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THE PROCESS IN WHICH NUCLEOTIDE IS BURIED INTO THE ACTIVE SITE OF HEAVY MEROMYOSIN

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SUMMARY. The process in which nucleotide is buried into the active site of heavy meromyosin was studied with stopped-flow apparatus by monitoring the time-course of the large fluorescence increase of 1,N^6-ethenoadenosine triphosphate (ε-ATP) when it binds from acrylamide-containing solutions. We have recently reported that free ε-ATP fluorescence is effectively quenched by acrylamide while bound ε-ATP is resistent to quenching by acrylamide. In the present study it was found that in the first step the phosphate moiety binds at a high rate, while the adenine moiety is still on the rim of the active site; the adenine moiety is then pulled into a crevice, and finally ε-ATP hydrolysis occurs.

INTRODUCTION. Myosin ATPase kinetics has been extensively studied either by measuring ADP (or Pi) production (1) or by measuring absorption (2) and fluorescence (3) changes of a tryptophanyl residue in myosin. These studies have shown that there are several intermediate states between the initial (collisional) binding of ATP and the release of the product, ADP (4). However, it has remained unknown how ATP is buried into the crevice of the active site. Does the phosphate moiety of ATP bind first, and then the adenine ring? Is the adenine ring already buried in the crevice in the collisional binding step?

A neutral fluorescence quencher, acrylamide, has recently been employed to study exposure of tryptophanyl residues of several kinds of proteins (5). When a tryptophanyl group is deeply buried in the protein matrix, its fluorescence is resistant to quenching by acrylamide. And we have recently shown that in the steady-state myosin subfragment-1 ε-ATPase reaction conducted in an acrylamide solution the bound ε-ATP is highly fluorescent while free ε-ATP is much less fluorescent (6). That is, in the main ES complexes the ε-adenine ring of ε-ATP is well buried in the active site. So

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it is of interest to see how the ε-adenine ring is buried in the course of the enzymatic process. In the present work we studied the rate of fluorescence increase of ε-ATP (as a function of ε-ATP concentration) after mixing ε-ATP with heavy meromyosin (HMM) in the presence of acrylamide. We found that the fluorescence increases with time in a single exponential manner, and that the maximum rate at infinite concentration of ε-ATP is finite. That is, the fluorescence does not increase in a second order manner, but in a first order manner. In other words, in the collisional complex (second order process) the ε-adenine moiety is still exposed to the solvent. Therefore, we postulate that when ε-ATP collides with HMM, the ε-adenine moiety is still on the rim of the active site, is pulled into a crevice, and then hydrolysis occurs.

MATERIALS AND METHODS. Myosin was prepared from rabbit skeletal muscle (7). HMM was obtained by digesting myosin with α-chymotrypsin (8).

HMM Mg^{2+}-ε-ATPase activity was measured as descrived previously (6). ε-ATP was purchased from Molecular Probes.

The rate of formation of an HMM·ε-ATP complex, in which the ε-ATP fluorescence is resistant to quenching by acrylamide, was measured by monitoring the rate of fluorescence increase after mixing 2 μM HMM with various concentrations of ε-ATP in 0.2 M acrylamide, 20 mM TES (pH 7.5), 2 mM $MgSO_4$, 0.5 mM DTT and $KCOOCH_3$ (0.15 M or 1.0 M) at 21°C, using a Durrum *D*-117 stopped-flow spectrophotometer. 330 nm light was used for excitation, and emission at 400 nm (selected by an interference filter) was was observed. The trace of the fluorescence signal was stored on the Tektronix Type 2A63 storage oscilloscope, and photographed using Polaroid Type 42 film.

RESULTS. Fig.1(a) shows the trace of the fluorescence intensity change immediately after mixing 40 μM ε-ATP (final) and 0.2 M acrylamide with 2 μM HMM. The semilog plots of this fluorescence transient produced a fairly straight slope which gives the rate (see Fig.1(b)). The double-reciplocal plot of the rate of fluorescence increase, v_f, as a function of ε-ATP concentration, is shown in Fig.2. The extrapolation of the linear v_f^{-1} vs. $[\varepsilon\text{-ATP}]^{-1}$ plot does not go through the origin, but has a non-zero intercept. Therefore, this fluorescence transient derives from a first-order reaction process. Thus, before the quench-resistant enzyme-substrate complex, ES^*, is formed, there exists a previous enzyme-substrate complex, ES, which is susceptible to quenching. This ES must be formed very rapidly, since there is no lag-phase in the fluorescence transient. Moreover, as can be seen in

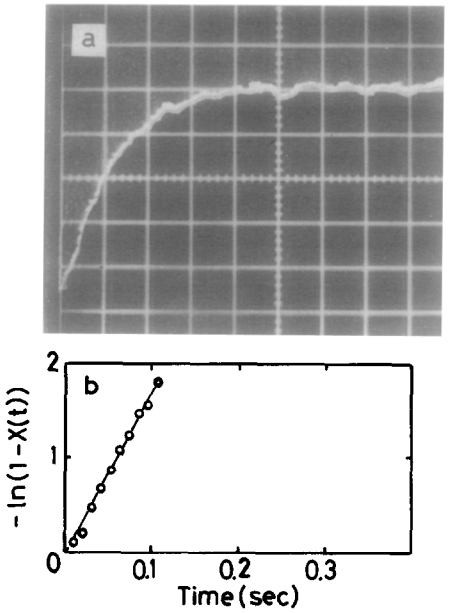

Fig.1(a): Memory oscilloscope trace of the fluorescence change after mixing HMM with ε-ATP in acrylamide. The fluorescence was measured at 400 nm when excited at 330 nm. One syringe contains 80 μM ε-ATP, 0.4 M acrylamide, 0.15 M $KCOOCH_3$ and buffer A (20 mM TES (pH 7.5), 2 mM $MgSO_4$ and 0.5 mM DTT). The other syringe contains 4 μM HMM, 0.15 M $KCOOCH_3$ and buffer A. Both solutions were thermostated at 21°C. X-Y scales are 50 msec/div. and 0.1 V/div., respectively. (b): Semilog plot of the above trace.

Fig.2, the apparent dissociation constant, K_f, (obtained from the X-intercept of the linear v_f^{-1} vs $[\varepsilon\text{-ATP}]^{-1}$ plot) is quite sensitive to the ionic strength, while the maximum rate of fluorescence change, V_F, seems to be insensitive. The higher ionic strength gave the larger K_f. Therefore the ES susceptible to quenching is likely to be a collisional complex. From these observations a following reaction mechanism is deduced:

$$E + S \underset{K_f}{\rightleftharpoons} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES^* \xrightarrow{k_3} E + \varepsilon\text{-ADP} + Pi$$

The following relations can be derived from the above reaction scheme:

$$v_f = k_{-2} + k_3 + \frac{k_2}{1 + K_f/[\varepsilon\text{-ATP}]} \quad (1)$$

$$V_F = k_2 + k_{-2} + k_3 \quad (2)$$

$$K_m = \frac{k_{-2} + k_3}{k_2} \cdot K_f \quad (3)$$

$$V_m = k_3 \quad (4)$$

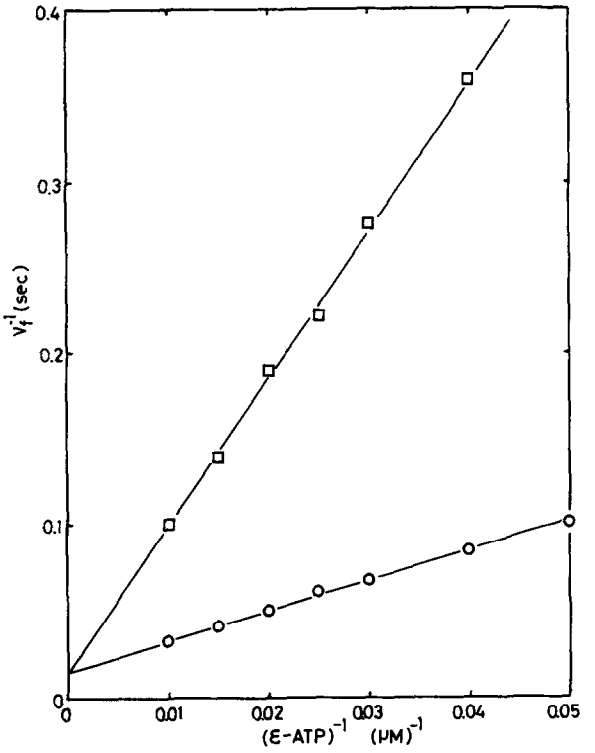

Fig.2: Double-reciprocal plot of dependence on ε-ATP concentration of the rate of ε-ATP fluorescence increase after mixing HMM with ε-ATP in acrylamide. The rates, v_f, were obtained from slopes of semilog plots as shown in Fig.1(b). Various concentrations of ε-ATP in 0.4 M acrylamide, 0.15 M $KCOOCH_3$ (○) or 1.0 M $KCOOCH_3$ (□) and buffer A were mixed with 4 μM HMM in 0.15 M $KCOOCH_3$ (○) or 1.0 M $KCOOCH_3$ (□) and buffer A at 21°C. Buffer A is same as that in Fig.1.

, where K_m and V_m are respectively the Michaelis constant and the maximum ε-ATPase activity. To complete the assignment of the rate constant, k_2, k_{-2}, and k_3 in the case of 0.15 M $KCOOCH_3$, the steady state HMM-ε-ATPase activities were measured in various concentrations of ε-ATP (see Fig.3). From Fig.2 and Fig.3, V_F, K_f, K_m and V_m were estimated to be V_F=66.7 s^{-1}, K_f=115.4 μM, K_m=4.5 μM, and V_m=0.476 (μmole Pi/sec·μmole myosin heads), respectively. From these values and relations (2), (3) and (4), k_2=64.2 s^{-1}, k_{-2}=2.0 s^{-1} and k_3=0.476 s^{-1} were obtained.

DISCUSSION. An ATP molecule consists of a relatively hydrophobic region, adenine (A), and a hydrophilic region, ribose-triphosphate (P). How do the two parts bind to the active site of myosin? Is A first, and then P, or the opposite? The present study suggests an answer to this question. The

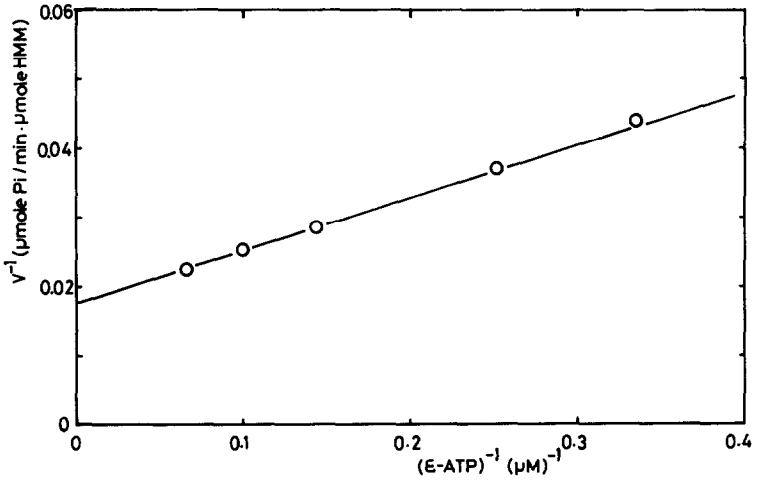

Fig.3: Lineweaver-Burk plot of HMM-Mg^{2+}-ε-ATPase activity. The reaction was started by adding HMM (0.075 μM final) to a solution containing various amounts of ε-ATP, 0.2 M acrylamide, 0.15 M $KCOOCH_3$, 2 mM $MgSO_4$, 2 mM DTT, 20 mM TES (pH 7.5), 14 μg/ml pyruvate kinase, 16 μg/ml lactate dehydrogenase, 1 mM phosphoenole pyruvate, 0.2 mM NADH at 21°C. The activity was measured by monitoring the 340 nm absorbance change of NADH.

transient from the exposed state of A to the buried state of A follows a first-order reaction. Therefore, there must exist a previous transition which follows a second-order reaction. We showed that the equilibrium constant of the previous transition is quite sensitive to ionic strength of the solution. Since P has some negative charges and the myosin active site has some positive charges (9), P-binding should be sensitive to ionic strength. Thus, we suggest that when ATP binds to HMM, initially P binds at a high rate, and then A is slowly pulled (at 64 s^{-1}) into the crevice site where acrylamide molecules cannot easily reach.

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REFERENCES

1. Trentham, D.R., Bardsley, R.G., & Eccleston, J.F. (1972) Biochem. J. 126, 635-644.
2. Morita, F., & Yagi, K. (1966) Biochem. Biophys. Res. Comn. 22, 297-302.
3. Werber, M.M., Szent-Györgyi, A.G., & Fasman, G.D. (1972) Biochemistry 11, 2872-2883.
4. Trentham, D.R., Eccleston, J.F., & Bagshaw, C.R. (1976) Quart. Rev. Biophys. 9, 217-281.
5. Eftink, M.R., & Ghiron, C.A. (1981) Anal. Biochem. 114, 199-227.
6. Ando, T., Duke, J.A., Tonomura, Y., & Morales, M.F. (1982) Biochem. Biophys. Res. Comn. 109, 1-6.
7. Tonomura, Y., Appel, P., & Morales, M.F. (1966) Biochemistry 5, 515-521.
8. Weeds, A.G., & Pope, B. (1977) J. Mol. Biol. 111, 129-157.
9. Miyata, H., & Asai, H. (1981) J. Biochem. 90, 133-139.