Enthalpy Changes for Intermediate Steps of the ATP Hydrolysis Catalyzed by Myosin Subfragment-1*

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The heat production during the ATP hydrolysis catalyzed by myosin subfragment 1 in 0.1 M KCl containing 0.01 M MgCl2 and 0.02 M Tris/HCl (pH 7.8) at 25°C was followed using a microcalorimeter with an improved time resolution. The results indicate that: (a) the binding of ATP to Subfragment 1 is exothermic (ΔH = -90 kJ mol⁻¹), all the ΔH values are corrected for protonation heat of Tris), (b) The actual cleavage of ATP on Subfragment 1 is endothermic (ΔH = +83 kJ mol⁻¹), which contrasts to the situation in free solution where ATP hydrolysis is exothermic, ΔH = -23 kJ mol⁻¹. Thus binding and cleavage together, which form the myosin-product complex at a rate which greatly exceeds the rate of decay of the complex (Pₐ release), is marginally exothermic (ΔH = -7 kJ mol⁻¹). (c) The slow Pₐ release is strongly exothermic (ΔH = -88 kJ mol⁻¹), as previously suggested by Yamada et al. (Yamada, T., Shimizu, H., and Suga, H. (1973) Biochem. Biophys. Acta 305, 643-653). (d) The dissociation of ADP from Subfragment 1 is endothermic (ΔH = +72 kJ mol⁻¹), being consistent with our recent result on the ADP binding to Subfragment 1 and heavy meromyosin (Kodama, T., Watson, I. D., and Woledge, R. C. (1977) J. Biol. Chem. 252, 8085-8087).

Thus in the myosin-catalyzed hydrolysis of ATP large enthalpy changes, as compared with the enthalpy change for the overall reaction, accompany several intermediate steps of the enzyme catalytic cycle. This is probably connected with the energy transduction role of myosin in muscle contraction.

Materials and Methods

Biochemical Procedures—S-1 prepared from rabbit skeletal muscle myosin as described by Weeds and Taylor (6), was precipitated by ammonium sulfate (65% saturation) and dissolved in a small volume of 0.1 M KCl, 20 mM Tris/HCl (pH 7.8). The concentrated S-1 solution was then dialyzed three times against 100 volumes of the same buffer. Protein concentrations were determined spectrophotometrically as previously described (5). The molecular weight of S-1 was taken as 1.15 × 10⁶ (7). ATP was purchased from P-L Biochemicals; ATPγS and AMP-PNP from Boehringer Mannheim. All nucleotides were used without further purification, but were analyzed by thin layer chromatography on PEI-cellulose plates (Macherey-Nagel) developed with 1.5 M K2HPO4 (8). Spots corresponding to the various adenosine derivatives were marked under incident UV light, and the cellulose powder of each spot was scraped and extracted with 0.1 M HCl. The extracts were neutralized with Tris, and then absorbance at 259 nm was measured. Total adenine nucleotide concentration was determined by measuring absorbance at 259 nm using ε = 15.4 (mM⁻¹ cm⁻¹). The steady state rate of ATP hydrolysis was measured in a reaction mixture containing 0.1 M KCl, 0.01 M MgCl₂, 20 mM Tris/HCl (pH 7.8), and 1 mM ATP at 25°C as previously described (4). The time course of the change in the ultraviolet difference spectrum of S-1 induced by ATP was studied at 288 nm in an Aminco DW-2 spectrophotometer in collaboration with Professor Y. Ogawa. The reaction mixture contained 0.1 M KCl, 0.01 M MgCl₂, 20 mM Tris/HCl (pH 7.8), and 15 μM S-1 in a total volume of 3 ml. The reaction was started by the addition of 30 μM ATP.

Calorimetric Experiments—Full details of the calorimeter used in the present work will be published elsewhere. In essence the wall of the cylindrical reaction vessel, diameter of 1 cm and total capacity of 2 ml, is a polyimide/Teflon film of 25-μm thickness. In close contact with the external surface of this wall is a silver-constantan thermopile with 15 to 20 junctions. The calorimeter was placed in a thermostated water bath (23°C). The reaction mixture of 1 ml was stirred continuously by vertical oscillations (5 Hz; amplitude, 5 mm) of a Teflon disk. The reaction was started by adding over a period of 1 s 4.2 μl of nucleotide solution from a motor-driven micrometer syringe. Additions of nucleotide to buffer solutions without protein in control experiments caused no detectable heat production. The calorimeter was calibrated by the reaction between half-neutralized Tris in excess phosphate; S-1, myosin Subfragment 1; M, the catalytic site contained in each S-1 head of myosin; PEI-cellulose, polyethyleneimine cellulose.
and a known amount of HCl. The heat of neutralization of Tris was taken to be $-47.5$ kJ mol$^{-1}$ (8). When 4 ml of heat was produced by this reaction the voltage output of the calorimeter rose with a time constant of 1.5 s to reach a maximum value of 0.5 μV. Heat loss from the calorimeter was exponential with a time constant of 350 s. Correction of data for heat loss and instrument lag were made by the methods of Hill (10), using 5-s time intervals.

RESULTS

Interaction of S-1 with ATP—When ATP is added to excess myosin it binds rapidly and essentially irreversibly (Reaction 1 in Fig. 1) and is cleaved, forming the $M\cdot ADP \cdot P_i$ complex (Reaction 2) at a rate which greatly exceeds the rate of its subsequent decay to $M\cdot ADP$ (Reaction 3) (11). For the conditions considered here the $M\cdot ATP$ which is formed would not dissociate because the equilibrium of Reaction 4 strongly favors the bound state (2, 4, 5). Thus the reaction can be divided into two phases, a rapid phase leading to the formation of $M\cdot ADP \cdot P_i$, and a slower phase leading to the formation of $M\cdot ADP$. Traces a in Fig. 2 show an observation of the heat produced during this reaction sequence, as recorded (A), after correction for instrumental distortion (B), and plotted semi-logarithmically (C). Essentially identical results were obtained in two observations as shown in Table I. The total heat produced was 153 kJ mol$^{-1}$, and as is apparent from the line drawn in Fig. 2C most of this heat (128 kJ mol$^{-1}$) was produced in a single exponential phase with a rate constant of 0.040 s$^{-1}$. In addition there was a smaller rapid phase which produced 25 kJ mol$^{-1}$. The identification of the slow phase with the dissociation of $M\cdot ADP \cdot P_i$ to $M\cdot ADP$ is confirmed by the observation that the rate constant is the same as the values (0.040 s$^{-1}$) obtained in a separate experiment in which the spectrophotometric change known to accompany this reaction (12) was followed, and is also similar to the steady state rate of ATP hydrolysis we have observed (0.042 s$^{-1}$). Thus this experiment suggests that $\Delta H_1 + \Delta H_2 = -25$ kJ mol$^{-1}$, and $\Delta H_3 = -128$ kJ mol$^{-1}$.

These results are consistent with the results obtained on addition of ATP to $M\cdot ADP$. The reaction sequence is similar to that just described but preceded by the dissociation of $M\cdot ADP$ (Reaction 4). The overall reaction is just the hydrolysis of ATP. As shown in Traces b of Fig. 2 there is a fairly rapid heat absorption at the start of this reaction sequence, followed by a slower heat production, bringing the total heat to 71 kJ mol$^{-1}$. The time course of the second slower phase is approximately the same as when M is the starting material (Traces a). But the first heat-absorbing phase is slow because its rate is controlled by the rather slow dissociation of $M\cdot ADP$ and competition of ADP with ATP for M. The graphic analysis shown in the semilog plot in Fig. 2C shows that the slow phase has a rate constant of 0.042 s$^{-1}$ and the intercept on the t = 0 axis is 144 kJ mol$^{-1}$. The fast phase is also approximately exponential and has a rate constant of about 0.2 s$^{-1}$ from the dotted line in Fig. 2C. For two successive exponential processes, a fast process (a), and a slow process (b), with rate constants $k_a$ and $k_b$, the intercept of a line drawn through the slow phase only will be $\Delta H_2/k_b/(k_a - k_b)$. Thus for $k_a = 0.042$ s$^{-1}$ and $k_b = 0.2$ s$^{-1}$, $\Delta H_2 = -115$ kJ mol$^{-1}$ in this case. A duplicate observation gave the same result (Table I). The following results are thus obtained: from the slow phase, $\Delta H_3 = -115$ kJ mol$^{-1}$; and by subtraction from the total heat, $\Delta H_4 + \Delta H_1 + \Delta H_2 = +44$ kJ mol$^{-1}$.

From a comparison of these two experiments a value of $\Delta H_1$ can be obtained. In the first experiment the total heat is $\Delta H_{ATP} - \Delta H_4$. In the second experiment the total heat is $\Delta H_{ATP}$ (the heat for the hydrolysis of ATP). Thus the difference (81 kJ mol$^{-1}$) is equal to $\Delta H_4$. This is in reasonable agreement with the result obtained at 12°C for this reaction by Kodama et al. (5), which is $\Delta H_4 = +83$ kJ mol$^{-1}$. From this result we can calculate another value for $\Delta H_1 + \Delta H_2$, which is $-37$ kJ mol$^{-1}$.

Thus these experiments yield values for $\Delta H_1 + \Delta H_2$, $\Delta H_3$, and $\Delta H_4$. For the second type of experiment, however, the $\Delta H$ values obtained depend on the value of the rate constant for the fast process which cannot be established precisely from these observations. Thus we prefer the values from the first type of experiment described for $\Delta H_1 + \Delta H_2$ and for $\Delta H_3$. The separate evaluation of $\Delta H_1$ and $\Delta H_2$ is not possible using ATP itself as the cleavage reaction is too fast (>160 s$^{-1}$)? Thus these experiments yield values for $\Delta H_1 + \Delta H_2$, $\Delta H_3$, and $\Delta H_4$. For the second type of experiment, however, the $\Delta H$ values obtained depend on the value of the rate constant for the fast process which cannot be established precisely from these observations. Thus we prefer the values from the first type of experiment described for $\Delta H_1 + \Delta H_2$ and for $\Delta H_3$. The separate evaluation of $\Delta H_1$ and $\Delta H_2$ is possible using ATP itself as the cleavage reaction is too fast (>160 s$^{-1}$)? (9). With these results we can calculate another value for $\Delta H_1 + \Delta H_2$, which is $-37$ kJ mol$^{-1}$.

Interaction of S-1 with ATPyS—Bagshaw et al. (13, 14) found that when ATPyS reacts with M the binding occurs at the same rate as for ATP, but its cleavage rate is reduced 1000-fold and product release takes place much faster. The cleavage step is thus the predominant rate-determining step in ATPyS hydrolysis. The $\Delta H$ for ATPyS binding to M can thus be found by analyzing the thermogram of the reaction of ATPyS with excess M, since the nucleotide can be expected to form M•ATPyS quantitatively and cleavage and dissociation of products then occur slowly. A typical thermogram for this reaction is shown in Fig. 3A. There is a large, rapid heat production followed by a slower heat production bringing the total to 135 kJ mol$^{-1}$ (Fig. 3B). Logarithmic extrapolation of the slower phase (Fig. 3C) leads to an estimate of 91 kJ mol$^{-1}$.

TABLE I

Summary of calorimetric experiments

<table>
<thead>
<tr>
<th>Experiments</th>
<th>N°</th>
<th>Heat produced</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Rapid phase</td>
<td>Slow phase</td>
</tr>
<tr>
<td>I. M + ATP</td>
<td>2</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>II. M + ATP</td>
<td>2</td>
<td>(44 ± 2) a</td>
</tr>
<tr>
<td>III. M + ATPyS</td>
<td>4</td>
<td>96 ± 5</td>
</tr>
<tr>
<td>IV. M + AMP-PNP</td>
<td>4</td>
<td>40 ± 4</td>
</tr>
</tbody>
</table>

2 See below for a discussion of the effects on this interpretation of the equilibrium constant of Reaction 2.

- Number of measurements.
- Heat absorption.
Fig. 2. Calorimetric records of heat production during the reactions of S-1 and ATP at 23°C. A, the thermograms. The reaction mixture contained 0.15 mM S-1, 100 mM KCl, 10 mM MgCl₂, and 20 mM Tris adjusted to pH 7.8 with HCl in a total volume of 1 ml. After thermal equilibration, ATP solution (4.2 μl) was added from a motor-driven syringe containing 18.6 mM ATP, 100 mM KCl, 20 mM MgCl₂, and 20 mM Tris (pH 7.8). Trace a is for the first addition of ATP. A very similar result (not shown) was obtained for the second addition. Trace b is for the fourth addition of ATP. A very similar trace (not shown) was obtained on the fifth addition. Traces denoted by a and b in B and C correspond to those a and b in A, respectively. B, corrected thermograms. Data from traces in A were analyzed by correcting for heat loss and instrument lag (10). The heat produced in the rapid phase of the heat production, and that the rapid phase consists of the heat of binding might be somewhat different. It does not seem likely, however, that the difference could be so great as to eliminate the striking contrast of this estimate of ΔH, to a rate constant of 0.2 s⁻¹. C, semilogarithmic plots of corrected thermograms. The arrow indicates the intercepts on the t = 0 axes of the extrapolated lines shown as dotted. The points (Δ) in b are the differences between extrapolated (— — — ) and observed (— — — ) values; the line drawn through them has a slope corresponding to a rate constant of 0.2 s⁻¹.

Interaction of S-1 with AMP-PNP—AMP-PNP is an ATP analogue which is not hydrolyzed by myosin ATPase but can dissociate actomyosin and is a competitive inhibitor with respect to ATP (15). The binding constant of the analogue to S-1 is about 10⁴ M⁻¹ (13) so that more than 99% of added nucleotide will be bound under the conditions employed. In a typical experiment, in which 51 nmol of AMP-PNP was mixed with 180 nmol of S-1 in the calorimeter, 4.8 ml of heat was produced with a time course hardly distinguishable from that for Tris/HCl interaction, indicating that all the heat was produced within the dead time of the calorimeter. The thin layer chromatographic analysis revealed the presence of a nucleotide (18%) which ran slightly faster than AMP-PNP on the PEI-cellulose plate. The nucleotide is probably AMP-PN resulting from the hydrolysis of the terminal phosphate of AMP-PNP as described by Yount et al. (16). The calorimetric result given in Table I was corrected assuming that the parameters for AMP-PN binding to S-1 are the same as for ADP. This yielded the ΔH value of −92 kJ mol⁻¹ for this analogue.

**DISCUSSION**

Energy Profile of the Myosin ATPase Reaction—Table II summarizes the calorimetric results obtained. ΔH can only be estimated by comparison with the results for the ATP analogues. The round value of −100 kJ mol⁻¹ is therefore taken for this quantity, and it must be admitted that, as ATP is cleaved so much more rapidly than the analogues studied, the heat of binding might be somewhat different. It does not seem likely, however, that the difference could be so great as to eliminate the striking contrast of this estimate of ΔH to the experimental value for ΔH₁ + ΔH₂ (−25 kJ mol⁻¹). It follows that the cleavage step is endothermic (ΔH₂ = +75 kJ mol⁻¹) in contrast to the situation for splitting of ATP in free solution which is of course exothermic (17).

Two corrections to the ΔH values tabulated are required before the signficance of the results can be discussed. The ΔH values are likely to be somewhat lower, and the calculated value for ΔH₁ + ΔH₂ may be even lower.
Some outstanding features of the energy profile emerge from the present work. Firstly, comparison of the ATP binding with the ADP binding is of interest, since in the myosin ATPase reaction the favorable free energy change of ATP hydrolysis in solution is expressed primarily in the difference between free energy changes for the binding of ATP and ADP to M (19). As shown in Table II and Fig. 4, a strongly favorable free energy change for the ATP binding is dominated by a large enthalpy change accompanying cleavage means a marked increase in

mixed with ATP, ADP, or ATPyS, Bagshaw et al. (20) estimated the fluorescence intensities relative to S-1 to be 1.10 for M-ATP (a larger value of 1.16 was recently obtained by Johnson and Taylor (21)) and 1.07 for M-ADP. Similar values were obtained by Werber et al. for M-ADP (22). In view of these results it seems that more detailed studies of nucleotide binding which can correlate spectral and other structural changes within the S-1 molecule with thermodynamic quantities may bring us to a deeper understanding of the role of myosin ATPase reaction in energy transduction during muscle contraction.

Secondly, it is apparent that the thermodynamic driving force for the rapid initial phase of the myosin ATPase reaction, M + ATP → M-ADP-Pi, is a large positive entropy change, which is ascribed to the peculiar nature of the cleavage step. Since this step is characterized kinetically as having a small equilibrium constant of 9 (18) which is equivalent to a small standard free energy change, a strong positive enthalpy change accompanying cleavage means a marked increase in

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**Table II**

<table>
<thead>
<tr>
<th>Reaction no. and Step</th>
<th>1 ATP binding</th>
<th>2 Cleavage</th>
<th>3 P dissociation</th>
<th>4 ADP dissociation</th>
<th>1+2+3+4 ATP hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔH (kJ mol⁻¹)</td>
<td>-25</td>
<td>-128</td>
<td>82</td>
<td>-71</td>
<td></td>
</tr>
<tr>
<td>ΔG° (kJ mol⁻¹)</td>
<td>-100</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected for K₈ = 9</td>
<td>100</td>
<td>163</td>
<td>196</td>
<td>182</td>
<td></td>
</tr>
<tr>
<td>Corrected for proton/buffer reaction</td>
<td>-90</td>
<td>83</td>
<td>-88</td>
<td>72</td>
<td>-23</td>
</tr>
<tr>
<td>Equilibrium constants (K)</td>
<td>From Table II of Ref. 2</td>
<td>7.9 × 10¹¹ M⁻¹</td>
<td>9</td>
<td>0.114 M</td>
<td>9.45 × 10⁻⁷ M</td>
</tr>
<tr>
<td>ΔG° (kJ mol⁻¹)</td>
<td>-68</td>
<td>-5</td>
<td>+5</td>
<td>+34</td>
<td></td>
</tr>
<tr>
<td>Corrected for proton/buffer reaction</td>
<td>-51</td>
<td>-5</td>
<td>-12</td>
<td>+6</td>
<td>-62</td>
</tr>
<tr>
<td>TΔS° (kJ mol⁻¹)</td>
<td>-22</td>
<td>+88</td>
<td>-93</td>
<td>+38</td>
<td></td>
</tr>
<tr>
<td>Corrected for proton/buffer reaction</td>
<td>-39</td>
<td>+88</td>
<td>-76</td>
<td>+96</td>
<td>+39</td>
</tr>
</tbody>
</table>

First of these is for the fact that the equilibrium constant of Reaction 2 is rather low, i.e. 9 (18). Thus the rapid phase of the reaction sequence on mixing M and ATP includes only about 90% of Reaction 2 and the slow phase does 10% of this reaction. The appropriate correction is made in the third row of the Table II. The second correction is for the heats of the reaction of the protons absorbed or released with the Tris buffer used. Bagshaw and Trentham (14) showed that at pH 8.0 1.0 mol of H⁺ is released concomitant with the Pᵢ dissociation (Reaction 3), and that about 0.2 mol of H⁺ is released by Reaction 1 and the same amount is taken up by Reaction 4. Using these values the correction is made in the fourth row, and the corrected ΔH values are then used to calculate TΔS°. The values of TΔS° and ΔG° are calculated for the usual standard state of 1 M, ADP = 10⁻³ M, ADP = 10⁻⁵ M, and Pᵢ = 10⁻³ M, which are pertaining to in vivo conditions of muscle and give values of more physiological interest. The energy and entropy profiles of the ATP hydrolysis reaction are shown in Fig. 4.

Some outstanding features of the energy profile emerge from the present work. Firstly, comparison of the ATP binding with the ADP binding is of interest, since in the myosin ATPase reaction the favorable free energy change of ATP hydrolysis in solution is expressed primarily in the difference between free energy changes for the binding of ATP and ADP to M (19). As shown in Table II and Fig. 4, a strongly favorable free energy change for the ATP binding is dominated by a large enthalpy change. The same applied to the ADP binding, but the cancellation effect of the entropy decrease is much larger in this case. The kinetic difference between M·ATP and M·ADP is that although both complexes are formed by two-step reactions

\[
\begin{align*}
M + ATP &\rightleftharpoons M\cdot ATP \rightleftharpoons M\cdot ADP \\
M + ADP &\rightleftharpoons M\cdot ADP \rightleftharpoons M\cdot ADP
\end{align*}
\]

where M·ATP and M·ADP are the binary complexes, the rate constant for M·ATP → M·ATP is much smaller than for M·ADP → M·ADP while other rate constants are similar for both cases (20). Thus ATP binds with a much higher affinity (K = 10¹ⁱ M⁻¹) than ADP (K = 10⁷ M⁻¹). These two complexes are also different in spectral properties. Based on the measurement of the protein fluorescence when S-1 was

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Reactivity Heat of Intermediate Steps of Myosin ATP Hydrolysis


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REFERENCES

Enthalpy changes for intermediate steps of the ATP hydrolysis catalyzed by myosin subfragment-1.
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