In writing eq A2–A5, the spin systems are considered weakly coupled, i.e., the spin–spin coupling constant between any pair of nuclei is small compared to the chemical shift difference between the nuclei (see data in Table I). The matrix elements may be calculated in the spin product basis of the different spin systems. ρ^{col} is the density matrix at the instant that particular complex is formed. The notation $\tilde{\rho}^{col}$ is introduced to distinguish between the two types of reactions (A6 and A7) leading to the formation of E·A'B' or E·A''B''. These terms introduce exchange coupling between density matrix elements representing transitions of different complexes. The matrix elements of ρ^{col} and $\tilde{\rho}^{col}$ are obtained as follows:

$$\langle abc|\rho^{col}_{ABC}|a'b'c'\rangle = (1/2)\langle ab|\rho_{A'B'}|a'b'\rangle\delta_{cc'} + (1/4)\sum_d \langle dc|\rho_{A''B''}|dc'\rangle\delta_{aa'}\delta_{bb'} \quad (A8)$$

$$\langle d|\rho^{col}_D|d'\rangle = \sum_c \langle dc|\rho_{A''B''}|d'c\rangle \quad (A9)$$

$$\langle ab|\rho^{col}_{A'B'}|a'b'\rangle = \sum_c \langle abc|\rho_{ABC}|a'b'c\rangle \quad (A10)$$

$$\langle ab|\rho^{col}_{A''B''}|a'b'\rangle = (1/2)\langle a|\rho_D|a'\rangle\delta_{bb'} + (1/2)\sum_{c,d} \langle cdb|\rho_{ABC}|cdb'\rangle\delta_{aa'} \quad (A11)$$

$$\langle ab|\tilde{\rho}^{col}_{A'B'}|a'b'\rangle = \langle ab|\rho_{A''B''}|a'b'\rangle \quad (A12)$$

$$\langle ab|\tilde{\rho}^{col}_{A''B''}|a'b'\rangle = \langle ab|\rho_{A'B'}|a'b'\rangle \quad (A13)$$

The basis states in eq A8–A13 are the spin product states associated with the respective spin systems.

The complete set of equations (for a given value of ω) may be written as

$$\mathbf{A}\chi = \mathbf{B}, \text{ so that } \chi = \mathbf{A}^{-1}\mathbf{B} \quad (A14)$$

where χ and $\mathbf{B}$ are 21-dimensional column vectors and $\mathbf{A}$ is a 21×21 matrix, which is in general complex and unsymmetrical. The dimension of eq A14 equals the total number of allowed transitions for all the spin systems: 12 for ABC, 1 for D, and 4 each for A'B' and A''B'', for a total of 21. Although the matrix is large and complicated, the following schematic representation will clarify the general features:

$$\mathbf{A} = \begin{vmatrix} \text{MgATP transitions } 12 \times 12 & & (\tau_1^{-1}) & (\tau_1^{-1}) \\ & \text{AMP } 1 \times 1 & & (\tau_1^{-1}) \\ (\tau_2^{-1}) & & \text{MgADP transitions } 4 \times 4 & (\tau_3^{-1}) \\ (\tau_2^{-1}) & (\tau_2^{-1}) & (\tau_4^{-1}) & \text{ADP transitions } 4 \times 4 \end{vmatrix} \quad (A15)$$

In eq A15, terms involving the transition frequencies of the spin systems along with their line widths and lifetimes, as given by the first terms in eq A2–A5, occur along as diagonal elements of $\mathbf{A}$. Off-diagonal elements of $\mathbf{A}$ arise from ρ^{col} terms in eq A2–A5 and occur in the various off-diagonal blocks in eq A15; e.g., in the block designated (τ_1^{-1}), the nonvanishing off-diagonal matrix elements occurs when a transition belonging to the reactants is coupled by exchange to a transition of the products of the reaction and vice versa.

The matrix $\mathbf{A}$ is diagonalized and inverted by using computer programs. The calculation need not be repeated for each value of ω, since ω occurs only in the diagonal elements. For

details of the computer calculation, see Vasavada et al. (1980). The theoretical spectrum is obtained by calculating $\mathcal{J}(\omega)$ for a number of closely spaced values of ω spanning the width of the observed spectrum.

Registry No. ATP, 56-65-5; ADP, 58-64-0; AMP, 61-19-8; ATP·Mg, 1476-84-2; ADP·Mg, 7384-99-8; adenylate kinase, 9013-02-9.

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Ligand Binding Site Interaction in Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase Catalytic Subunit: Circular Dichroic Evidence for Intramolecular Transmission of Conformational Change†

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ABSTRACT: Blue Dextran has been shown to interact specifically with the nucleotide binding site of the catalytic subunit of cAMP-dependent protein kinase. By observing changes in the induced dichroism associated with the absorbance of the bound chromophore, one can monitor conformational changes in the immediate vicinity of the ATP binding site. With this technique, it has been possible to demonstrate that attachment of ligand at the protein substrate binding site of the enzyme results in a conformational change at the ATP binding site. This alteration takes place in at least two steps, one of which appears to be dependent on the presence of a phosphorylatable hydroxyl group on the substrate and the other being triggered by the "basic subsite" (usually one or more arginine residues) to the N-terminal side of the target serine or threonine. Competition experiments suggest that the change induced results in closure over the substrate protein after the initial electrostatic binding; the movement initiated by the presence of a serine hydroxyl group may also involve interaction with a tyrosine residue at the surface of the ATP binding site.

The precise method through which the phosphorylation of selected substrate proteins is carried out by cAMP-dependent protein kinase (EC 2.7.1.37) has been a surprisingly durable problem in research on this enzyme. It has been known for some time that the holoenzyme, consisting of two regulatory and two catalytic subunits, dissociates in the presence of cAMP to form a regulatory dimer and two active catalytic subunits (Langan, 1967). As the intracellular mediator of a number of hormonally regulated processes, the active catalytic subunit was expected to display a fair degree of substrate specificity. In vitro, however, it acts relatively nonspecifically, the only limiting factors at the molecular level being (a) the presence of a serine or threonine hydroxyl group in an exposed position on the protein and (b) the presence of one or more arginine residues to the N-terminal side of the phosphorylated site (Daile et al., 1975; Kemp et al., 1977; Yeaman et al., 1977; Kemp, 1978; Feramisco et al., 1979; Meggio et al., 1981). The search for further control mechanisms operating within the cell has implicated a number of possible processes. The most direct of these involves alteration of the tertiary structure of the enzyme itself: inhibition or modulation of activity by the binding of appropriate ligands, often small, acid-stable proteins (Beale et al., 1977; Demaille et al., 1977; Szmigielski et al., 1977; Ferraz et al., 1979; Hashizome & DeGroot, 1979). Recent work in this laboratory has shown that such modulators

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