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A KINETIC STUDY OF THE ENERGY STORING ENZYME–PRODUCT COMPLEX IN THE HYDROLYSIS OF ATP BY HEAVY MEROMYOSIN

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SUMMARY

The hydrolysis of ATP by heavy meromyosin was studied by means of the measurement of the development of enthalpy. The results were compared with the rate of change in the intensity of the ultraviolet difference spectrum of heavy meromyosin. It is shown that as far as the enthalpy change is concerned: (1) most of the excess energy associated with ATP does not directly dissipate into the solution during the rapid hydrolysis of ATP in the initial stage of the reaction but is stored in stable form in an enzyme–product complex, (2) the ultraviolet difference spectrum of heavy meromyosin is due specifically to the reaction *via* the enzyme–product complex, suggesting that a local conformation of heavy meromyosin is changed because of the excess energy stored in the complex, and (3) the complex dominantly exists during the steady splitting of ATP.

INTRODUCTION

The reaction mechanism of the hydrolysis of ATP by myosin has been extensively studied by Tonomura and Oosawa[1]. The reaction mechanism proposed by them may be simplified as follows:

$$E+\mathrm{ATP}\rightleftharpoons E\cdot\mathrm{ATP}\rightarrow E+\mathrm{ADP}+\mathrm{P_i}+\mathrm{H^+}$$
$$\downarrow \qquad\qquad \uparrow$$
$$E\cdot\mathrm{ADP}\cdot\mathrm{P_i}\text{——}\text{┘}$$

Recently, Taylor[2] proposed a much simpler mechanism than this,

$$E+\mathrm{ATP}\rightleftharpoons E\cdot\mathrm{ATP}\overset{\mathrm{H^+}}{\rightleftharpoons}E\cdot\mathrm{ADP}\cdot\mathrm{P_i}\rightarrow E+\mathrm{ADP}+\mathrm{P_i}$$

No objection seems to be raised to the existence of a reaction process *via* the myosin–ADP–P_i complex, though there are still many problems about the reaction mechanism of myosin–ATPase in the presence of Mg^{2+}. Several lines of study have shown that ATP induces a change in a local conformation of the enzyme. Morita[4] and Morita and Yagi[3] found that a change produced by ATP in the ultraviolet absorption of the tryptophan region differed from that given by ADP. After depletion of ATP the change dropped to the value produced by ADP alone. ATP and ADP also differ in their effect on the fluorescence of ANS (8-anilino-1-naphthalene sulfonate) bound to

myosin[5]. The mobility of a spin label bound to a particular thiol group of myosin is increased by ATP[6]. A change in the electron spin resonance spectrum of the label persisted as long as ATP was present. It was observed recently that a change of the intrinsic fluorescence of tryptophan residues in heavy meromyosin, myosin and S-1 subfragment is largely increased by ATP and cannot be imitated by ADP[7].

One of the important problems to be studied at present will be the process of energy transfer from ATP in the reaction mediated by myosin. We report here a calorimetric study of the hydrolysis of ATP by heavy meromyosin. Our attention is particularly directed to the following points: whether or not the myosin–ADP–P_i complex stores excess energy and whether or not the conformation change of the enzyme induced by ATP is related with the excess energy. Our answer to the first question is that the enzyme–product complex formed *via* ATP hydrolysis stores most of the excess energy caused from ATP over a few minutes. A comparative study of the ultraviolet difference spectrum with the calorimetric measurement infers an answer to the second problem; that the conformational change of heavy meromyosin observed through the spectrum in the tryptophan region is due to the excess energy stored in the complex.

MATERIALS AND METHODS

Heavy meromyosin was prepared by the method of Lowey and Cohen[8]. Crude heavy meromyosin thus obtained was fractionated by precipitation in 45–55% saturated ammonium sulfate. Ammonium sulfate was extensively dialysed against 50 mM KCl and 20 mM Tris–HCl at pH 8.0. Heavy meromyosin was then lyophilized by the method of Yount and Koshland[9] in the presence of sucrose, and stocked in a desiccator in a cold room. The preparation was dissolved and dialysed against buffers to remove sucrose, and centrifuged for 2 h at $100000 \times g$ before use. All the data in the present paper were obtained from a single heavy meromyosin preparation.

ATP, ADP, trypsin and soybean trypsin inhibitor were obtained from Sigma Chemical Co. The concentration of the nucleotides in solutions was determined by their ultraviolet absorption using the value of 15.4 $cm^2/\mu mole$ at 260 nm (ref. 10). The concentration of heavy meromyosin was also determined by means of its ultraviolet absorption by utilizing the value of 647 cm^2/g at 280 nm (ref. 11). The molecular weight of heavy meromyosin was assumed to be 320000 (ref. 8). The concentration of phosphate was determined by the method of Martin and Doty[12].

All the calorimetric measurements were made by an LKB-8700 precision calorimeter at 20.0 ± 0.1 °C. The accuracy of the calorimeter was $\pm 0.1\%$ which was obtained by measuring the heat of reaction of Tris (Standard Reference Material 724) with HCl. The time constant of the apparatus was about 5 s for our experiments. After about 1 h thermal equilibrium was established between the heavy meromyosin solution of 100 ml in a reaction vessel, and the nucleotide solution of 0.8 ml in an ampoule. Then, the reaction of the two solutions was initiated by breaking the thin glass wall of the ampoule. The change in the temperature of the solution was monitored by a thermistor, and the unbalanced voltage in a Wheatstone bridge circuit was amplified and recorded. The energy equivalent of the calorimeter was calibrated by supplying a known amount of electrical energy to the built-in heater. The calibration was made before and after each reaction. The energy equivalent was un-

changed during reaction. The mean value of two different measurements was used for the analysis of the data. A typical recorder trace is shown in Fig. 1.

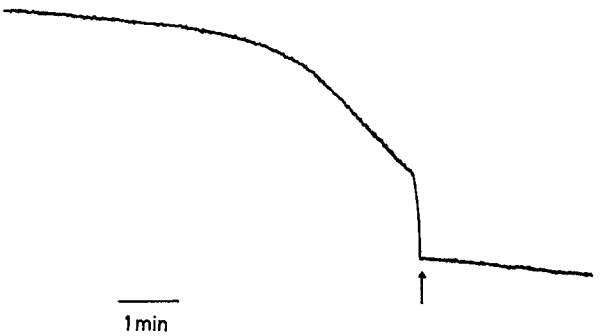

Fig. 1. Recorder trace illustrating the enthalpy development obtained by the mixing of ATP solution with heavy meromyosin solution in a precision calorimeter. The slope of the baseline reflects a small amount of heat influx into the reaction vessel from the external heat bath. The deviation from the baseline beginning at the position indicated by an arrow comes from the enthalpy developed from the reaction: 13.2 mg/ml heavy meromyosin, 165 μM ATP, 0.3 M KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C.

The enthalpy change due to the mixing of ATP with heavy meromyosin may be attributed mainly to the enthalpy of dilution and to the enthalpy of the hydrolysis of ATP. pH was maintained throughout the present experiment at 8.00 ± 0.02 by employing Tris–HCl buffer at 20 °C. The concentration of electrolytes and pH of the nucleotide solution were carefully adjusted as much as possible to make conditions equivalent to those of the enzyme solution, and to minimize undesired enthalpy change. In order to estimate the enthalpy of dilution including the effect of binding between heavy meromyosin and nucleotide, ADP solution of the same molar concentration as the ATP solution was used as a reference. It is noted here that phosphates were absent in the ADP solution, since the enthalpy and entropy due to phosphates liberated in the hydrolysis of ATP may be included in the free energy of the ATP solution.

A fast development of enthalpy was observed only at the initial stage when the ADP solution was mixed with the heavy meromyosin solution. Subtraction of the total amount of the enthalpy developed in the ADP–heavy meromyosin system from that in the ATP–heavy meromyosin system gave an enthalpy change of about -16 kcal/mole of nucleotide for all the experiments performed. The same value was also obtained when the ATP and ADP solutions, respectively, were mixed with the heavy meromyosin solution containing twice molar ADP. In addition, the value is almost the same as the enthalpy change expected from the hydrolysis of ATP in the same conditions, if the data (-16.2 kcal/mole of ATP) obtained by Podolsky and Morales[13] in which the enthalpy of the neutralization of protons yielded in the hydrolysis of ATP by buffer (-11.6 kcal/mole of proton) is included. On the other hand, the binding constant between heavy meromyosin and ADP is so large that a heavy meromyosin molecule can bind a maximum of two ADP molecules in a stable way after depletion of ATP. These results indicate that the net amount of

enthalpy for the hydrolysis of ATP is estimated reasonably well by subtracting the enthalpy developed in the ADP–heavy meromyosin system from that in the ATP–heavy meromyosin system.

The enthalpy development in the mixing of the ADP solution with the heavy meromyosin solution was also used to estimate the time resolution of the apparatus. No appreciable influence was given to our calorimetric observations from the time resolution of the apparatus which gave rise to an apparent delay of about 15 s for the overall development of the enthalpy in the reactions.

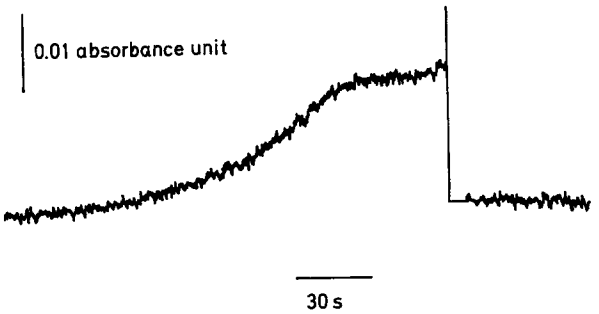

Fig. 2. Recorder trace illustrating the intensity change of the difference spectrum of heavy meromyosin at 288 nm in the reaction with ATP: 2.6 mg/ml heavy meromyosin, 32 μM ATP, 0.3 M KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C. The trace begins 10 s after the addition of ATP to heavy meromyosin.

We studied also the kinetics of the above reaction by measuring the ultraviolet difference spectrum of heavy meromyosin at 288 nm by a Cary model 14 spectrophotometer at 20 °C. Four matched 1-cm cells, two reference cells and two sample cells were used. A full scale sensitivity of 0.1 absorbance unit was employed. A typical recorder trace of the intensity change of the difference spectrum is shown in Fig. 2.

RESULTS

Calorimetric studies

The amount of enthalpy developed when the ATP and heavy meromyosin solution are mixed is shown in Figs 3a, 5a, and 7a together with the data for the mixing with the ADP solution. Both the KCl concentration and the relative concentration of ATP or ADP to heavy meromyosin were taken as parameters. The KCl concentration used was 0.1 and 0.3 M, which will be tentatively represented by the notation d (dilute) and c (concentrated). The relative concentration of ATP was equimolar, twice molar and four-fold molar with respect to heavy meromyosin, and will be referred by symbols I, II and IV, respectively. For instance, IVc means the four-fold molar ATP solution with 0.3 M KCl. The amount of phosphate liberated under the same experimental condition is also shown in Figs 3b, 5b and 7b for the cases I, II and IV, respectively.

For the sake of convenience of discussion the quantity $\log(\Delta H_t - \Delta H_\infty)$ is plotted *vs* time t in Figs 4, 6 and 8 for the cases I, II and IV, respectively, where ΔH_t refers to the amount of the enthalpy developed per mole of heavy meromyosin in the

reaction up to the time t and ΔH_∞ to the final amount of the enthalpy developed over the whole course of the reaction, both being corrected by the enthalpy of dilution.

The ATP in the solution was hydrolyzed completely by heavy meromyosin within 10 s in I. As indicated by Fig. 5b the size of the initial burst of the liberation of phosphate is about 1.3 moles per mole of heavy meromyosin in II. Fig. 7b shows that the early burst of phosphate amounts to 1.1 moles per mole of heavy meromyosin and is followed by a steady splitting of ATP whose rate is 0.038 s^{-1}.

A remarkable point in the development of enthalpy common to the cases I, II and IV is that no heat burst is observed corresponding to the early rapid hydrolysis of ATP after correcting the enthalpy of dilution. The enthalpy is gradually developed in a few minutes. Figs 4 and 6 indicated that the enthalpy is developed as a single exponential process,

$$\Delta H_t = \Delta H_\infty [1 - \exp(-\kappa t)],$$

where ΔH_∞ was -16.1 and -32.4 kcal/mole of heavy meromyosin for I and II, respectively, and κ was 0.019 s^{-1} for Ic and 0.033 and 0.020 s^{-1} for IId and IIc, respectively. In contrast to I and II, a steady development of enthalpy was observed in IV from the beginning of the reaction, by correcting the enthalpy of dilution and the time resolution of the apparatus. The thermal process shown in Fig. 8 may be represented as

$$\Delta H_t = \kappa_1 \Delta H_{ATP} t \qquad \text{for } t \leqq t_0$$

$$= \kappa_1 \Delta H_{ATP} t_0 + \Delta H^* [1 - \exp\{-\kappa_2 (t - t_0)\}] \qquad \text{for } t > t_0$$

where ΔH_{ATP} represents the enthalpy change of the hydrolysis per mole of ATP, ΔH^* the total amount of the enthalpy developed per mole of heavy meromyosin throughout the late exponential process, κ_1 and κ_2 the rate constant of the steady

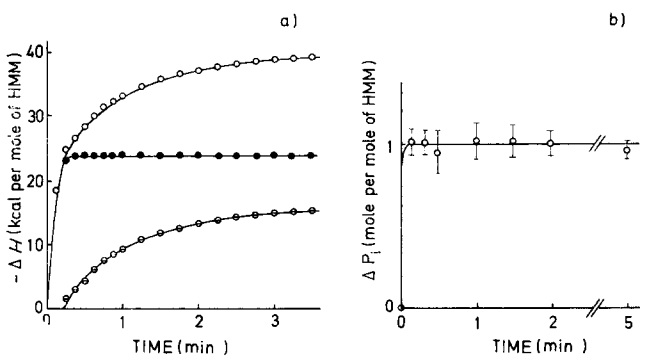

Fig. 3 (a) The development of enthalpy from the mixing of equimolar of ATP with heavy meromyosin (HMM) (○) and that from the mixing of equimolar of ADP with HMM (●). The difference of the two curves (⊖) represents the net amount of the enthalpy development associated with the hydrolysis of ATP by heavy meromyosin. It gives the enthalpy change of -16.1 kcal/mole of heavy meromyosin at the end of reaction, which is equivalent to the enthalpy change associated with the hydrolysis of ATP: 13.2 mg/ml HMM, 41 μM nucleotide, 0.3 M KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C. (b) The phosphate liberation from the mixing of equimolar of ATP with heavy meromyosin: 7.7 mg/ml heavy meromyosin, 24 μM ATP, 0.3 M KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C (three assays).

process and the late exponential process, respectively, and t_0 a time conveniently introduced to connect the steady and late exponential processes. The magnitude of ΔH^* was about -32 kcal/mole of heavy meromyosin and was nearly the same as the enthalpy change due to 2 moles of ATP. The time t_0 was about 60 s and κ_1 and κ_2 were estimated as 0.034 and 0.023 s^{-1} in IVc by assuming -16 kcal/mole for the enthalpy change for the hydrolysis of ATP as described in Materials and Methods.

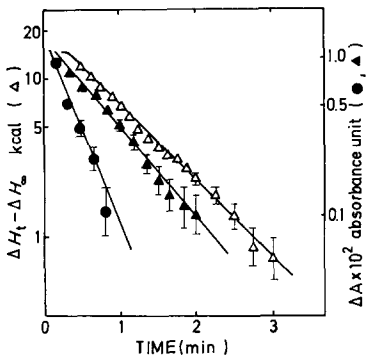

Fig. 4. A semilog plot of the difference between the enthalpy developed per mole of heavy meromyosin up to time t ($\triangle H_t$) and the enthalpy developed per mole of heavy meromyosin to the end of the reaction ($\triangle H_\infty$) after correcting the enthalpy of mixing shown in Fig. 3a (△); and a semilog plot of the difference $\triangle A$ between the intensity of the difference spectrum at 288 nm at time t ($\triangle A_t$) and that at the end of the reaction ($\triangle A_\infty$) in the case of the mixing of equimolar of ATP with heavy meromyosin in 0.1 M KCl (●) and that in 0.3 M KCl (▲): 2.6 mg/ml heavy meromyosin, 8 μM ATP, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C.

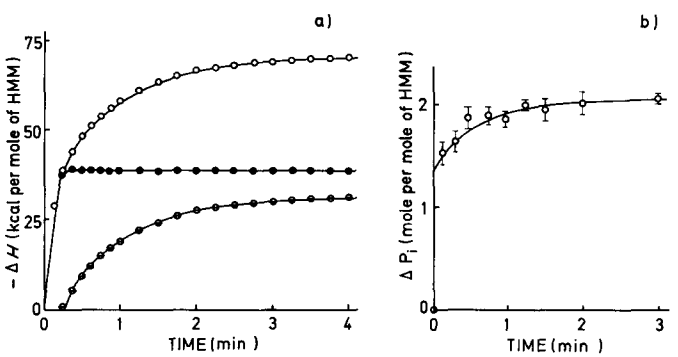

Fig. 5. (a) The development of enthalpy from the mixing of two-fold molar ATP with heavy meromyosin (HMM) (○) and that from the mixing of two-fold molar ADP with heavy meromyosin (●). The difference of the two curves (⊖) represents the net amount of the enthalpy development associated with the hydrolysis of ATP by heavy meromyosin. It gives the enthalpy change of -32.4 kcal/mole of heavy meromyosin at the end of reaction, which is equivalent to the enthalpy change associated with the hydrolysis of ATP: 13.2 mg/ml heavy meromyosin, 82 μM nucleotide, 0.3 M KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C. (b) The phosphate liberation from the mixing of two-fold molar ATP with heavy meromyosin: 7.7 mg/ml heavy meromyosin, 48μM ATP, 0.3 M KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C. The burst is about 1.3 moles of P_i per mole of heavy meromyosin (three assays).

The value 0.034 s^{-1} of κ_1 is nearly the same as the rate, 0.038 s^{-1}, obtained from the steady liberation of phosphate, and κ_2 is the practically the same as κ for II. The development of enthalpy in IVd was the same except κ_1, κ_2 and t_0, which were 0.070 s^{-1}, 0.038 s^{-1} and about 30 s, respectively.

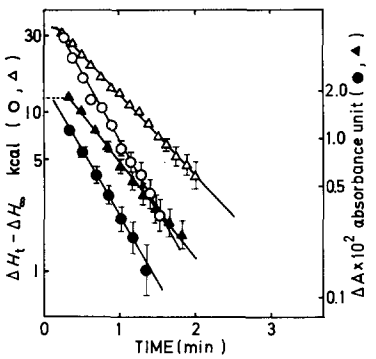

Fig. 6. A semilog plot of the difference between the enthalpy developed per mole of heavy meromyosin up to time t ($\triangle H_t$) and the enthalpy developed per mole of heavy meromysosin up to the end of reaction ($\triangle H_\infty$) in the mixing of two-fold molar of ATP with heavy meromyosin after correcting the enthalpy of mixing: in 0.1 M KCl (○) and in 0.3 M KCl (△). Other conditions as described in Fig. 5a. And a semilog plot of the difference $\triangle A$ between the intensity of the difference spectrum at 288 nm at time t ($\triangle A_t$) and that at the end of the reaction ($\triangle A_\infty$) in the case of the mixing of two-fold molar of ATP with heavy meromyosin in 0.1 M KCl (●) and that in 0.3 M KCl (▲): 2.4 mg/ml heavy meromyosin, 16 μM ATP, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C.

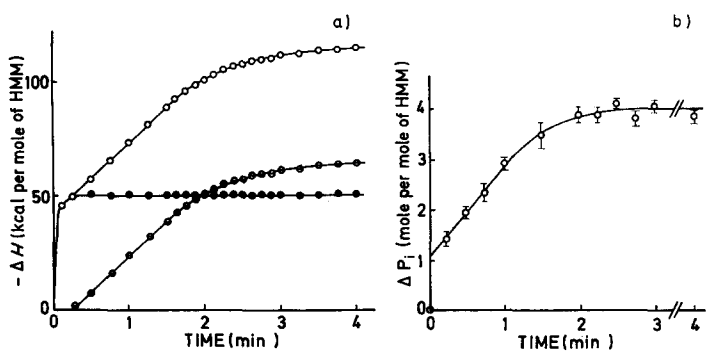

Fig. 7. (a) The development of enthalpy from the mixing of four-fold molar ATP with heavy meromyosin (HMM) (○) and that from the mixing of four-fold molar of ADP with heavy meromyosin (●). The difference of the two curves (⊖) represents the net amount of the development of enthalpy associated with the hydrolysis of ATP by heavy meromyosin. It gives the enthalpy change of −64.7 kcal/mole of heavy meromyosin at the end of the reaction, which is equivalent to the enthalpy change associated with the hydrolysis of ATP; 13.2 mg/ml heavy meromyosin, 165 μM nucleotide, 0.3 M KCl, 2 mM $MgCl_2$, and 20 mM Tris–HCl (pH 8.0) at 20 °C. (b) The phosphate liberation from the mixing of four-fold molar ATP with heavy meromyosin; 7.2 mg/ml heavy meromyosin, 90 μM ATP, 0.3 M KCl, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C. The burst is about 1.1 moles of P_i per mole of heavy meromyosin (three assays).

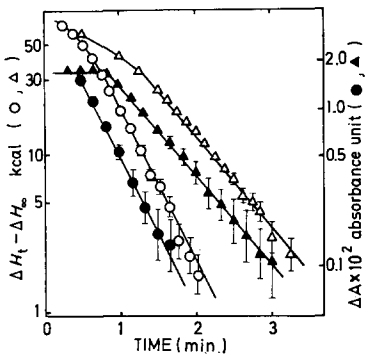

Fig. 8. A semilog plot of the difference between the enthalpy developed per mole of heavy meromyosin up to time t ($\triangle H_t$) and the enthalpy developed per mole of heavy meromyosin up to the end of the reaction ($\triangle H_\infty$), in the case of the mixing of four-fold molar ATP with heavy meromyosin after correcting the enthalpy of mixing: in 0.1 M KCl (○) and in 0.3 M KCl (△). Other conditions as described in Fig. 7a. And a semilog plot of the difference $\triangle A$ between the intensity of the difference spectrum at 288 nm at time t ($\triangle A_t$) and that at the end of the reaction ($\triangle A_\infty$) in the case of the mixing of four-fold molar ATP with heavy meromyosin in 0.1 M KCl (●) and that in 0.3 M KCl (▲): 2.6 mg/ml heavy meromyosin, 32 μM ATP, 2 mM $MgCl_2$ and 20 mM Tris–HCl (pH 8.0) at 20 °C.

Difference spectral studies

The ultraviolet difference spectrum of heavy meromyosin specific to the ATP hydrolysis persisted during the steady state as has been already observed by Morita[4], and it decreased after depletion of ATP to the level proper to ADP alone. The change of the intensity was studied at 288 nm for the sake of comparison under the same conditions as that of the corresponding calorimetric study except for the concentration of heavy meromyosin. The difference between ΔA_t, the absorbance at 288 nm of the difference spectrum at time t, and ΔA_∞, the value after depletion of ATP, in I, II and IV are plotted respectively in a semilog form in Figs 4, 6 and 8.

The difference spectrum shows a single exponential decrease,

$$\Delta A(t) = \Delta A_t - \Delta A_\infty = \Delta A_0 \exp(-\kappa t),$$

in I and II, whereas

$$\Delta A(t) = \Delta A_0 \qquad \text{for } t \leqq t_0$$

$$= \Delta A_0 \exp(-\kappa t) \qquad \text{for } t > t_0$$

in IV. ΔA_0 was about 0.013, 0.030 and 0.017 absorbance unit, in I, II and IV, respectively. The corresponding difference molar extinction coefficients were 1800, 3700 and 2100 $M^{-1} \cdot cm^{-1}$ for I, II and IV, respectively. The first value is nearly the same as the data obtained by Morita[14] in 0.1 M KCl and 2 mM $MgCl_2$ at 10 °C by means of a stopped-flow method. The second one is much larger than that in the steady state[4]. It may indicate that the exponential decrease of the intensity of the difference spectrum takes place slightly after the mixing (about 5–10 s). The last one is about the same with the value obtained by Morita[4] in the steady state in the presence of 10 mM $MgCl_2$ at pH 8 and 20 °C. The rate constant κ was 0.040 and 0.022 s^{-1} in Id and Ic,

TABLE I

RATE CONSTANTS OBTAINED FROM THE ENTHALPY DEVELOPMENT AND FROM THE DECREASE OF THE INTENSITY OF THE DIFFERENCE SPECTRUM

The rate constants described as $k(\triangle H_{exp})$ and $k(\triangle A_{228\ nm})$ are determined from the linear slope in the semilog plot of the enthalpy development and from that of the decrease of the intensity of the difference spectrum at 288 nm, respectively. The rate constant described as $k(\triangle H_{11near})$ is determined from the steady phase of the enthalpy development observed in the case of the mixing of four-fold molar of ATP with heavy meromyosin. In every cases measurements were made under 2 mM $MgCl_2$, and 20 mM Tris–HCl (pH 8.0) at 20 °C) varying KCl concentration. Heavy meromyosin concentration is about 13.2 mg/ml for the enthalpy measurements, and about 2.6 mg/ml for the measurements of the difference spectrum.

KCl (*M*)	*ATP/heavy meromyosin* (*mole/mole*)	$k(\triangle H_{exp})$ (s^{-1})	$k(\triangle A_{288})$ (s^{-1})	$k(\triangle H_{11near})$ (s^{-1})
(d) 0.1	(I) 1.0	—	0.040	—
	(II) 2.0	0.033	0.032	—
	(IV) 4.0	0.038	0.038	0.070
(c) 0.3	(I) 1.0	0.019	0.022	—
	(II) 2.0	0.020	0.023	—
	(IV) 4.0	0.023	0.023	0.034

0.032 and 0.023 s^{-1} in IId and IIc, and 0.038 and 0.023 s^{-1} in IV d and IVc, respectively. The time t_0 was about 30 and 50 s in IVd and IVc, respectively, and was about the same with that from the measurement of enthalpy. The values of κ from the difference spectrum show a good agreement with those from the calorimetric measurement as shown in Table I.

DISCUSSION

It has been well established that a small amount of ATP is immediately hydrolyzed when added to myosin and an enzyme–product complex, myosin–ADP–P_i, is formed[1,2,15–17]. Our data of phosphate liberation in Figs 3b, 5b and 7b show that ATP up to about one mole is immediately hydrolyzed by a mole of heavy meromyosin under the present conditions. However, the calorimetric measurements indicate that no detectable amount of enthalpy develops within the initial 15–20 s of the reaction, correlating with the initial rapid hydrolysis of ATP by heavy meromyosin when corrections are made for the time resolution of the apparatus. The development of enthalpy takes place gradually over a few minutes. It demonstrates that most of the excess energy originating from ATP is not directly transferred to the solution as heat during the initial rapid hydrolysis of ATP, but it is stored in the enzyme–product complex. The low rate of the development of enthalpy indicates that the enzyme–product complex with the stored energy (the energy rich complex) is thermally stable. It suggests that the excess energy is trapped in the complex molecule.

Morita[14] concluded by the analysis of the spectral change utilizing the equation of Chance[18] that the ultraviolet difference spectrum of heavy meromyosin specific to ATP hydrolysis arose from the enzyme–substrate complex. In the present experi-

ments the decrease of the intensity of the ultraviolet difference spectrum of heavy meromyosin is well correlated with the development of enthalpy, namely, with the relaxation of the excess energy stored in the enzyme–product complex. It is therefore reasonable to conclude that the major contribution to the change of the difference spectrum of heavy meromyosin specific to ATP hydrolysis under the present conditions comes from the enzyme–product complex storing the excess energy. The difference spectrum was attributed by Morita[4] to a red shift of the absorption spectrum of tryptophan residue. It may indicate again that the excess energy stored in the enzyme–product complex induces a local conformation change of the enzyme around the tryptophan residues. The trapping and localization of the excess energy may be suggestive that free energy is possibly stored in the energy rich complex.

Assuming that the difference spectrum specific to steady ATP hydrolysis arises mainly from the energy rich complex, we may conclude that the conformation of most of heavy meromyosin is also changed during the steady ATP splitting. This conclusion is consistent with the result that a local conformation is changed in the enzyme during the steady state obtained by the difference spectrum measurement[3,4]; by the fluorescence probe method[5]; by the ESR spectrum of the labeled spin[6] and by the study of the intrinsic fluorescence of tryptophan[7]. Thus it is possible that the energy rich complex dominantly persists during the steady state. This is further confirmed in the case IV where the late exponential process appears in the development of enthalpy and difference spectrum almost at the same time with nearly the same rate constant. This fact can be correlated with the disappearance of the energy rich complex from solution.

Our results confirmed most directly that the enzyme–product complex formed *via* ATP hydrolysis is different from that formed on the simple combination of ADP (*plus* P_i) with myosin, with respect to its stored energy.

Finlayson and Taylor[19] observed an early burst of proton in the hydrolysis of ATP by myosin, and proposed a reaction mechanism of myosin–ATPase where the proton liberation took place when the enzyme–product complex was formed. Our calorimetric measurements show no detectable amount of the enthalpy develops in the initial rapid hydrolysis of ATP. It leads to a conclusion that no appreciable amount of proton is liberated in the present cases in the initial rapid hydrolysis of ATP, since the enthalpy of neutralization of proton by the buffer is fairly a large exothermic reaction (see Materials and Methods). Thus our present findings are inconsistent with the reaction scheme of myosin–ATPase proposed by Taylor with respect to the step of the proton liberation. On the other hand, Tokiwa and Tonomura[20] observed a rapid absorption and a subsequent rapid liberation of the same amount of proton in the early phase of the hydrolysis of ATP by myosin. However, since the time constant of the present calorimetric measurements was about 5 s, it presents information only about a net change of the concentration of proton in the early phase. Our calorimetric measurements are consistent with the result obtained by a pH-stat measurement by Imamura *et al.*[21], indicating that no proton change occurred in the early phase.

The overall development of enthalpy, including the enthalpy of neutralization of proton produced in the reaction by the buffer, can be well expressed by a single exponential process in I. The excess energy stored in the energy-rich complex relaxes together with the neutralization of proton produced in the hydrolysis of ATP.

In other words, the major amount of proton yielded in the reaction is liberated, being coupled with the relaxation of the excess energy. The present result is, therefore, consistent with the route *via* forming the enzyme–product complex in the reaction mechanism by Tonomura and Oosawa[1]. By considering the reaction scheme of myosin–ATPase proposed by Tonomura and collaborators and that by Taylor and collaborators, the reaction process for the ATP hydrolysis by myosin *via* the enzyme–product complex may be described as follows:

$$E+\mathrm{ATP}\rightleftharpoons E\cdot\mathrm{ATP}\rightarrow E^{*}\begin{matrix}\nearrow \mathrm{ADP}\\ \searrow \mathrm{P_i}\end{matrix}\xrightarrow{\mathrm{H^+}} E\text{–}\mathrm{ADP}+\mathrm{P_i}\rightleftharpoons E+\mathrm{ADP}+\mathrm{P_i}$$

where the asterisk refers to the conformation changed state of the enzyme with stored energy. Recently Viniegra-Gonzalez and Morales[22] predicted the existence of the enzyme product complex storing energy in the myosin–ATPase. The present experiment demonstrates the existence of essentially the same species in the form of the enzyme–product complex.

It is remarkable that F-actin activates the process of the product release from the enzyme–product complex, myosin–ADP–P_i, in the reaction mechanism of actomyosin–ATPase proposed by Tonomura and Oosawa[1] as well as by Taylor and collaborators[2]. Considering the above discussions we propose that the excess energy stored in the enzyme–product complex is utilized in yielding work through the interaction between myosin and F-actin in muscular contraction.

McClare[23] recently presented a model for muscular contraction where the free energy from ATP was stored in the myosin head and was utilized to work through the interaction, by forming an excimer, with the actin fiber. Independently, we proposed a "convention model"[24,25] for muscular contraction in which a marginal state is assumed in each molecular engine composed of a cross bridge and F-actin, corresponding to a state storing a certain amount of the internal energy, the residual energy, that is not able to be changed to mechanical work. Only when excess energy is further given to the molecular engine, it is converted to the mechanical work. Our model presumes the existence of a marginal state to give an ordered motion to each molecular engine for continuous operation of muscle contraction.

In any case, the existence of energy storing state in the myosin head seems essential for the mechano-chemical conversion where the chemical energy is believed to be directly transferred to the mechanical work without being converted once in the form of thermal energy like in the case of conventional thermal engines. We propose that the excess energy of ATP is stored in the myosin head with high efficiency, making a marginal state there.

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