The interaction of myosin Subfragment 1 with ATP in 0.1 M KCl containing 0.01 M MgCl$_2$ and 0.02 M Tris/HCl (pH 8.0) was studied by microcalorimetry at temperatures of 4, 12, and 23°C so that values of the heat capacity change ($\Delta C_p$) could be obtained for intermediate steps of the ATPase cycle. The $\Delta C_p$ values are large compared to the value for the overall cycle, indicating that large changes in the hydrophobic effect are involved in transitions between different intermediate states. However, the heat capacity changes themselves show peculiar temperature dependences. Thus bindings of ATP and ADP to Subfragment 1, both of which are strongly exothermic processes, take place with large negative $\Delta C_p$ of about $-3$ kJ K$^{-1}$ mol$^{-1}$ between 4 and 12°C but with very small $\Delta C_p$ of 0.3-0.4 kJ K$^{-1}$ mol$^{-1}$ between 12 and 23°C. On the contrary, the $\Delta C_p$ for the endothermic hydrolysis of ATP bound to Subfragment 1 at 4°C is positive ($=1$ kJ K$^{-1}$ mol$^{-1}$) in the lower temperature range but strongly negative ($=-4$ kJ K$^{-1}$ mol$^{-1}$) in the higher temperature range. The magnitude of $\Delta C_p$ for the slow P$_i$ dissociation process is similar but its sign is just opposite to that for the hydrolysis. These anomalous changes in the heat capacity may be due to the temperature-induced changes in a balance between large opposing effects which result from distinct, local conformation changes within the Subfragment 1 molecule.

Kinetic and spectroscopic studies of the myosin-catalyzed hydrolysis of ATP have indicated that the myosin heads undergo a series of local conformation changes during the enzyme catalytic cycle (for reviews, see Refs. 1 and 2). Recently Kodama and Woledge (3) described calorimetric experiments on ATP hydrolysis catalyzed by myosin Subfragment 1 using a calorimeter with an improved time resolution. The results show that enthalpy changes, which are large compared with the enthalpy change for the overall reaction (ATP hydrolysis), are involved in the accompanying transitions between different intermediate states. In particular, the hydrolysis of ATP bound to SF-1 is strongly endothermic, in contrast to the reaction in free solution which is, of course, exothermic. The implication of this finding is that the hydrolysis of bound ATP is characterized by a large increase in entropy since the equilibrium constant for this step obtained by kinetic measurements is very small (4, 5). One possible explanation for the large entropy increase, which might be compatible with other evidence for the protein conformation change, would be a decrease in hydrophobic regions of SF-1 molecule exposed to water when the bound ATP is hydrolyzed. If this explanation is correct, we can predict that there should be a decrease in heat capacity for this reaction (3, 6).

In the present work, we have repeated the calorimetric experiments as previously described (3) at different temperatures so that heat capacity changes for intermediate steps of the ATP hydrolysis by SF-1 can be evaluated.

**EXPERIMENTAL PROCEDURES**

**Biochemical Procedures**—The buffer contained 100 mM KCl and 20 mM Tris (Sigma), and its pH was adjusted with HCl at 23 ± 1°C to such a value that at the temperature in the calorimeter it would be 8.0. Thus, when prepared the pH value of the buffer was 7.80 for experiments at 12°C and 7.60 for experiments at 4°C. SF-1 was prepared from rabbit skeletal muscle myosin by the method of Weeds and Taylor (7) and concentrated as previously described (3). The concentrated SF-1 solution (20-30 mg of protein ml$^{-1}$) was dialyzed three times against 100 volumes of the buffer with an appropriate pH value and stored on ice. Before use, MgCl$_2$ was supplemented to give a final concentration of 10 mM. The molecular weight of SF-1 was taken as 1,15 × 10$^5$ (8) with $\varepsilon$ of 7.5 cm$^{-1}$ (9). The ATP (P-L Biochemicals) solution consisted of 12-16 mM ATP, 100 mM KCl, 20 mM MgCl$_2$, and 20 mM Tris, and the pH was adjusted as described above. The total adenine nucleotide concentration of the ATPyS (Boehringer Mannheim) solution was also 12-16 mM in the buffer (pH 8.0) containing 100 mM KCl, 20 mM MgCl$_2$, 20 mM Tris, and 2 mM dithiothreitol. The purity of nucleotides was checked by thin layer chromatography on polyethyleneimine cellulose plates (Macherey-Nagel) developed with 0.5 mM LiCl/2 mM formic acid mixture (1-1, v/v) (3, 10). No detectable amount of ADP or AMP was found in the ATP solution. The presence of 3% ADP and 1% AMP was noted in the freshly prepared solutions of ATPyS. The nucleotide containing less than 5% ADP could be obtained by DEAE-Sephadex A-25 column chromatography with a triethylammonium bicarbonate gradient (11). This preparation was used in a later stage of the work. Total adenine nucleotide concentration was determined by measuring absorbance at 269 nm with $\varepsilon = 15.4$ mm$^{-1}$ cm$^{-1}$ at pH 7.5.

**Calorimetric Experiments**—An outline of the design of the calorimeter used has been described elsewhere (12). Methods for stirring the reaction mixture and starting the reaction were the same as described in a previous paper (3). Full details of the instrument will be published separately. The calorimeter was placed in a water bath thermostatted by circulating water containing 20% (v/v) ethylene glycol from a Haake F2C circulator. The temperature of the bath fluctuated within a range of ±0.02°C.

**Determination of Proton Release**—The SF-1 solution used in this experiment had been dialyzed against a buffer with a reduced concentration of Tris (5 mM). The pH changes were measured by using a Radiometer TTA60 titration assembly with a glass and calomel electrode pair. Nitrogen gas was flushed over the reaction mixture which was contained in a thermostatted glass vessel. The pH change generated by the addition of a known quantity of acid (10 µl of 4 M HCl) to the reaction mixture was used as a calibration value for the conversion of pH increments into moles of H$^+$ released. The turnover number for ATP and ATPyS hydrolysis was determined by using
these procedures. The final concentrations of ATP and ATPyS added were 0.1 and 6.0 mm, respectively. Measurements were made within a pH change of 0.1. The calculation was based on the fact that 1.0 mol of $H^+$ is released/mol of ATP hydrolyzed at pH 8.0. This stoichiometry also holds in the case of ATPyS hydrolysis (cf. Table II).

The Kinetic Scheme of ATP Hydrolysis by Myosin and the Reaction Heat for the Intermediate Steps—As a basis for the interpretation of the heat changes that occur when ATP or ATPyS is mixed with SF-1 in the calorimeter, the following scheme was used. The hydrolysis of ATP to ADP and Pi by SF-1 consists of at least six steps as revealed by kinetic studies (cf. Refs. 1 and 2) but can be simplified as follows in the present context:

$$1 \rightarrow 2 \rightarrow 3 \rightarrow 4 \rightarrow 5 \rightarrow 6$$

where $M$ is SF-1, $T$ is ATP, and $D$ is ADP. In this scheme, when ATP interacts with SF-1, the $M \cdot T$ complex is formed rapidly and essentially irreversibly (step 1). Then there occurs a rapid hydrolysis of ATP to form $M \cdot D \cdot P$, but the complexes $M \cdot T$ and $M \cdot D \cdot P$ are in equilibrium (step 2). The equilibrium constant $K_1$ ranges between 1 and 10, depending on the experimental conditions (5, 13). The hydrolysis is followed by very slow decay of $M \cdot D \cdot P$, to $M \cdot D$ (step 3). The equilibrium of the subsequent ADP dissociation (step 4) strongly favors the $M \cdot D$ complex (3, 14). Thus, when ATP is added to excess SF-1, a mixture of $M \cdot T$ and $M \cdot D \cdot P$, complexes is formed instantaneously in terms of the time scale of the present work. The heat produced by this rapid phase, $h_{fast}$, is described by Equation 1:

$$\Delta h_{fast} = h_1 + h_2$$

where $h_1$ and $h_2$ refer to the reaction heats for steps 1 and 2, respectively. It should be noted that $K_2/(K_1 + 1)$ can be theoretically approximated to the magnitude of the initial phosphate burst (2, 5).

Since the dissociation of $M \cdot D$ would be negligible under the conditions used in the present work, the heat produced during the slower phase, $h_{slow}$, is given by Equation 2:

$$\Delta h_{slow} = 1/(K_2 + 1) \cdot h_3 + h_3$$

where $h_3$ denotes the reaction heat for step 3. The rate of this slow heat production would be controlled by the rate constant of $K_3$. The heat produced ($h$) by time $t$ after the mixing of SF-1 with ATP is described by Equation 3:

$$h = \Delta h_{fast} + \Delta h_{slow} \cdot [1 - \exp(-K_2 \cdot t)]$$

Since the heat for the hydrolysis of ATP ($\Delta H_{ATP}$) is equal to the sum of the heats of four intermediate steps, the total heat produced ($\Delta H_{total}$) is expressed by Equation 4:

$$\Delta H_{total} = \Delta h_{fast} + \Delta h_{slow} = \Delta H_{ATP} - h_4$$

where $h_4$ is the reaction heat for step 4. This equation indicates that $h_4$ can be determined from $\Delta h_{total}$ to $\Delta H_{ATP}$ is evaluated in a separate experiment. Thus, it is clear that calorimetric experiments, in which ATP is mixed with excess SF-1, will yield estimations of $h_3$ and $h_4$, and also, provided that $K_3$ value is known, estimates of $h_1 + h_2$ and $h_3$.

Since separate evaluation of $h_1$ and $h_2$ is not possible using ATP itself, ATPyS is used to estimate the heat for step 1. The binding of this analogue is known to be as fast as that of ATP but its hydrolysis rate is reduced several hundred-fold (15-17). The heat for ATPyS binding to SF-1 can then be found by analyzing the thermograms of the reaction of ATPyS with excess SF-1 since the nucleotide can be expected to form $M \cdot T \cdot D \cdot P$ rapidly and quantitatively; hydrolysis and dissociation of products then occurs slowly. The rapid phase of heat production thus corresponds to step 1 alone. The method of analysis is essentially the same as for the reaction with ATP as described above (cf. Fig. 3).

**RESULTS**

Interaction of SF-1 with ATP—Typical thermograms of the interaction of ATP with excess SF-1 at different temperatures are shown in Fig. 1A. As can be seen, there is a rapid initial heat production followed by a slower heat production at each temperature, confirming our previous observation (3).

It can be seen that the heat produced during the rapid phase is greater at higher temperatures. To evaluate the values of $h_{fast}$ and $h_{slow}$, in Equation 3, data were plotted semilogarithmically after correction for instrumental distortion (Fig. 1B). The slow heat production can be fitted by a single exponential. Analysis of Fig. 1B gives estimates of $h_{fast}$, $h_{slow}$, and a rate constant for each temperature, which are listed in Table I.

The interaction of SF-1 with ATP was also investigated by measuring the $H^+$ release. A representative result (23°C) from this experiment is shown in Fig. 2. ATP was added to an excess of SF-1 under the same conditions used for calorimetry and the time course of $H^+$ release was measured. As can be seen, there is a rapid $H^+$ release followed by a slower release, bringing the total to 1.2-1.3 mol of $H^+$ released/mol of ATP added. Logarithmic extrapolation (Fig. 2B) leads to an estimate of 0.2-0.3 mol of $H^+$ released/mol of ATP for the rapid phase. ATP was also added to SF-1 saturated with ADP (trace b in Fig. 2A). In this case monophasic release of 1 mol

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1. The backward reaction in step 3 could be negligible since the concentration of $P_i$ was very low under experimental conditions.

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**Fig. 1.** Calorimetric records of heat production during the reaction of SF-1 and ATP at different temperatures. A, the thermograms. The reaction mixtures of 1 ml containing SF-1 (208 nmol at 4°C, 235 nmol at 12°C, 217 nmol at 23°C) were incubated in the calorimeter. After thermal equilibration, indicated amounts of ATP in 4.2 μl were added from a motor-driven syringe. --- ---, baselines assumed for calculation of the heats produced. B, semilogarithmic plots of corrected thermograms. Heats are expressed in kJ/mol of ATP added. Calorimetric experiments similar to those in A were repeated at each temperature. The thermograms were analysed by correcting for instrumental distortion (18). The points of 5-s time intervals refer to averaged results from multiple determinations. For the plot at each temperature, the linear part is extrapolated to intercept the heat axis for the estimation of $h_{fast}$ (see Equation 3).
TABLE I
Summary of calorimetric experiments on the reaction of SF-1 with ATP and ATPγS at different temperatures

The heat values were obtained by analyzing the semilogarithmic plots in Figs. 1B and 3B and show the averaged results of multiple determinations, the numbers of which are given in parentheses. The data from individual experiments were also plotted and analyzed in the same way, and the means and standard errors of the heat and rate constants were then calculated. The means were very close to the values obtained for the averaged results, and the standard errors are given. As for the results for ATPγS, a correction for the heat produced by binding of ADP (present as impurity in the ATPγS) to SF-1 was made as described in the text. Heat values are expressed in terms of moles of H+ released/mol of SF-1 site, and the values are summarized in Table I.

| Nucleotides | Temperature °C | Heat produced H+ released | Rate constant of slow phase s⁻¹ |
|-------------|----------------|----------------------------|-------------------------------|
| ATP         | 4 (5)          | 132 ± 2                    | 0.031 ± 0.001                 |
|             | 12 (6)         | 152 ± 2                    | 0.034 ± 0.001                 |
|             | 23 (8)         | 151 ± 2                    | 0.035 ± 0.002                 |
| ATPγS       | 4 (4)          | 125 ± 4                    | 0.044 ± 0.003                 |
|             | 12 (4)         | 149 ± 3                    | 0.065 ± 0.004                 |
|             | 23 (5)         | 147 ± 3                    | 0.17 ± 0.01                   |

ND, not determined since the rate of the slow phase was too fast to measure with a glass electrode.

TABLE II
Proton release by the interaction of SF-1 with ATP and ATPγS

Data were obtained from experiments similar to that shown in Fig. 2. In the case of the experiments with ATPγS, a part of the proton released during the interaction should have been attributed to the binding of ADP (present as impurity in the ATPγS) to SF-1. This was corrected by using the value of 0.26 mol of H⁺ released/mol of ADP bound to SF-1. The values are averages of results of duplicate determinations at each temperature.

| Nucleotide | Temperature °C | H⁺/ATP ratio Total | Rate constant of slow phase s⁻¹ |
|------------|----------------|--------------------|--------------------------------|
| ATP        | 4              | 0.28               | 0.038                          |
|            | 12             | 0.22               | 0.037                          |
|            | 23             | 0.26               | 0.037                          |
| ATPγS      | 4              | 0.23               | 0.045                          |
|            | 12             | 0.27               | 0.053                          |
|            | 23             | 0.27               | ND*                            |

* ND, not determined since the rate of the slow phase was too fast to measure with a glass electrode.

TABLE III
Effect of temperature on steady state rates of ATP and ATPγS hydrolysis

| Temperature °C | Turnover rate ATP s⁻¹ | ATPγS s⁻¹ |
|---------------|------------------------|-----------|
| 4             | 0.016                  | 0.010     |
| 12            | 0.022                  | 0.035     |
| 23            | 0.033                  | 0.16      |

of H⁺ at the same rate as in trace a was seen (Fig. 2B). These results are consistent with previous observations (1, 2, 19) that a fraction of a proton is released in the transient phase of SF-1/ATP interaction and 1.0 mol of H⁺/mol of SF-1 site is released at the same rate as for fluorescence decay and other phenomena associated with the dissociation of M·D·P to M·D + P. Similar experiments were made at different temperatures and the results are summarized in Table II.

Since the rate constants for the slow phase both in heat production and H⁺ release are very similar, the identification of the slow phase with the dissociation of M·D·P to M·D (step 3) is obvious. It is of interest to note that the rate constant of this step shows little temperature dependence between 4 and 23°C, and the value is comparable to the steady state rate at 23°C (Table III). At lower temperatures, however, the steady state rate becomes smaller than k₂ due to a marked temperature dependence of the rate of the process controlling ADP dissociation from SF-1, as already analyzed in detail by Bagshaw and Trentham (16). The rate constant of this temperature-sensitive process (step 4) was calculated by their method with the values shown in Tables I and III to be 1.2, 0.18, and 0.08 s⁻¹ at 23, 12, and 4°C, respectively. These values are in good agreement with those obtained by Bagshaw and Trentham (16).

In summary, the time course of heat production and H⁺ release during the interaction of SF-1 and ATP can be divided into two phases, the rapid phase leading to the formation of an equilibrium mixture of M·T and M·D·P₀ which is accompanied by a burst of heat and release of a fraction of a proton/SF-1 site, and the slower phase leading to the formation of M·ADP associated with a further large heat production and release of 1 mol of H⁺. Thus, our results conflict in part with the observation made by Swenson and Ritchie (20). They saw...
a rapid heat absorption and a subsequent slow heat production upon the addition of ATP to myosin. It remains very hard to account for this discrepancy at present.

Interaction of SF-1 with ATP$_7$S—This reaction was also studied at different temperatures by calorimetry (Fig. 3, Table I) and measuring the H$^+$ release (Table II).

The commercial samples of ATP$_7$S contained about 30% ADP but were first used without further purification. In the calorimetric experiments, therefore, a correction should have been made for the heat produced by binding of the ADP to SF-1. This was done with the use of the heat of ADP binding ($-\Delta H$) calculated by Equation 4 and the measured amount of ADP in the ATP$_7$S. The calculated heat due to ADP binding was then subtracted from the total heat and $h_{\text{corr}}$ (see below), and the results thus obtained are given in Table IV. A similar correction was made to the results of H$^+$ release measurement.

At a later stage in the work a column-purified ATP$_7$S preparation was brought into the reaction mixture by a single addition of ATP$_7$S to the nucleotide solution in the motor-driven syringe. The data from calorimetric experiments similar to those in Fig. 1A were pooled and analysed as described in Fig. 1B. The plots were made on the basis of total adenine nucleotide added.

**Fig. 3.** Calorimetric records of heat production during the reaction of SF-1 and ATP$_7$S at different temperatures. The experimental conditions were essentially the same as for Fig. 1 except that ATP was replaced with ATP$_7$S and the reaction mixture and the nucleotide solution in the motor-driven syringe were supplemented with 2 mM dithiothreitol. Total adenine nucleotide concentration in the syringe was 12.9 mM. The thin layer chromatographic analysis showed the presence of 31% ADP and 1% AMP in the ATP$_7$S used. Thus, 37 nmol of ATP$_7$S and 17 nmol of ADP should have been brought into the reaction mixture by a single addition of ATP$_7$S solution. A, the thermograms. B, semilogarithmic plots of corrected thermograms. The data from calorimetric experiments similar to those in A were pooled and analysed as described in Fig. 1B. The plots were made on the basis of total adenine nucleotide added.

**Table IV.**

| Temperature °C | Reaction steps | Heat production $kJ \text{ mol}^{-1}$ |
|---------------|----------------|----------------------------------------|
|               |                | a | b | c | d |
| 1             | ATP hydrolysis | 27 | 65 | 65 | 52 |
| 2             | ATP hydrolysis | -38 | -54 | -54 |
| 3             | ATP hydrolysis | 105 | 120 | 73 |
| 4             | ATP hydrolysis | -67 | -67 | -54 |
| 5             | ATP hydrolysis | 65 | 17 |
| 6             | ATP hydrolysis | 92 | 79 |
| 7             | ATP hydrolysis | -53 | -69 | -69 |
| 8             | ATP hydrolysis | 113 | 129 | 81 |
| 9             | ATP hydrolysis | -85 | -85 | -72 |
| 10            | ATP hydrolysis | 67 | 19 |
| 11            | ATP hydrolysis | 89 | 76 |
| 12            | ATP hydrolysis | -33 | -38 | -38 |
| 13            | ATP hydrolysis | 95 | 100 | 52 |
| 14            | ATP hydrolysis | -83 | -83 | -70 |
| 15            | ATP hydrolysis | 68 | 20 |

The effect of temperature on reaction heats for intermediate steps of ATP hydrolysis by SF-1

Table a shows the heat values obtained by experiments with ATP, and column b shows heats for steps 1 and 2 estimated by experiments with ATP$_7$S (Table I). In column c heats for steps 2 and 3 have been corrected with values of initial phosphate burst estimated from the data reported by Taylor (5). Column d shows heats for intermediate steps of the ATP hydrolysis by SF-1 after correction for the heats of the reaction of the protons released or absorbed with Tris buffer used. The calculation is based on the assumption that 0.26 mol of H$^+$ is released by step 1 and the same amount is taken up by step 4 and that 1.0 mol of H$^+$ is released concomitant with step 3. The protonation heat of Tris was taken to be $-47.5 \text{ kJ mol}^{-1}$ (25). The values of heat for ATP hydrolysis are from Ref. 24. Negative signs refer to "heat absorption (endothermic process)" for convention of this table and Fig. 4.

**DISCUSSION**

**Treatment of Calorimetric Data**

Table IV shows the calorimetric results, as obtained (columns a and b) and corrected (columns c and d). Several remarks are required before the implication of the results is discussed.

**Estimation of the Heats for ATP Binding and Hydrolysis**—Although the calorimeter used in the present work has a response time of about 1 s which is much faster than that of earlier designs (12), the hydrolysis is too fast even at low temperatures (25 °C). The thermograms show that the rapid phase leading to the formation of $M$-ATP$_7$S complex is associated with a large heat burst and a release of a fraction of a proton/SF-1 site. It should be pointed out that the rate of the slower phase leading to the end product, $M$-ADP complex, shows a marked temperature dependence.

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sequent dissociation of products. Since the binding heat is very similar for ATPyS and ADP regardless of the difference in structure of the phosphate group, we assume that the binding heat for ATP is also the same for these nucleotides: $h_1 = h_{\text{ref}}$ for ATPyS. Calculation of $h_2$ is then made by subtracting $h_1$ from the $h_{\text{ref}}$ obtained for ATP (column b in Table IV). It should be noted here that the validity of the assumption should be verified using ATP itself by further improvements of calorimeter, as difference in the terminal phosphate group between ATP and ATPyS may affect some other parameters as discussed by Chock et al. (21). In fact, Goody and Hofmann (17) also showed that the nucleotide is less effective for dissociation of acto/SF-1 than ATP, suggesting that the binding constant of ATPyS to myosin may be smaller than that of ATP, about $10^{-11} \text{ M}^{-1}$ (22, 23), although the effect need not be due only to the enthalpic term.

**The Initial Phosphate Burst**—The results obtained by Taylor (5) are used to estimate the values for $h_1$ and $h_2$ according to Equations 1 and 2 (column c in Table IV). This data was chosen because it covers a range of temperature similar to that in the present work and because the measurements were made by several methods so they seem the most reliable results at present available. The estimated values of the phosphate burst at 4, 12, and 23°C are 0.70, 0.77, and 0.86, respectively.

**The Heat for the ATP Hydrolysis** ($\Delta H_{\text{ATP}}$)—Accurate values of the $\Delta H_{\text{ATP}}$ are crucial in the interpretation of the calorimetric results, in particular for the calculation of the heat for ADP dissociation, $h_4$. As described elsewhere (24), the fragmented sarcoplasmic reticulum of rabbit or frog skeletal muscle has proved to be useful for determination of $\Delta H_{\text{ATP}}$ under various conditions, values which are now available for a range of temperature between 4 and 23°C. When ATP is mixed with excess SF-1 saturated with ATP in the calorimeter, the net reaction is the hydrolysis of ATP, as described in our previous paper (3). The values obtained in this way were 18 and 22 kJ mol$^{-1}$ at 4 and 23°C, respectively. These are reasonably close to the values obtained with the fragmented sarcoplasmic reticulum (see Table IV) and support the use of the latter values as $\Delta H_{\text{ATP}}$ values in the present work. Values of $h_4$ calculated with $\Delta H_{\text{ATP}}$ and $h_{\text{total}}$ for ATP (Equation 4) are given in column a in Table IV. At 12°C, $h_4$ is in good agreement with the result obtained by a calorimetric titration method (26). The results at 4 and 23°C are also confirmed by the same method.1

**The Release of H$^+$**—The release of H$^+$ is associated with the rapid phase of heat production is taken to be 0.26 mol of H$^+$/mol of ATP (the average value for results for ATP and ATPyS independent of temperature). It is not yet certain, however, whether this fractional release of H$^+$ may be ascribed to step 1 or to step 2. In the earlier papers by Bagshaw and Trentham (16) and Koretz and Taylor (27) it was suggested that the release of H$^+$ was associated with the binding rather than the hydrolysis of ATP. On the other hand, Chock (19) recently indicated that the rate of the H$^+$ release is equal to the rate of the hydrolysis which is slower than that of the two-step binding of ATP. However, here we tentatively assign the rapid H$^+$ release to the binding step since ATPyS was used to estimate $h_1$ and the rapid phase of its interaction with SF-1, which corresponds to the binding, is in fact accompanied by the H$^+$ release as shown in Table II. No matter what step is responsible for the rapid H$^+$ release, the same amount of H$^+$ should be reabsorbed when the M-D complex dissociates into $M + D$ (step 4) since at pH 8.0 the overall reaction of ATP, produces 1 mol of H$^+$/mol of ATP. Correction for the heat produced by interaction of the released protons with Tris has been made in column d of Table IV.

**Heat Capacity Profile of the Myosin ATPase Reaction**

The effect of temperature on the reaction heats for intermediate steps of the SF-1 ATPase reaction is shown in Fig. 4. Large variations in the reaction heats with temperature (heat capacity change) are apparent. In addition, the heat capacity changes themselves show peculiar temperature dependences. The slope of the temperature/heat curve for each intermediate step more or less reverses at 12°C, which is fortuitously very close to the actual deflection point of 13 ± 1°C indicated by our preliminary calorimetric experiments on the ADP binding to SF-1, done at smaller temperature intervals.

As can be seen in Fig. 4, the reaction heats for the binding of ATP and ADP to SF-1 (the latter is the reversal of ADP dissociation) becomes more strongly negative as temperature rises from 4 to 12°C. The $\Delta C_p$ is calculated to be about $-3 \text{ kJ K}^{-1} \text{ mol}^{-1}$, which is consistent with our previous result for the ADP binding to myosin obtained by a calorimetric titration method (26). The binding heats, however, show little temperature dependence between 12 and 23°C (within the range of experimental error, $\Delta C_p = 0$). On the other hand, the $\Delta C_p$ for the endothermic hydrolysis of ATP bound to SF-1 is positive in the lower temperature range ($\Delta C_p = 2 \text{ kJ K}^{-1} \text{ mol}^{-1}$) but strongly negative in the higher temperature range ($\Delta C_p = -3 \text{ kJ K}^{-1} \text{ mol}^{-1}$).

Of several possible sources the hydrophobic effect seems likely to make the greatest contribution to the heat capacity changes for reactions involving proteins (28). It is generally accepted that the hydrophobic effect results from the formation of cages of structured water of abnormally high heat capacity and low entropy around nonpolar groups (29-31). Thus, it seems plausible to assume in the following discussion that the large change in heat capacity during the SF-1 ATPase reaction cycle is a reflection of conformation changes in the SF-1 molecule which affect the extent of interaction between water and the protein hydrophobic groups.

Apart from the temperature dependence of the heat capacity changes, therefore, the present results suggest that large changes in the hydrophobic effect accompany several inter-
mediate steps of the enzyme catalytic cycle. In particular, the hydrolysis step at high temperatures is now characterized as having a large increase in entropy and a large decrease in heat capacity, indicating a decrease in the hydrophobic effect as previously suggested (3). In other words, the SF-1 molecule would probably take up a more compact conformation when the bound ATP is hydrolyzed, and the subsequent slow product dissociation would reverse the effect.

**Explanation of the Anomaly of Heat Capacity Changes**

An outstanding problem then is how to explain the temperature-dependent anomalies of the heat capacity changes for each intermediate step of the ATPase cycle. One should bear in mind that the magnitude and sign of the heat capacity change reflects a balance between the opposing effects which result from the distinct, local conformation changes of the SF-1 molecule in the accompanying transitions between different intermediates. (The same applies in theory to any other thermodynamic parameters.)

Reconstituting a three-dimensional image from the electron micrograph of the complex of actin filaments and SF-1 or heavy meromyosin molecules, Wakabayashi et al. (32–34) have recently shown that the myosin head is composed of several structural domains. In view of this structural information, we are led to propose a hypothesis which consists of three major elements to account for the present results. First, it is assumed that of these domains of the myosin head at least one is for binding and hydrolysis of nucleotide (catalytic domain) and that sequential changes in the conformation take place in this domain through catalysis. Second, the effect caused by a conformation change in the catalytic domain would be transmitted to the other domain(s) (effector domain), where it would probably induce the conformation change of the opposite effect. Third, certain structural distortion of peptide chains would be responsible for such a transmission of the conformation change. This mechanism could work at high temperatures but not properly below some critical temperature (<13°C).

According to the hypothesis presented here, the nucleotide binding presumably induces a conformation change in the catalytic domain with a decrease in the heat capacity over the temperature range studied in the present work. However, the effect would be cancelled by a change of the opposite effect induced in the effector domain at high temperatures (ΔC_p > 0) but not at lower temperatures (ΔC_p < 0), due to an ineffectiveness of the transmission mechanism. The reversal of the sign of heat capacity change for the ATP hydrolysis step below 12°C would also be ascribed to the lack of a large opposing change at low temperatures.

Although we cannot exclude other possible explanations of the present results, the multiple domain hypothesis with a concept of the cancelling effects between domains appears to be attractive as a means of understanding the energy transduction role of the myosin head in muscle contraction. Thus, this hypothesis could provide a way to see how the chemical energy of ATP is converted to conformation changes within the protein molecule which are in turn transformed to mechanical work through interaction with actin. It should be noted that Morales and Bots (35) recently proposed a very similar hypothesis for the interaction between the myosin head and actin in the presence of nucleotide.

**Acknowledgments**—I express my sincere gratitude to Professor Y. Ogawa for his interest and support which enabled this work to be done and Dr. R. C. Wolede for many helpful discussions and comments on the manuscript. I am also grateful to H. Harafuji for his assistance in protein preparation.

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