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Chemistry

^{31}P nuclear magnetic resonance kinetic measurements on adenylate kinase

(kinetic rates/double resonance/equilibrium exchange)

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ABSTRACT **By using ^{31}P double-resonance nuclear magnetic resonance, preliminary kinetic measurements were made on the equilibrium reaction 2ADP ⇄ ATP + AMP catalyzed by adenylate kinase. The double-resonance method used consisted of selectively inverting the spin population in a particular chemical environment and observing the transfer of the inverted spin to another chemical environment. The chemical transfer time between AMP and ADP, free in solution, was proportional to the substrate-to-enzyme ratio, and a transfer rate of 95 mol of AMP/sec per mol of enzyme was obtained. In the same series of experiments, the life-time of AMP in solution was determined from the extra broadening of its resonance line due to the active enzyme. This gave a rate 3.2 times faster than the overall transfer rate given above. From these rates and other nuclear magnetic resonance measurements we have calculated the individual rate constants between the ternary complexes, AMP–enzyme–ATP and ADP–enzyme–ADP. In addition, we obtained one of the ADP off-rates from this latter complex. The rates on the enzyme are approximately 1250 and 500 mol/sec per mol of enzyme for the forward and reverse directions, respectively. The ADP has an off-rate of 450 sec^{-1}.**

The determination of reaction rates has been of great use in understanding reaction pathways and developing models for reaction mechanisms. A great variety of techniques have been applied to this problem, ranging from stopped flow (1) and initial velocity measurements to radioisotope exchange (both in and out of equilibrium) and various nuclear magnetic resonance (NMR) and electron spin resonance (ESR) methods such as linewidth analysis (2) and saturation transfer (3). Kinetic measurements are of particular importance in the case of enzymatically catalyzed reactions because, in many cases, they provide the only information available on possible mechanisms.

Of the available NMR techniques for obtaining kinetic information, one of the most direct is saturation transfer, whereby the rate constants for chemical exchange between various species are determined by observing the direct transfer of a saturated spin between them. This has been used by a number of workers (3–5), notably Gupta and Redfield (3) who obtained electron exchange times in cytochrome *c*. We report there the use of a modification of this technique in which, instead of selectively saturating a given spin, it is inverted. We think that this modified saturation transfer, which we shall refer to as "inversion transfer," has great potential for enzymatic kinetic measurements. Although suggestions for obtaining chemical rate constants by selective inversion were made as long ago as 1957 (6), this technique apparently has not been applied to enzyme kinetics. A recent application by Dahlquist *et al.* (7), for example, measured the conformational exchange time of pyrolloparacyclophane.

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We have initially applied this inversion transfer technique to the ATP/ADP/AMP system as equilibrated by adenylate kinase for several reasons. First, because the equilibrium constant is near unity and all the reactants contain phosphorus, they are easily observed by ^{31}P NMR. In addition, because the catalysis occurs at the phosphate groups themselves, we are able to follow the transfer of a specific phosphate from one molecule to another, thus observing the reaction pathways in some detail. In this preliminary report, we concentrate on the exchange between AMP and ADP and will report on the full reaction later because it is quite complex.

The enzyme, adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3), which equilibrates the reaction AMP + ATP ⇄ 2ADP, has been studied by a number of workers. A comprehensive review has been provided by Noda (8). More recently, the x-ray structure, to 3 Å resolution, of the porcine enzyme (molecular weight, 22,000) has been reported by Schulz *et al.* (9). Several NMR studies of this enzyme have been reported by Cohn and coworkers (10–12).

METHOD

An inversion transfer measurement is made by applying a low-power selective 180° radio-frequency pulse to the sample prior to the standard 90° sampling pulse, the selectivity being achieved by setting the 180° pulse frequency to the resonant frequency of the spins to be inverted. By varying the delay time between the 180° and 90° pulses, one can observe the rate of transfer of the inverted spins from their initial chemical environments to others with which they are in exchange.

As in the case of all selective NMR spin "labeling" techniques, the inverted spin (the label) decays with the time constant of the spin-lattice relaxation time T_1. Also, the selective labeling requires the chemical exchange between the different species to be in the slow exchange limit with respect to the differences in chemical shift. Hence, the reaction times measurable by inversion transfer typically range from a second to a few milliseconds.

To make the 180° pulse as selective as possible, its width and strength are typically adjusted so that the nearest spins in frequency are rotated 360° around the effective field during the pulse (which rotates the selected spins only 180°) and thus are again pointing along the direction of the applied field when the pulse is turned off. In a spectrum of only two exchanging spins, this means that either spin can be inverted in only $\sqrt{3}/\delta$ in which δ is the difference in their chemical shifts in hertz.

Our experimental measurements have been on the exchange among the phosphate moieties of AMP, ADP, and ATP in equilibrium at 15°C in the presence of adenylate kinase. They have been carried out at 145.7 MHz on a Bruker HX360 NMR

Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance.

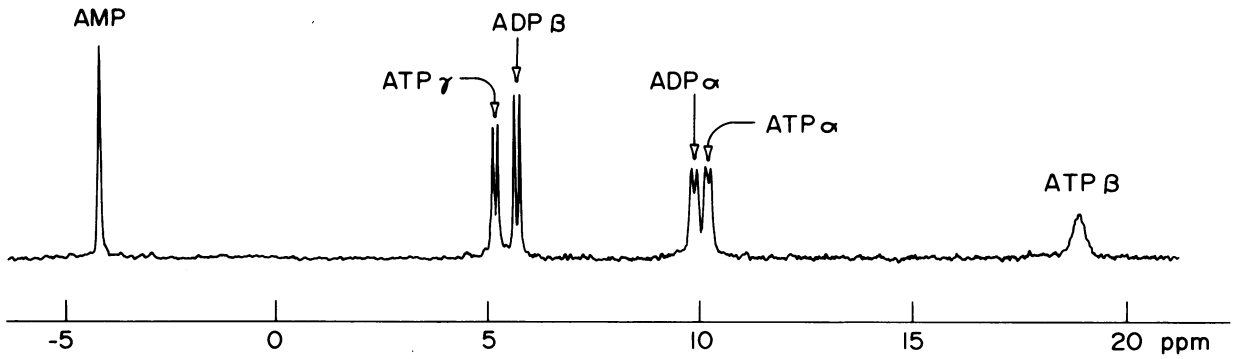

FIG. 1. ^{31}P NMR spectrum of a mixture of AMP, ADP, and ATP, initially 22 mM in ADP with 11 mM $MgSO_4$ in 0.2 M Tris/0.2 M tetramethylammonium chloride/5 mM dithioerythritol, pH 8.0, at 15°C equilibrated by a small amount of adenylate kinase. The free induction decay consisted of 400 scans, every 5 sec. The pulse angle was 90°. The equilibrium constant, $[ADP]^2/[AMP][ATP]$, was 1.3.

spectrometer, modified to produce the 180° selective pulse by using the decoupling channel circuitry driven by an independent frequency synthesizer at the resonant frequency of the selected spins. The pulses are given at a constant repetition time of 1.8 sec so as to keep any saturation of uninverted spins fixed as the delay between the two pulses is varied.

MATERIALS

The porcine enzyme was prepared in this laboratory essentially by the method of Heil *et al.* (13). All the enzyme used in these experiments had a specific activity between 1800 and 2000 units/mg at 20°C as assayed in the AMP + ATP → 2ADP direction by the pyruvate kinase and lactate dehydrogenase enzyme system (12). The nucleotides were of the highest commercially available grades and were passed through Chelex 100 (Bio-Rad) in order to remove any divalent metal ions. In all experiments the enzyme was dissolved in 0.2 M Tris/0.2 M tetramethylammonium chloride/4 mM dithioerythritol, pH 8, at a concentration between 3 and 5 mM as measured by optical absorption at 280 nm and calculated by using an extinction coefficient of 1.1×10^4 (8). The nucelotides were added from fresh stock solutions in the same buffer and were checked for ^{31}P impurities by NMR (these were <3% in all cases). In the experiments presented here, only ADP was added and the enzyme was allowed to equilibrate the nucleotide mixture. $MgSO_4$ was added from a 1 M stock solution used throughout the experiments. Samples were kept under nitrogen and there was no appreciable decay of enzymatic activity for 2 days at 15°C.

RESULTS

Fig. 1 shows a ^{31}P NMR spectrum of the three nucleotides AMP, ADP, and ATP equilibrated by a small amount of adenylate kinase under the solvent conditions used in the present study. The equilibrium constant calculated from the integrals of the peaks in Fig. 1 was 1.3, favoring ADP slightly.

In Fig. 1 the chemical exchange time between the various nucleotides is of the order of minutes and thus has no effect on the individual NMR resonances. Increasing the enzyme concentration so that the exchange time was substantially shorter than 1 sec enabled us to observe the transfer of phosphate spins among the various nucleotides by both inversion transfer and line broadening due to the shortened lifetime of the individual species.

Fig. 2 shows a typical example of this; the sample consisted of 3 mM adenylate kinase together with its substrates AMP, ADP, and ATP, added initially as 60 mM ADP together with 30 mM $MgSO_4$ to activate the enzyme. The initial inverting pulse was applied midway between the positions of the α phosphate resonances of ADP and ATP, inverting both. Note that the presence of the enzyme broadened the resonances considerably.

As the time delay between the 180° and 90° pulses was increased, there was clearly a transfer of the inverted spins from the α resonances to the AMP resonance. Note that there is no sign of transfer to any of the other uninverted resonances. This shows that the reaction pathway does not involve the transfer of a phosphate away from the sugar ring. In this connection, the exchanges we have observed upon inverting the terminal phosphates of ATP and ADP indicate similarly that the mode of action of the enzyme is to transfer the ATP γ phosphate to the AMP as expected.

In Fig. 2, the exchange of spins occurs initially between AMP and ADP as can be seen by inspecting the 10, 20, and 30 msec spectra in which only the left side of the inverted peak has been affected. As the delay was increased, the ATP α spin started to exchange, equilibrating by 150 msec. Further increases in the delay showed no changes other than a slow uniform recovery, in agreement with the expected T_1 values.

Fig. 3 presents the exchange data of Fig. 2 as a semilog plot of the delay time versus the differences in magnetization between the chemically equilibrated spectrum at 150 msec delay and the spectra at shorter delays. We extrapolated the 150 msec spectrum to earlier delay times by using the later T_1 recovery to calculate the correct values at earlier times. This caused less than 5% change in the measured exchange rates. Fig. 3 shows clearly the initial exponential exchange between AMP and ADP together with the slow involvement of ATP. By analyzing these curves in detail we could extract both the AMP/ADP exchange rate and also the ADP/ATP exchange rate, which must be several times slower because there is little involvement of the ATP until the AMP and ADP are practically equilibrated. By combining these data with inversion transfer measurements in which we inverted the various other possible spins, we should be able to construct a complete kinetic model of adenylate kinase. Thus far, in our preliminary analysis, we have concentrated on the AMP/ADP exchange, excluding the other possible exchanges for the present. These will be discussed in detail in a future report.

In the following analysis, we treat the exchange rates between AMP and ADP as pseudo-first-order rate constants. In this case the exponential exchange time obtained from the AMP and ADP slopes in Fig. 3 is the inverse of the sum of the individual unidirectional rate constants. Because the ratio of the rate constants must be the inverse of the ratio of the concentrations, we can calculate the unidirectional rate constant from AMP to ADP. The inverse of this is presented in Fig. 4 as a function of

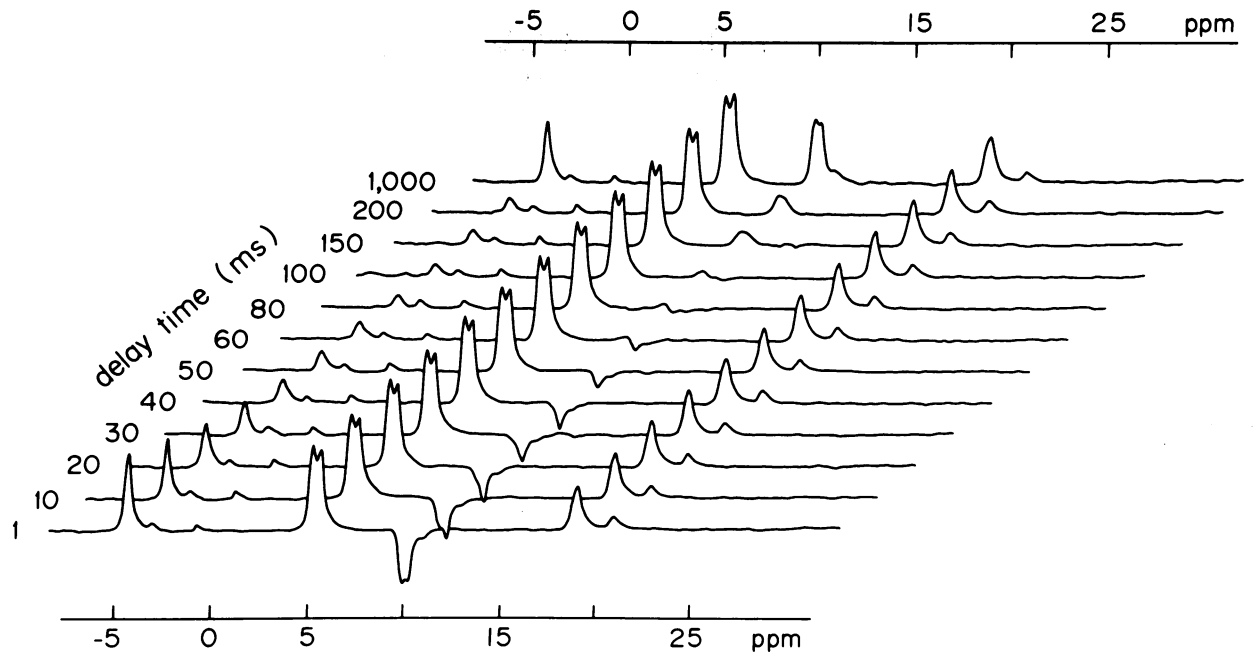

FIG. 2. A typical inversion transfer sequence. Each spectrum is 200 scans taken every 1.8 sec. The 180° pulse was applied to the α phosphates of ADP and ATP. The delay between this pulse and the 90° pulse varied from 1 ms to 1 sec and is indicated by the number to the left of each spectrum. The small peaks at −0.5 and −3.0 ppm are a marker and inorganic phosphate, respectively. The small peaks at 11 and 21 ppm are an unknown impurity which shows no sign of inversion transfer to any of the other peaks.

substrate-to-enzyme ratio. The data have a slope corresponding to a rate of 95 mol/sec per mol of enzyme for AMP converted to ADP.

Another time available from the NMR measurements is the lifetime of AMP as determined by its excess linewidth in the presence of the active enzyme. This is also presented in Fig. 4 and has a slope corresponding to a rate of 300 mol/sec per mol of enzyme. (Unfortunately, because of the nearness of the ATP α resonance we are unable to measure the excess linewidth for the ADP α line and thus cannot estimate its lifetime.) The linear

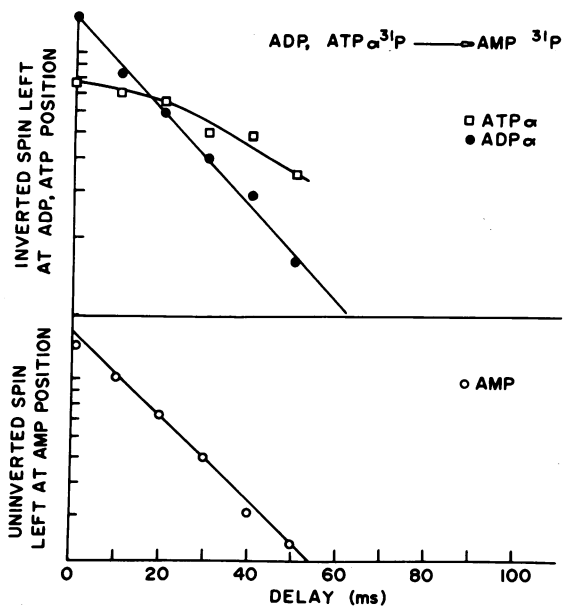

FIG. 3. Semilog plot of the delay time dependent magnetization after subtracting T_1 decay contribution. The delay time is the time between the selective 180° pulse and the sampling 90° pulse. The AMP/ADP exchange rate is 33 sec^{-1}.

dependence of these two times upon the substrate-to-enzyme ratio shows definitively that we are observing an exchange mediated by the adenylate kinase. Both the inversion transfer and the extra line broadening require the presence of Mg^{2+}. The extra broadening is homogeneous, and the AMP resonance shows no appreciable shift when the substrate-to-enzyme ratio was varied. We also studied the binding of AMP to the enzyme in absence of Mg^{2+}, both with and without ATP. The bound AMP never shifted more than about 60 Hz downfield nor broadened more than 15 Hz when fully complexed to the enzyme. This AMP binding in the absence of Mg^{2+} would contribute less than 10% of the observed excess linewidth in the presence of Mg^{2+}. Therefore, the dominant contribution to the excess linewidth comes from the conversion of AMP into some state, further down the reaction pathway, which for simplicity is assumed to be ADP on the active enzyme.

By comparing the AMP lifetime to the AMP/ADP transfer time, we can probe different parts of the path by which free AMP reacts with enzyme-complexed ATP to give free ADP plus enzyme-complexed ADP (the free ADP coming from the free AMP). Fig. 5 presents a diagram of this reaction. As indicated, the chemical transfer time yields the overall rate, whereas the AMP lifetime is determined by a step that must be slow compared to the chemical shift difference before and after. We believe this step to be the conversion of AMP to ADP *on the enzyme* for the reasons given above. It is of course possible that the relevant step is one to an enzyme-bound intermediate in the reaction path between AMP and ADP. If this were the case, then the rate constants calculated below become averages of the rate constants into and out of the intermediate state. For simplicity, we will continue to discuss this as though there were no such intermediate, bearing in mind the above caveat.

In order to extract individual rate constants from our measurements, we have used the following expressions for the rates k_{LT} and k_{MD} in mol/sec per mol of enzyme, associated with the AMP lifetime and the transfer from AMP and ADP, respectively:

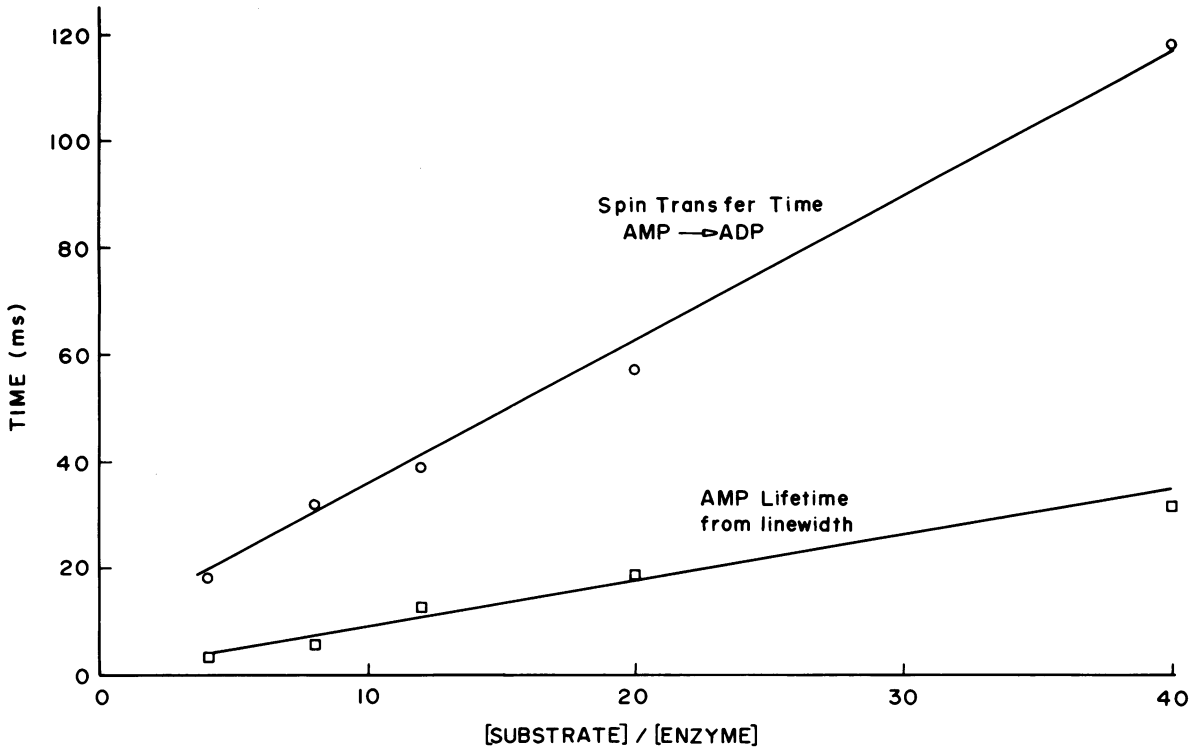

FIG. 4. Spin inversion transfer time and AMP lifetime versus total adenine-to-enzyme ratio. In each case the adenine was added initially as ADP. A ±5 Hz error is estimated for the linewidth data; the inversion transfer times have a possible systematic error of ±10%. The AMP lifetime was calculated from the extra AMP linewidth in the presence of the active enzyme. The extra broadening was homogeneous and there was no appreciable shift in line position.

$$k_{LT} = k_{-1} \times \frac{k_1}{k_{-1} + k_1} \times \frac{k_{-2}}{k_{-1} + k_{-2}} \quad [1]$$

$$k_{MD} = k_{LT} \times \frac{k_{-3}}{k_{-3} + [k_1 k_{-2}/(k_{-1} + k_{-2})]} \quad [2]$$

k_1, k_{-1}, k_{-2}, and k_{-3} are defined in Fig. 5. These expressions are derived in the high-substrate-concentration limit with steady-state kinetic equations and the assumption that all of the doubly complexed enzyme is accounted for by the two ternary complexes. In fact, Eq. 1 can be simply derived as follows. The total number of AMP-E-ATP complexes being converted to ADP-E-ADP complexes per second is simply $k_{-1} \times$ [AMP-E-ATP] which is $k_{-1} \times k_1 E^0/(k_1 + k_{-1})$ (we assume all of the enzyme is in one of the two ternary forms), E^0 being the total enzyme present. For one of these conversions to contribute to the AMP linewidth, the AMP-E-ATP complex must have just come from its free constituents rather than from the ADP-E-ADP complex. This fact then adds the extra factor of $k_{-2}/(k_{-1} + k_{-2})$, giving Eq. 1. Eq. 2 has a similar derivation,

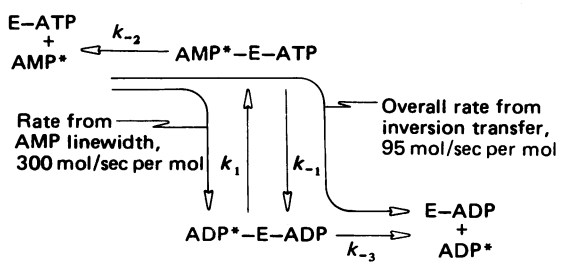

FIG. 5. Diagram of the reaction path from free AMP to free ADP. * represents the inverted spin. The individual rate constants are: k_1 = 1330, k_{-1} = 530 mol/sec per mol of enzyme; k_{-2} = 2000 sec^{-1}, k_{-3} = 450 sec^{-1}.

resulting from the competition between the ADP off-rate, k_{-3}, and the rate back to the AMP-E-ATP complex, k_1.

Because we have four rate constants and only two measurements, we need at least two more pieces of information to determine the individual rates. These are (*i*) the internal equilibrium constant on the enzyme, k_1/k_{-1} between AMP-E-ATP and ADP-E-ADP, and (*ii*) an estimate of the AMP off-rate from our AMP binding studies mentioned above. From the ratio of AMP to ADP under excess enzyme conditions (enzyme ~5 mM, substrate ~3 mM), we estimate $k_1/k_{-1} = 2.5 \pm 0.5$. From the fact that the AMP line broadens no more than 5 Hz in the middle of the titration range, we can calculate a lower limit to the AMP off-rate in the absence of Mg^{2+}. Taking the full chemical shift upon binding to be 80 Hz, we obtain an off-rate of 2000 sec^{-1} for AMP leaving the ternary complex. We assume below that this is unchanged in the presence of Mg^{2+}.

Using these two estimates, together with Eq. 1, we obtain k_1 = 1330 and k_{-1} = 530 mol/sec per mol of enzyme. These values are not very sensitive to k_{-2}, the AMP off-rate. If this were taken to be infinitely fast, we would get k_1 = 1050 and k_{-1} = 430 in the same units. From Eq. 2, we can now calculate the ADP off-rate, k_{-3}; it is 450 sec^{-1}.

In a simpler system we could now compare these rates with V_{max} measurements for consistency. However, in this system, until we have analyzed the other possible exchanges we are unable to include the effects of the ATP off-rates into the calculation of the one-way maximum velocities. The measured rates, however, are comparable with our V_{max} measurements of a turnover number of 600 sec^{-1} for the reverse direction at 20°C.

We should emphasize that the above numbers for the rate constants depend on the internal equilibrium constant, the estimation of which assumes the absence of any dead-end complexes such as an enzyme molecule with AMP and ADP bound

to it. A full analysis of the kinetics by the present technique should allow us to measure the amount of enzyme tied up in these complexes and thus to determine the rates unambiguously.

In conclusion, we have used standard NMR techniques plus a new method, inversion transfer, to separate various steps in the reaction pathway from AMP to ADP catalyzed by adenylate kinase. In general, this method appears to have applicability to any equilibrium exchange measurements in which the individual substrates are observable by NMR and have exchange rates between their differential chemical shift and the inverse of the T_1 of the observed spins.

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