Protein-Bound Adenosine 5′-Triphosphate: Properties of a Key Intermediate of the Magnesium-Dependent Subfragment 1 Adenosinetriphosphatase from Rabbit Skeletal Muscle†

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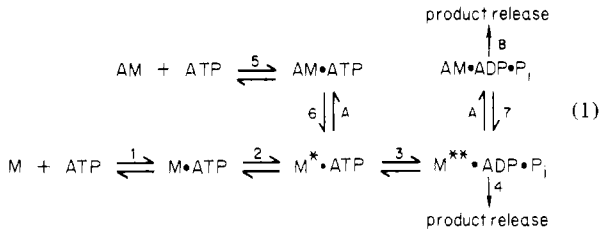
ABSTRACT: The time course of formation and decay of protein-bound adenosine 5′-triphosphate (ATP) has been monitored during single turnovers of the myosin subfragment 1 ATPase with nonspectrophotometric techniques. The rate constant controlling the ATP cleavage step increases markedly with ionic strength, so that in low salt the protein–ATP complex is observed transiently at higher concentration than the protein–products complex. The kinetics of the ATP cleavage step in a single turnover of the actosubfragment 1 ATPase indicates that under appropriate conditions this step is partially rate limiting during overall steady-state ATPase activity. It follows that a binary subfragment 1–ATP complex is a significant component of the steady-state intermediate of the actosubfragment 1 ATPase. Transient kinetic studies of ATP and adenosine 5′-(3-thiotriphosphate) [ATP(γS)] binding show directly that a substrate-induced protein isomerization accompanies ligand binding. The rate constant of the isomerization is 170 s^{-1} at pH 7.0, 15 °C, and 0.01 M ionic strength. Under these conditions nucleotide binding appears to be accompanied by a protein fluorescence increase that is 50% of the increase associated with magnesium-dependent steady-state ATPase activity.

Investigations of the myosin subfragment 1 (M) and actosubfragment 1 (AM) ATPase[1] mechanisms using rapid reaction techniques have led to the following minimum scheme of the mechanism prior to the release of reaction products ADP and P_i:



(1)

$K_1 = 4.5 \times 10^3\ M^{-1}$, $k_{+2} = 400\ s^{-1}$, $k_{-2} = 1 \times 10^{-4}\ s^{-1}$ (at pH 7), $K_3 = 9$, $k_3 + k_{-3} \geq 160\ s^{-1}$, and $k_{+4} = 0.06\ s^{-1}$ at pH 8 and 21 °C and 0.1 M ionic strength. k_{+i}, k_{-i}, and K_i ($=k_{+i}/k_{-i}$) are the forward and reverse rate constants and the equilibrium constants for the ith step (Trentham et al., 1976; Cardon & Boyer, 1978). The apparent second-order rate constant resulting in actosubfragment 1 dissociation (K_5k_{+6}) is $1.5 \times 10^6\ M^{-1}\ s^{-1}$, and that of actin association (k_{+7} or K_7k_{+8}) is $2.0 \times 10^5\ M^{-1}\ s^{-1}$ (White & Taylor, 1976).

The work described here examines the kinetics of ATP binding and cleavage directly by measuring the concentrations of the subfragment 1–ATP complex, M*·ATP, and of M**·ADP·P_i at various times. The rationale of the experiments has been to obtain a better understanding of the steps associated with these two processes, emphasizing chemical as opposed to spectrophotometric techniques since the former are more unambiguously interpreted. When the isomerization step in the subfragment 1 ATPase was first inferred, the relative values of k_{+2} and k_{+3} were an open question and the possibility existed that $k_{+3} > k_{+2}$. More recent work has suggested that under appropriate conditions $k_{+3} < k_{+2}$ (Johnson & Taylor, 1978; Chock et al., 1979). Aside from the importance of knowing the relative values of k_{+2} and k_{+3}, the fact that k_{+3} may be less than k_{+2} means that, when this occurs, it should be possible to identify a first-order process in the ATP binding process that is kinetically distinct from ATP cleavage. This has not been done previously. If such a first-order process is found, it will provide additional support for the proposed isomerization M·ATP ⇌ M*·ATP. Hitherto characterization of the isomerization step, M·ATP ⇌ M*·ATP, has relied predominantly on analysis of the association kinetics of ATP and of ATP analogues to subfragment 1 detected by the enhancement of protein fluorescence that accompanies this process. While this approach is important, it has its limitations. For example, in the case of ATP, assigning what fraction of protein fluorescence increase is due to ATP binding and what fraction to ATP cleavage has proved difficult to resolve. Secondly, while protein isomerization may be clear-cut for nonhydrolyzable ATP analogues, there is an element of doubt as to the validity of any argument that assigns by analogy the same isomerization to ATP binding (Chock et al., 1979).

The Lymn–Taylor (1971) hypothesis linking the biochemical events of the actomyosin ATPase mechanism with the cross-bridge cycle suggests that the process M*·ATP ⇌ M**·ADP·P_i is common to both the myosin and actomyosin ATPases. Earlier work has shown that the equilibrium constants of the cleavage steps of the subfragment 1 and actosubfragment 1 ATPases are the same (Eccleston et al., 1975). Johnson & Taylor (1978) have compared the rate constants of the cleavage in the subfragment 1 and actosubfragment 1 ATPases under conditions in which cleavage occurs subsequent to essentially complete ATP binding. They found that the rate constant of ATP cleavage was the same in the two cases. The

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[1] Abbreviations: ATP(γS), adenosine 5′-(3-thiotriphosphate); ATPase, adenosine-5′-triphosphatase; DEAE, diethylaminoethyl; UV, ultraviolet; HPLC, high-performance liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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work described here also examines the important question of ATP cleavage kinetics in the actosubfragment 1 ATPase but under conditions in which ATP binding is not so well resolved kinetically from ATP cleavage. In addition such transient kinetic data indicate to what extent ATP cleavage is the rate-limiting process of the actosubfragment 1 ATPase mechanism. If ATP cleavage is partially rate limiting and the actosubfragment 1 ATPase mechanism is as described in eq 1, then M*·ATP will be a component of the steady-state complex. For this reason alone actosubfragment 1 will be partially dissociated during ATPase activity.

Materials and Methods

Proteins. Generally myosin subfragment 1 was prepared from rabbit skeletal muscle myosin by using papain digestion essentially as described by Lowey et al. (1969). In experiments carried out at 15 °C and pH 7 [conditions used by Chock et al. (1979)], chymotryptic subfragment 1 was prepared as described by Weeds & Taylor (1975). The protein was purified by ion-exchange chromatography on DEAE-cellulose and used within 24 h of column elution. Concentrations are quoted on the basis of a molecular weight of 115 000 and an $E^{1\%}_{280nm} = 7.9$ cm^{-1}. The active-site concentration was measured from the stoichiometry of the bound nucleotide with subfragment 1 as described in the experiments in Table II and Figure 4. The protein active-site concentration was typically 70–85% of the protein concentration determined from $E^{1\%}_{280nm}$.

F-Actin was prepared from an acetone-dried powder as described by Lehrer & Kerwar (1972). Concentrations were determined from the extinction coefficient $E^{1\%}_{280nm} = 11.08$ cm^{-1} (West et al., 1967).

Reagents. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). [8-^{14}C]ATP was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. ATP(γS) (from Boehringer, Mannheim, West Germany) was further purified by chromatography at 3 °C and pH 7.5 with a DEAE column (40 cm × 5 cm^2) with a linear gradient of triethylammonium bicarbonate from 10 to 500 mM. ATP(γS) was preincubated with a small molar excess of dithioerythritol prior to the chromatography. The UV-absorbing peak containing ATP(γS) was concentrated to dryness. Excess triethylammonium bicarbonate was removed by repeated evaporation of methanol added to the solid. ATP(γS) was stored as a concentrated solution at neutral pH and −20 °C. In experiments involving ATP(γS) all solutions contained 1 mM dithioerythritol. [8-^{14}C]ATP(γS) was prepared essentially as described by Goody et al. (1972) with nucleoside diphosphate kinase and purified by DEAE chromatography.

The purity of ATP(γS) was determined analytically by HPLC with a Waters SAX column and with an eluting solvent of 10% methanol and 90% 0.6 M ammonium phosphate adjusted to pH 4.0 with HCl. This analysis showed that DEAE column chromatography resolved ATP(γS) cleanly from other nucleotides. In addition [^{14}C]ATP(γS) was shown to be 99% pure by analysis of its ^{14}C elution profile on the HPLC column. Nucleotide concentrations were based on a molar extinction coefficient of 1.54 × 10^4 M^{-1} cm^{-1} at 259 nm (Bock et al., 1956). Doubly glass distilled water was used throughout.

Rapid Reaction Equipment. The quenched-flow apparatus was similar to that described by Gutfreund (1969). Experiments at 3 °C were performed in a cold room at 3 °C. For experiments at 15 °C the flow block and reaction syringes were immersed in a thermostatically controlled circulating water bath. The time range for quenching solutions following continuous flow was from 5 to 200 ms. For reaction times of 2 s the reactants were mixed by using the apparatus, and the quenching reagent was added manually from a third syringe.

The rate constant of the substrate-induced protein isomerization that was measured with this apparatus was found to be 170 s^{-1}. Since this is close to the upper limit of rate constant that can be determined by using the quenched-flow technique, it is important to describe the expected limitations and reliability of the measurement. Gutfreund (1969) has defined carefully the mixing time and dead time of a quenched-flow apparatus. The mixing block was the same type as that in the fluorescence stopped-flow apparatus for which mixing was shown to be at least 99% complete at 1 ms even in the presence of subfragment 1 (Bagshaw et al., 1974). The dead time of the quenched-flow apparatus was measured as described by Gutfreund (1969) and was 3 ms. This time was incorporated as a correction into the calculated time points. There was a second mixing when the reactants were added to acid (to quench the reaction) or to a solution of nonradioactive nucleotides (in "cold-chase" experiments). In the former case the mixing time was unlikely to introduce a significant error since the reaction will have been quenched well before total equilibration with the acid was achieved, because the protein is inactivated below pH 5. In the latter case the binding of radioactive ATP to the protein will have been suppressed during the mixing of nonradioactive ATP with the protein solution.

The major point at issue in characterizing the substrate-induced protein isomerization is whether the rate constant controlling the process is as slow as 170 s^{-1}, since the process had been observed to be essentially complete within the time resolution of the apparatus (13 ms) in other laboratories (Chock et al., 1979). Underestimating the dead time (the probable direction of error) or inefficiency at the second mixing will both lead to an observed value of the rate constant that is larger than the true value. Inefficiency at the first mixing would lead to a low value for the observed rate constant. As noted above this is unlikely to be a problem given the high efficiency of the first mixing.

Analysis of Protein-Bound Nucleotide. On mixing [γ-^{32}P]ATP or [^{14}C]ATP with myosin subfragment 1, we measured one or more of the following as a fraction of the total nucleotide: free ATP, ATP tightly bound to protein (M*·ATP), ADP or P_i formed (generally as M**·ADP·P_i), or total bound nucleotide (M*·ATP + M**·ADP·P_i).

For analysis of [^{14}C]ADP or [^{32}P]P_i formed, the reactants were quenched into an equal volume of ice-cold 7% $HClO_4$. The pH was adjusted to between 4.0 and 4.5 by addition of a solution of 1.2 N sodium acetate and 2.8 N NaOH. Protein was removed by centrifugation. The ratio of [^{32}P]P_i to [γ-^{32}P]ATP was measured by thin-layer chromatography as described by Bagshaw & Trentham (1973). The ratio of [^{14}C]ADP to [^{14}C]ATP was measured by analyzing a sample with HPLC. Carrier ADP and ATP were used to help identify the radioactive peaks. This HPLC method is an improvement over the thin-layer approach, and the results were highly reproducible (e.g., Table II). Forty samples could be routinely prepared within 8 h for analysis in a liquid scintillation counter.

For analysis of protein-bound nucleotide (M*·ATP + M**·ADP.P_i) the reactants were added at predetermined times into a 50–1000-fold nucleotide excess of ATP. (In the HPLC experiments ADP was also present to serve as a marker.) After 2 min (an estimated seven reaction half-times) when radioactive protein-bound nucleotide will have become more than 99% [^{14}C]ADP or [^{32}P]P_i, the mixture was quenched with 7% $HClO_4$ and the radioactivity analyzed as described above. This gives the ratio of free ATP to M*·ATP + M**·ADP·P_i

and is the cold-chase technique similar to that described by Bagshaw & Trentham (1973). In experiments done in the absence of actin, bound nucleotide will not have dissociated from the protein as radioactive ATP during the cold chase, since the dissociation rate constant is much less than the catalytic center activity of the ATPase (Cardon & Boyer, 1978). One experiment was done in the presence of actin, and its influence on the measurement of protein-bound nucleotide is described in the legend to Figure 3. A combination of these two methods gives the quantities of free ATP, M*·ATP, and M**·ADP·P_i.

In many of the experiments the concentration of subfragment 1 exceeded that of the radioactive ATP. All the nucleotide was thus bound in a single turnover of the enzyme. This affords a particularly sensitive approach to measuring levels of M*·ATP.

Zero time measurements in cold-chase experiments were done by mixing radioactive nucleotide with protein buffer in the quenched-flow apparatus into a solution containing the nonradioactive ATP to which subfragment 1 had been added a few seconds previously. After 2 min the reaction was quenched with 7% $HClO_4$ as in the other measurements. Care was taken to see that for each assay in a cold-chase experiment the conditions of the cold chase were reproduced as precisely as possible. Typically zero time measurements showed 4–6% ATP hydrolysis in cold-chase experiments and 2–3% ATP hydrolysis in direct-quenching experiments. Results have been corrected for extraneous ATP hydrolysis on the basis of these zero time measurements.

In the [^{14}C]ATP(γS) experiments the bound nucleotide as a fraction of total nucleotide was measured by using the cold-chase technique. In practice the bound nucleotide present at the time of adding nonradioactive ATP will have been predominately ATP(γS) and committed to hydrolysis (Bagshaw et al., 1972; Goody & Mannherz, 1975). Additional ATP(γS) was not added as a marker in the HPLC analysis. Rather, the ADP and ATP peaks together with the ^{14}C radioactivity were used to identify the [^{14}C]ADP and [^{14}C]-ATP(γS). Due to the relatively long retention time of ATP-(γS), only 20 samples could be analyzed in 8 h.

The fluorescence stopped-flow equipment has been described by Bagshaw et al. (1972). Experiments at 15 °C were carried out in a temperature-controlled laboratory. Signals were stored on disc or tape with a PDP 1103 computer with facilities for computer averaging and calculation of the rate constants of exponential processes. Kinetic simulations were done with the system described by Hardman et al. (1978). Steady-state fluorescence measurements were made on a photon counting SLM spectrofluorometer.

Results

Concentration of Protein-Bound ATP. The first experiments in which excess protein was mixed with [γ-^{32}P]ATP were designed to determine the time course of protein-bound ATP (i.e., M*·ATP) formation and decay during a single turnover of the subfragment 1 ATPase. The results show (Figure 1A) that M*·ATP was formed rapidly and that its concentration then decayed relatively slowly over the first 200 ms. On the other hand, free ATP and M**·ADP·P_i decayed and formed, respectively, in an approximately exponential fashion with a rate constant of 17 s^{-1}. The ratio of M*·ATP to M**·ADP·P_i varied more than 6-fold between 5 and 200 ms of the reaction time. This is consistent with the sum of the rate constants, $k_{+3} + k_{-3}$, being in the range 100–150 s^{-1} (Figure 1B). The data are not sufficiently precise to observe any lag phase in M**·ADP·P_i formation. Nevertheless the concentration of M*·ATP can be measured in a rather sensitive

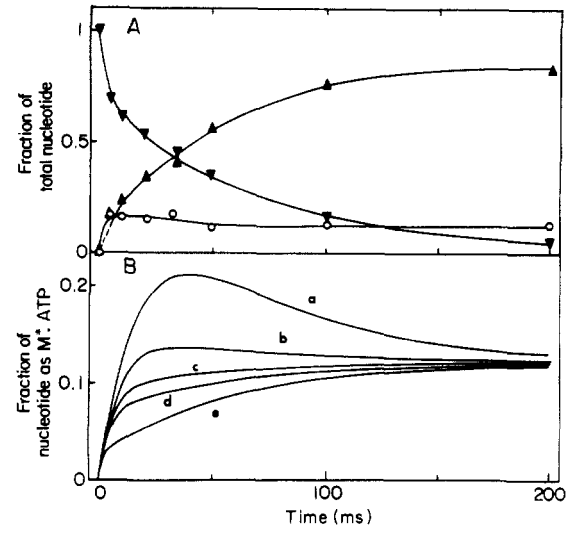

FIGURE 1: Time course of a single turnover of the subfragment 1 ATPase. (A) 19 μM subfragment 1 was mixed with 5 μM [γ-^{32}P]ATP in a solution of 50 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris adjusted to pH 8.0 with HCl at 20 °C (i.e., ionic strength 93 mM). The fraction of nucleotide as free ATP (▼), M*·ATP (○), and M**·ADP·P_i (▲) is shown. (B) Computer simulation of fraction of nucleotide as M*·ATP for various values of k_b in the scheme

$$A \xrightarrow{k_a} B \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} C$$

Values of k_a = 17 s^{-1} and K_b ($=k_b/k_{-b}$) = 7 were determined from the time course of the disappearance of free ATP and the ratio (M**·ADP·P_i/M*·ATP) at 200 ms. In simulations a, b, c, d, and e, k_b takes values of 50, 100, 150, 200, and 500 s^{-1}, respectively.

way, and the result of computer modeling shows that $k_{+3} + k_{-3}$ can be determined even when its value is 1 order of magnitude greater than the observed rate constant of the binding step.

A principal aim of the work described in this paper is to analyze the kinetics of ATP binding to subfragment 1 by using the cold-chase technique. Hence it is important to validate this approach by comparing results using this method with those of other approaches. Experiments at pH 6.9 and 3 °C have been carried out by Sleep & Taylor (1976) and Johnson & Taylor (1978) in which the kinetics of ATP binding and the values of k_{+3} and k_{-3} were determined by fluorescence stopped-flow and quenching techniques similar to those used here. A cold-chase experiment (Table I) under these conditions showed that after 200 ms M*·ATP was present in considerable excess over M**·ADP·P_i. Further analysis of the data shows that the results are compatible with those of Sleep & Taylor (1976) in that the rate constants lead to a reasonable fit with the observed yields of M**·ADP·P_i (Table I). From the data we conclude that measurement of M*·ATP provides a sensitive probe to distinguish the binding of ATP from its subsequent cleavage.

Ionic Strength Dependence of ATP Cleavage. In a single turnover experiment at an ionic strength of 10 mM the concentration of M*·ATP reached its maximum value within 20 ms (Figure 2A), and like the previous experiment (Figure 1) the ratio of M*·ATP to M**·ADP·P_i fell severalfold during the reaction. The rate of M**·ADP·P_i formation could be described as a single exponental process with a rate constant of 28 s^{-1}. Computer simulation as the Figure 1 indicated the sum of the rate constants, $k_{+3} + k_{-3}$, to be in the range 30–50 s^{-1}, and hence, k_{+3} is in the range 25–40 s^{-1}.

By raising the concentration of reactants sufficiently high, it should be possible to make the binding process of ATP to subfragment 1 so fast that it is well resolved from ATP

Table I: Time Course of a Single Turnover of Subfragment 1 ATPase at 3 °C[a]

expt	subfragment 1 (μM)	time (s)	% of nucleotide as free ATP	M*·ATP	M**·ADP·P_i	[M**·ADP·P_i]/[M*·ATP]	% of M**·ADP·P_i predicted
1	20	0.20	86.7	8.4	4.9	0.58	2.9–4.1
		2.0	34.5	31.7	33.8	1.07	
2	40	0.20	71.0	22.5	6.5	0.29	5.6–9.3
		2.0	6.4	37.5	56.1	1.50	

[a] Free ATP, M*·ATP, and M**·ADP·P_i were measured when 20 or 40 μM subfragment 1 was mixed with 5 μM [γ-^{32}P]ATP in 50 mM KCl, 5 mM $MgCl_2$, and 10 mM Mes, adjusted to pH 6.9 with Tris at 3 °C. The experiments were done with two different subfragment 1 preparations. The results are the average of duplicate measurements, which agreed to within 1 and 2% in experiments 1 and 2, respectively. The predicted values of M**·ADP·P_i at 0.2 s were calculated from the data of Sleep & Taylor (1976) for which K_3 is in the range 1–2 and $(k_{+3} + k_{-3})$ is in the range 5–7 s^{-1}. The pseudo-first-order association rate constant of ATP binding to subfragment 1 (needed to predict the percentage of M**·ADP·P_i) was calculated from the rate of ATP disappearance and was 0.71 s^{-1} and 1.7 s^{-1} in experiments 1 and 2, respectively.

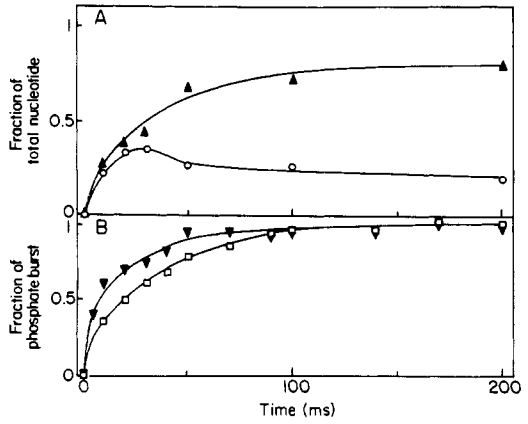

FIGURE 2: (A) Time course of a single turnover of the subfragment 1 ATPase. 22 μM subfragment 1 was mixed with 5 μM [γ-^{32}P]ATP in a solution of 2 mM KCl, 2 mM $MgCl_2$, and 4 mM Tris at pH 8.0 and 20 °C. The fraction of nucleotide as M*·ATP (○) and M**·ADP·P_i (▲) is shown. (B) Ionic strength dependence of M**·ADP·P_i formation during the initial phase of subfragment 1 ATPase activity. 150 μM [γ-^{32}P]ATP was mixed with 30 μM subfragment 1, and the time course of [^{32}P]P_i and hence M**·ADP·P_i formation was followed over the first 200 ms. The solution contained (▼) 50 mM KCl, 2 mM $MgCl_2$, and 50 mM Tris or (□) 2 mM KCl, 2 mM $MgCl_2$, and 4 mM Tris, both solutions at pH 8.0 and 20 °C. At ionic strength 0.08 M the M**·ADP·P_i formed at 200 ms corresponded to 16% of the total [γ-^{32}P]ATP present initially and at 0.01 M to 15%.

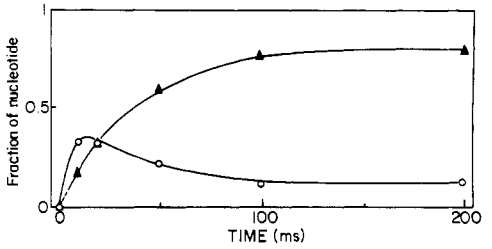

FIGURE 3: Time course of a single turnover of the actosubfragment 1 ATPase. Reaction conditions were identical with those in Figure 2A, except for the presence of 27 μM actin (reaction chamber concentration) in the subfragment 1 syringe. The fraction of nucleotide as protein-bound ATP (○) and total product (▲) is shown. On the basis of the findings of Sleep & Hutton (1978) it is possible that actin will promote the release of radioactive ATP back into the medium from M*·ATP during the cold-chase phase. Since the protocol in the cold-chase step was always the same, protein-bound ATP present at the time of adding cold nucleotide will partition to form either products or ATP, which returns to the medium in the same ratio in each assay. When the results at 200 ms are considered, the ratio of free ATP:protein-bound ATP:products is 6.6:12.6:80.8. It follows that, if all the free ATP found at 200 ms had been derived from protein-bound ATP, then the actual values of protein-bound ATP should be increased by 55% over those plotted in the figure. Extrapolating the data of Sleep & Hutton (1978) to the solvent conditions of the cold chase suggests that the actual values of protein-bound ATP should be increased by 20%. Either correction has a relatively small effect on the value of rate constant controlling ATP cleavage, which still falls in the range of 30–50 s^{-1}.

cleavage. Under these conditions the rate of formation of M**·ADP·P_i should be independent of reactant concentration. This was tested and shown to be the case when 100 or 200 μM [γ-^{32}P]ATP was mixed in 5-fold excess over subfragment 1 in the same solvent conditions as those described in Figure 2A. The rate of [^{32}P]P_i and hence M**·ADP·P_i formation in each case was the same and could be described as an exponential process with a rate constant of 32 s^{-1}. The similar time course of M**·ADP·P_i formation in these two experiments indicates that $k_{+3} + k_{-3} = 32$ s^{-1}.

In a further experiment the rate of P_i formation was measured by using the same protein preparation at ionic strengths of 10 and 80 mM. The observed rate constants were 28 s^{-1} and 100 s^{-1}, respectively (Figure 2B). Taken altogether, the results of these experiments (Figures 1 and 2) establish that the value of $k_{+3} + k_{-3}$ depends strongly on the ionic strength increasing 5-fold between 10 and 100 mM salt.

ATP Cleavage Step in the Actosubfragment 1 ATPase. The experiment described in Figure 2A was repeated but with the addition of 27 μM actin (1.13 mg/mL) (Figure 3). As in the previous single turnover experiments, the ratio of the concentration of protein-bound ATP to that of protein-bound products decreases over the first 200 ms, indicating that the value of the rate constant controlling the ATP cleavage step is comparable to or smaller than that controlling the binding of ATP. The catalytic center activity of the actosubfragment 1 ATPase was 12 s^{-1} at this actin concentration and ionic strength. Thus in contrast to the single turnover experiments described in Figures 1 and 2, the protein–products complex will have decayed significantly by 200 ms.

Simulation of the kinetics of protein-bound ATP formation and decay in Figure 3 showed that the rate constant controlling ATP cleavage is in the range 30–50 s^{-1} and comparable to that observed in the absence of actin (Figure 2A). The simulation was done by using the two-step scheme described in the legend to Figure 1 in which B and C represent M*·ATP and M**·ADP·P_i, respectively. K_b was determined from the ratio of protein–products complex to protein-bound ATP at 200 ms and was corrected for the decay of the protein–products complex. Thus the kinetic evidence from the single turnover experiment is compatible with eq 1 in which the cleavage step M*·ATP to M**·ADP·P_i is common to both the myosin and actomyosin ATPases. In addition to the dissociating pathway for ATP hydrolysis (eq 1), the results are also compatible with a significant fraction of ATP hydrolysis occurring via a nondissociating pathway with a comparable rate constant for the hydrolytic step in the two pathways (Stein et al., 1981).

At an F-actin concentration (6 mg/mL) approaching saturation, the actin-activated ATPase activity was 29 s^{-1} when measured at 0.01 M ionic strength in the presence of 2 mM MgATP at pH 8 and 20 °C. This catalytic center activity

Table II: Fraction of Subfragment 1 Active Sites Bound with Nucleotide 5 ms after Mixing with ATP[a]

initial ATP concn (μM)	% existing as M*·ATP + M**·ADP·P_i	SD	n
60	50.3	1.15	4
200	57.8	2.5	4

[a] [^{14}C]ATP was mixed with 22 μM subfragment 1 at 15 °C in 1.5 mM $MgCl_2$ and 7.8 mM imidazole adjusted to pH 7.0 with HCl. ATP binding was determined by the cold-chase technique. Bound nucleotide was measured at 36 and 110 ms, when the binding phase was 95 and 100% complete, respectively, to determine the protein active-site concentration. The fraction of nucleotide present as ADP in the zero time measurements was 4.2 ± 0.2%. The results are corrected for this ADP formation.

is comparable to the value of the rate constant of the step controlling ATP cleavage (Figure 3).

A Substrate-Induced Isomerization in the Subfragment 1 ATPase. The above experiments have shown that at an ionic strength below 0.1 M and sufficiently high protein or ATP concentration the rate constant controlling ATP binding to subfragment 1 is greater than that controlling ATP cleavage. Chock et al. (1979) showed this by using similar techniques to those described here in experiments carried out at 0.01 M ionic strength, pH 7.0, and 15 °C. Their data suggested that the binding of ATP was so rapid that at 80 μM ATP the subfragment 1 active site was more than 80% saturated with tightly bound ATP within 13 ms, the first measurable mixing time of their quenched-flow apparatus. This implies that any substrate-induced isomerization represented in eq 1 by the process M·ATP $\rightleftharpoons$ M*·ATP is controlled by a rate constant >100 s^{-1}. Chock et al. (1979) also showed that the protein fluorescence change on mixing 80 μM ATP with subfragment 1 occurred as an exponential process with a rate constant of 28 s^{-1}. Their very extensive data collected at pH 7.0 and 15 °C provided an excellent basis on which to carry out the experiments described below. We reproduced their kinetics of P_i formation and of the protein fluorescence changes on mixing ATP with subfragment 1 at 1.5 mM $MgCl_2$ and 7.8 mM imidazole, although it was difficult to be certain whether or not there was a small fast component of the protein fluorescence changes occurring at >80 μM ATP. Thus it appears that under these conditions the major part of the observed protein fluorescence change does not correspond to the substrate-induced isomerization.

Measurement of tightly bound ATP formation in a series of cold-chase experiments is, however, a possible approach to determine the kinetics of ATP binding and hence of the proposed substrate-induced isomerization. In principle the most accurate measurement of protein-bound nucleotide (i.e., M*·ATP + M**·ADP·P_i) formation is obtained when subfragment 1 is in excess of ATP since under these conditions all the nucleotide will be bound in a single turnover experiment. A series of cold-chase experiments was carried out with subfragment 1 in 8-fold excess of ATP. Over the range of subfragment 1 concentration used (3–40 μM) the observed rate constant of nucleotide binding increased from 9 to 70 s^{-1} and did not reach a plateau. To see whether a plateau could be observed, it was necessary to have ATP at greater concentrations than subfragment 1 or otherwise excessive protein was required. Preliminary data together with that of Chock et al. (1979) suggested that the best experimental design was to make several observations at single time points since the crucial measurements would be at zero time and within a few milliseconds of mixing ATP with subfragment 1.

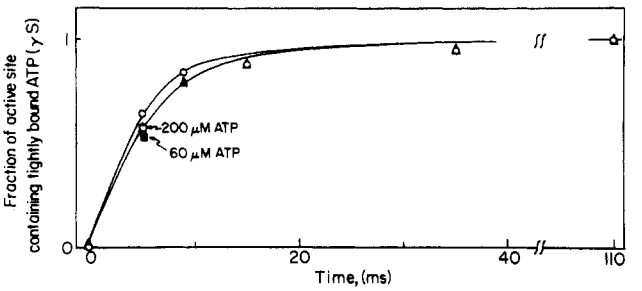

FIGURE 4: Fraction of subfragment 1 active sites bound with nucleotide after mixing with ATP(γS). 200 (○) and 60 μM (▲) [^{14}C]ATP(γS) were mixed with 40 μM subfragment 1 at 15 °C in 1.5 mM $MgCl_2$ and 7.8 mM imidazole adjusted to pH 7.0 with HCl. ATP(γS) was determined by the cold-chase technique. The fraction of nucleotide present as ADP in the zero time measurement was 10.5 ± 1.4%. The results are corrected for this ADP formation. Results from Table II are shown for comparison.

The results of one such experiment are recorded in Table II. The data show that at 5 ms approximately 50% of the subfragment 1 sites contained tightly bound nucleotide at both 60 and 200 μM ATP. This provides direct evidence that there is a substrate-induced isomerization associated with ATP binding. From the data in Table II the rate constant for the process, k_{+2} in eq 1, equals 170 s^{-1}.

This result is compatible with the observation of Chock et al. (1979) that 82% of the subfragment 1 contains tightly bound ATP at 13 ms when the protein is mixed with 80 μM ATP. We could not identify any slow phase in ATP binding; by 36 ms the rapid-binding phase was consistently 95 ± 2% complete. [Chock et al. (1979) reported that about 18% of the ATP binding was relatively slow with a $k_{obsd} \sim 20$ s^{-1}.]

The binding of ATP(γS) to subfragment 1 was analyzed in detail by the cold-chase technique, since the binding kinetics of this analogue provided much of the evidence on which the ATP-induced isomerization was identified originally (Bagshaw et al., 1974). As with ATP, studies were carried out with up to 30 μM subfragment 1 in excess of ATP(γS) with similar results.

We compared the kinetics of ATP(γS) binding to subfragment 1 with the nucleotide in excess at concentrations of 60 and 200 μM (Figure 4). The data show that the observed rate constant only increases by about 10% for a 3.3-fold increase in nucleotide concentration. Thus there is an ATP(γS)-induced protein isomerization controlled by a rate constant whose value is the same within experimental error as that found for ATP.

Nucleotide-Induced Protein Fluorescence Change. It was found that ATP(γS) induced less protein fluorescence change when added to subfragment 1 at 0.01 M ionic strength than when 1 M KCl was present in the solution (Figure 5). Thus the overall fluorescence change associated with ligand binding is ionic strength dependent, a result also shown by the work of Trybus & Taylor (1982). Addition of ATP to a solution of subfragment 1 containing bound ATP(γS) produced a further fluorescence change (as Figure 5) which, in view of the close similarity in the binding kinetics of ATP(γS) and ATP, can be assigned to the ATP cleavage step.

The kinetics of ATP(γS) binding to subfragment 1 were monitored by stopped-flow fluorescence at pH 7.0 and 15 °C in the same low ionic strength solvent as in Figure 5. The signal amplitude in the experiments was appreciably less than that predicted from the data in Figure 5. This point has been analyzed by Johnson & Taylor (1978) and is discussed below. The rate constant of ATP(γS) (and ATP) binding to subfragment 1 at low reagent (i.e., ~5 μM) concentration was

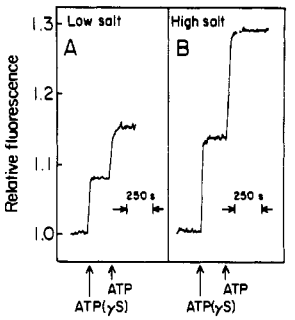

FIGURE 5: Dependence of the protein fluorescence change on salt concentration when ATP(γS) binds to subfragment 1. (A) 90 μM ATP(γS) was added to 2.0 μM subfragment 1 (A2 isoenzyme; Weeds & Taylor, 1975) in a solvent of 1.5 mM $MgCl_2$ and 7.8 mM imidazole at pH 7.1 and 15 °C. During the steady-state hydrolysis of ATP(γS) 200 μM ATP was added to the solution. (B) As in (A) except that the solvent contained 1.0 M KCl. Excitation was at 295 nm and emission at 340 nm. Under these conditions inner filter effects were negligible.

in the range (2.5–3) $\times 10^6$ M^{-1} s^{-1} for both nucleotides and was the same as that found by the cold-chase technique. A signal was also present at up to 200 μM ATP(γS) concentration, but even with computer averaging it was difficult to obtain reliable data in view of a poor signal to noise ratio. The apparent second-order rate constant for binding is comparable with the results of Chock et al. (1979) for ATP binding.

Discussion

The principal results we have described depend on a more detailed application of the cold-chase technique than has generally been applied in the analysis of mechanistic problems of the myosin ATPase. Under conditions when k_{+2} or $K_1k_{+2}/[ATP]$ is comparable with k_{+3}, the value of the technique becomes most apparent since it is difficult to apply spectrophotometric techniques alone to distinguish the intermediates and their rates of formation and decay. Furthermore the chemical nature of the approach allows for unambiguous characterization of the intermediates. Some of the apparent differences in results from various laboratories have arisen at least in part from the range of experimental conditions used. Thus we have taken care to reproduce conditions in other laboratories as closely as possible. When this is done, the results in general agree. There is also agreement with the conclusions of Johnson & Taylor (1978) and Chock et al. (1979) that over a relatively wide range of conditions it is possible to characterize the kinetics of the ATP cleavage step and that the observed rate constant for this process decreases with decreasing ionic strength.

When working at 0.01 M ionic strength at pH 7, we were able to reproduce the fluorescence stopped-flow observations of Chock et al. (1979) characteristic of the ATP interaction with subfragment 1, namely, that the rate constant of the observed signal equaled the measured rate constant of the cleavage step. This apparently straightforward result is surprising because the steady-state fluorescence measurement (Figure 5) suggests that some fluorescence signal is associated with ATP binding, which occurs at 150–170 s^{-1} above 60 μM ATP, and is thus well resolved from the cleavage step. As noted above we did observe indications of a faster phase in the protein fluorescence change. However, under different conditions, at pH 8, 20 °C, and 0.01 M ionic strength, the observed rate constant of the fluorescence change, 102 s^{-1} at 200 μM ATP, was almost 4-fold greater than the rate constant of the cleavage step (Geeves et al., 1979). In each case the fluorescence signals appeared to be monophasic exponential processes whereas biphasicity would have been expected. The problem may at least in part arise because the signal to noise ratios were insufficient to resolve the fluorescence into two phases. However, one cannot rule out that there is a more fundamental underlying cause. It could be that M·ATP has a fluorescence somewhat greater than M though this does not explain the above observation of Geeves et al., which was done under careful temperature control. Johnson & Taylor (1978) discuss this problem in considerable detail, and indeed at 5 °C they were able to detect biphasicity in the fluorescence signals. Using a thiol-modified subfragment 1, Sleep et al. (1981) were able to resolve and characterize the fluorescence changes associated with the ATP binding and ATP cleavage steps.

The slow phases observed in Figure 5 on adding ATP(γS) and ATP to subfragment 1 probably reflect the presence of more than one population of subfragment 1. This may have some bearing on the results of Johnson & Taylor (1978), who compared the fluorescence spectra of various forms of myosin and its proteolytic subfragments with and without bound nucleotide in a stopped-flow apparatus and a conventional spectrofluorometer. They were able to account for the smaller signal observed in the stopped-flow apparatus on the basis of the broad band-passes used. It does seem possible that some attenuation of the stopped-flow signal may also occur as a result of the slower phases illustrated in Figure 5 not appearing in the stopped-flow records.

This leads to the more general comment that one of the major difficulties encountered in working with subfragment 1 is that beside the fraction that gives the conventional phosphate transient (Lymn & Taylor, 1970) there is another fraction of subfragment 1 that gives no transient but that also has Mg^{2+}-dependent ATPase activity (Tonomura & Inoue, 1974). It is probably this fraction that contributes to many of the inconstant results that can occur with subfragment 1 such as are evident in the fluorescent signals and oxygen isotope studies (variable extents of oxygen exchange; Webb & Trentham, 1981). A still unsolved problem is to know the physiological significance, if any, of this fraction. However, it contributes greatly to the difficulty in knowing whether relatively small effects observed in the analysis of the subfragment 1 ATPase are or are not fundamental elements in the mechanism of the main protein fraction that exhibits the phosphate transient.

One of the most important postulates of the Lymn–Taylor model is that the ATP cleavage step is common to both the myosin and actomyosin ATPases. There is increasing evidence that this is so. The equilibrium constant for the ATP cleavage step is the same in either case (Eccelston et al., 1975), and the work described here indicates that the rate constant of the cleavage step is also unaltered. The data of Johnson & Taylor (1978) and Stein et al. (1981) show the same point at 10 and 15 °C, respectively. However, these data do not rule out the possibility that under appropriate conditions it is possible for ATP cleavage to occur when ATP is bound to an actomyosin complex (Inoue et al., 1979; Mornet et al., 1981; Stein et al., 1979, 1981).

Whether a "refractory" state exists in the actosubfragment 1 ATPase has been a problem of major interest in recent years (Eisenberg & Kielley, 1972; Taylor, 1979). Although the question is not yet resolved, there have been significant advances. Marston (1978), Wagner & Weeds (1979), and Wagner et al. (1979) have shown that at 20 or 25 °C the catalytic center activity of the actosubfragment 1 ATPase is only marginally greater if actin is limiting as catalyst compared to that if subfragment 1 is limiting. This means that the

predominant steady-state intermediate of the actosubfragment 1 ATPase is an actosubfragment 1 complex—probably an actosubfragment 1–products complex. At 0 °C the activity is almost 7-fold greater when actin is limiting, indicating a preponderance of an actin-free subfragment 1–nucleotide complex as the steady-state intermediate (Marston, 1978). However, the data of Marston (1978) and of Wagner et al. (1979) show that even at 20 °C a certain fraction of an actin-free subfragment 1–nucleotide complex contributes to the steady-state ATPase intermediate when subfragment 1 is limiting. The results in Figure 3 strongly suggest that M*·ATP is this intermediate at pH 8, 20 °C, and low ionic strength. However, at pH 8 and 15 °C data of Stein et al. (1979) suggest that the ATP hydrolysis step is too fast to permit this explanation. Further studies are needed, especially at 0 °C, to characterize the steady-state intermediate of the actosubfragment 1 ATPase more completely.

For a number of years there has been substantial spectroscopic evidence for an ATP-induced protein isomerization during myosin ATPase activity. However, the data of Chock et al. (1979) coupled with the other evidence that the ATP cleavage step significantly determines the rate of M**·ADP·P_i formation in transient kinetic experiments raised questions as to the validity of the arguments. The direct measurement of [M*·ATP] + [M**·ADP·P_i] formation with respect to time at various ATP concentrations gives new evidence that the isomerization does occur and that ATP(γS) mimics ATP binding very closely. Circular dichroism studies with ATP analogues (Eccleston & Bayley, 1980) and X-ray diffraction studies of muscle fibers (Goody et al., 1975) both strongly point to the similarity in detail between the protein-bound ATP (i.e., M*·ATP) and protein-bound ATP(γS) [i.e., M*·ATP(γS)] states.

Since the kinetics of ATP(γS) binding to subfragment 1 closely match those of ATP, it is probable that the magnitude of the fluorescence change associated with ATP(γS) binding mimics that of the M*·ATP state. In which case the data in Figure 5 show the change in fluorescence between native subfragment 1 and M*·ATP is likely to be similar to that between M*·ATP and M**·ADP·P_i. It is not clear what are the relative values of the fluorescence of M, M·ATP, and M*·ATP. This problem will most likely be solved by studies closer to 0 °C when the transformation M·ATP to M*·ATP will be slower. However, extrapolation from one set of experimental conditions must be done with caution in view of the large salt, temperature, and pH effects of several steps in the ATPase mechanism.

Finally since ATP cleavage can be temporally isolated from other elementary steps of the ATPase mechanism, it should now be possible to obtain greater chemical insight into the role of ionic strength, monovalent ions, pH, and temperature on the MgATP hydrolysis that appears to be associated with this step (Webb & Trentham, 1981).

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References

Bagshaw, C. R., & Trentham, D. R. (1973) *Biochem. J. 133*, 323–328.

Bagshaw, C. R., Eccleston, J. F., Trentham, D. R., Yates, D. W., & Goody, R. S. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 127–136.

Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., & Trentham, D. R. (1974) *Biochem. J. 141*, 351–364.

Bock, R. M., Ling, N. S., Morell, S. A., & Lipton, S. M. (1956) *Arch. Biochem. Biophys. 62*, 253–264.

Cardon, J. W., & Boyer, P. D. (1978) *Eur. J. Biochem. 92*, 443–448.

Chock, S. P., Chock, P. B., & Eisenberg E. (1979) *J. Biol. Chem. 254*, 3236–3243.

Eccleston, J. F., & Bayley, P. M. (1980) *Biochemistry 19*, 5050–5056.

Eccleston, J. F., Geeves, M. A., Trentham, D. R., Bagshaw, C. R., & Mwra, U. (1975) *Colloq. Ges. Biol. Chem. 26*, 42–52.

Eisenberg, E., & Kielley, W. W. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 145–152.

Geeves, M. A., Midelfort, C. F., Trentham, D. R., & Boyer, P. D. (1979) in *Motility in Cell Function* (Pepe, F. A., Sanger, J. W., & Nachmias, V. T., Eds.) pp 27–50, Academic Press, New York.

Glynn, I. M., & Chappell, J. B. (1964) *Biochem. J. 90*, 147–149.

Goody, R. S., & Mannherz, H. G. (1975) in *Protein-Ligand Interactions* (Sund, H., & Blauer, G., Eds.) pp 109–127, de Gruyter, Berlin.

Goody, R. S., Eckstein, F., & Schirmer (1972) *Biochim. Biophys. Acta 276*, 155–161.

Goody, R. S., Holmes, K. C., Mannherz, H. G., Barrington-Leigh, J., & Rosenbaum, G. (1975) *Biophys. J. 15*, 687–705.

Gutfreund, H. (1969) *Methods Enzymol. 16*, 229–249.

Hardman, M. J., Coates, J. H., & Gutfreund, H. (1978) *Biochem. J. 171*, 215–223.

Inoue, A., Takenaka, H., Arata, T., & Tonomura, Y. (1979) *Adv. Biophys. 13*, 1–194.

Johnson, K. A., & Taylor, E. W. (1978) *Biochemistry 17*, 3432–3442.

Lehrer, S. S., & Kerwar, G. (1972) *Biochemistry 11*, 1211–1217.

Lowey, S., Slayter, H. S., Weeds, A. G., & Baker, H. (1969) *J. Mol. Biol. 42*, 1–29.

Lymn, R. W., & Taylor, E. W. (1970) *Biochemistry 9*, 2975–2983.

Lymn, R. W., & Taylor, E. W. (1971) *Biochemistry 10*, 4617–4624.

Marston, S. (1978) *FEBS Lett. 92*, 147–151.

Mornet, D., Betrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306.

Sleep, J. A., & Taylor, E. W. (1976) *Biochemistry 15*, 5813–5817.

Sleep, J. A., & Hutton, R. L. (1978) *Biochemistry 17*, 5423–5430.

Sleep, J. A., Trybus, K. M., Johnson, K. A., & Taylor, E. W. (1981) *J. Muscle Res. Cell. Motil. 2*, 373–399.

Stein, L. A., Schwartz, R. P., Chock, P. B., & Eisenberg, E. (1979) *Biochemistry 18*, 3895–3909.

Stein, L. A., Chock, P. B., & Eisenberg, E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1346–1350.

Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 6*, 103–164.

Tonomura, Y., & Inoue, E. A. (1974) *Mol. Cell. Biochem. 5*, 127–143.

Trentham, D. R., Eccleston, J. F., & Bagshaw, C. R. (1976) *Q. Rev. Biophys. 9*, 217–281.

Trybus, K. M., & Taylor, E. W. (1982) *Biochemistry 21*, 1284–1294.

Wagner, P. D., & Weeds, A. G. (1979) *Biochemistry 18*, 2260–2266.
Wagner, P. D., Slater, C. S., Pope, B., & Weeds, A. G. (1979) *Eur. J. Biochem. 99*, 385–394.
Webb, M. R., & Trentham, D. R. (1981) *J. Biol. Chem. 256*, 10910–10916.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.
West, J. J., Nagy, B., & Gergely, J. (1967) *Biochem. Biophys. Res. Commun. 29*, 611–616.
White, H. D., & Taylor, E. W. (1976) *Biochemistry 15*, 5818–5826.

A Covalent Nicotinamide Adenine Dinucleotide Intermediate in the Urocanase Reaction†

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ABSTRACT: When imidazolepropionate, a competitive inhibitor of the nicotinamide adenine dinucleotide (NAD) dependent urocanase from *Pseudomonas putida*, binds to the enzyme, a chromophore is produced that exhibits an absorption maximum near 330 nm. In order to establish the identity of this chromophore, and particularly to determine whether this material might be enzyme-bound reduced NAD (NADH) formed by a hydride ion transfer from imidazolepropionate, fluorescence studies of the imidazolepropionate–enzyme complex were undertaken, along with isolation of the pyridine nucleotide coenzyme from the enzyme. The chromophoric material could be readily distinguished from NADH by its fluorescence emission spectrum and by its failure to react with pyruvate and lactate dehydrogenase after extraction of the coenzyme from urocanase. [^{14}C]Imidazolepropionate or urocanase containing [^{14}C]NAD^+ was used to generate a labeled enzyme–analogue complex. In both cases detergent treatment of the complex released a ^{14}C-labeled coenzyme that was then oxidized with phenazine methosulfate and purified by gel filtration and ion-exchange chromatography, whereupon it was established to be an addition product of NAD^+ and imidazolepropionate. Proton nuclear magnetic resonance spectroscopy of the oxidized pyridine nucleotide derivative revealed an attachment of the τ nitrogen of the imidazole ring of the analogue to the 4 position of the nicotinamide portion of NAD^+. The isolation of an oxidized NAD–imidazolepropionate addition complex provides support for a mechanism suggested earlier [Egan, R. M., Matherly, L. H., & Phillips, A. T. (1981) *Biochemistry 20*, 132–137] in which NAD^+ functions in urocanase catalysis through an intermediate involving the reversible addition of NAD^+ to a nucleophilic site on the imidazole ring of the substrate.

The recent identification of a very tightly bound NAD^+ (nicotinamide adenine dinucleotide) on the urocanases (EC 4.2.1.49) from *Pseudomonas putida* (Egan & Phillips, 1977; Keul et al., 1979) and bovine liver (Keul et al., 1979) has provided an opportunity for clarification of the chemistry presented by this unusual hydration reaction. During the urocanase-catalyzed addition of water across the conjugated system of urocanate to produce oxoimidazolepropionic acid (Figure 1), the hydrogen atom on carbon 5 of urocanate (I) is lost and hydrogen additions are made to the side chain α and β carbons plus carbon 4 in giving rise to the keto tautomeric form, oxoimidazolepropionic acid (II). Although hydride transfer involving transient NADH formation could be used to explain some of these hydrogen movements subsequent to water addition, such mechanisms are generally incompatible with the solvent origin of the hydrogens added to the side chain α and β carbons (Egan et al., 1981; Kaeppeli & Retey, 1971) and with the identification of the enol tautomer of oxoimidazolepropionic acid (III) as the true reaction product rather than the more stable keto form (Kaeppeli & Retey, 1971; Matherly & Phillips, 1981).

Hug et al. (1978) observed that the binding of imidazolepropionate, a substrate analogue, to urocanase gave rise to an increased UV absorbance with a maximum at 331 nm. The spectrum of the chromophore resembles that for borohydride-generated, enzyme-bound NADH (reduced NAD) [λ_{max} = 335 nm (Egan & Phillips, 1977)], but Hug et al. (1978) attributed this instead to an addition compound of imidazolepropionate with NAD. Also, incubation of enzyme with imidazolepropionate did not lead to irreversible inactivation, whereas treatment of urocanase with $NaBH_4$ did. Although imidazolepropionate is not hydrated by urocanase, both the analogue and urocanate undergo an enzyme-catalyzed exchange of the imidazole 5 hydrogen with solvent (Egan et al., 1981; Gerlinger & Retey, 1980). This would imply a similar mode of binding for the two compounds and suggests that a study of the chromophore produced upon imidazolepropionate binding might provide new mechanistic information regarding urocanase catalysis.

In this report, we present evidence that the chromophore generated on urocanase in the presence of imidazolepropionate is definitely not NADH but rather is a reversible covalent addition complex between the nicotinamide ring of the active site NAD and imidazolepropionate. A preliminary account of these findings has previously appeared (Phillips & Matherly, 1980). The following paper (Matherly et al., 1982) provides kinetic evidence that an analogous complex involving urocanate itself forms during catalysis.

Experimental Procedures

Chemicals. Deuterium oxide (100 atom %), urocanic acid, Sephadex G-15, beef heart lactate dehydrogenase, potassium

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