ENZYMATIC STUDIES ON THE INTERACTION OF MYOSIN AND HEAVY MEROMYOSIN WITH 1,N^6-ETHENOADENOSINE TRIPHOSPHATE (εATP), A FLUORESCENT ANALOG OF ATP

W. D. McCubbin, G. E. Willick and C. M. Kay

Department of Biochemistry, University of Alberta

Medical School, Edmonton, Canada

Received December 18, 1972

SUMMARY - The fluorescent analog of adenosine triphosphate (ATP)* 1,N^6-ethenoadenosine triphosphate, (εATP), has been utilized as a substitute for ATP in the myosin and heavy meromyosin ATPase systems. For myosin, the analog εATP replaced ATP with a somewhat larger K_m (2.6×10^{-4} mole ℓ^{-1} for εATP as opposed to 8.8×10^{-5} mole ℓ^{-1} for ATP), indicating that the apparent affinity of the enzyme for εATP is less than for ATP. Perhaps of more interest, further comparison yielded a V_{max} for εATP about two and one half times the value for ATP (20 μmole PO_4 sec^{-1} g $protein^{-1}$ as opposed to 8.1 μmole sec^{-1} g $protein^{-1}$). Results for the HMM-εATPase system were similar, yielding a K_m value of 1.47×10^{-4} mole ℓ^{-1} and a V_{max} of 54.2 μmole PO_4 sec^{-1} g $protein^{-1}$, as opposed to corresponding K_m and V_{max} values of 1.23×10^{-4} mole ℓ^{-1} and 20.4 μmole PO_4 sec^{-1} g $protein^{-1}$, respectively for the HMM-ATP interaction. The pH dependence of εATPase for both systems was comparable to ATP, suggesting a similarity in the mechanism of hydrolysis of the two nucleotides. Activation of εATPase by Ca^{2+} in the presence of 0.5 M KCl was comparable to ATPase for both systems, but inhibition by Mg^{2+} seemed to be more effective for εATPase. These results indicate that εATP is an excellent substitute for ATP in the myosin and heavy meromyosin systems and because of its insertion into the active site of these muscle proteins, it promises to be a very useful probe for conformation studies at this level.

The question of whether myosin undergoes a conformational change upon interaction with its substrate ATP has been a subject of considerable interest over the past few years. The most careful measurements of optical rotatory dispersion (1-3) and ultraviolet absorption spectra of myosin (3) as well as hydrogen exchange of heavy meromyosin (4,5) have failed to detect any gross conformational change as a result of interaction with ATP. Nevertheless, several lines of investigation show that ATP induces local changes in conformation in a rather unique manner. Seidel and

---

Abbreviations: adenosine triphosphate - ATP; 1,N^6-ethenoadenosine triphosphate - εATP; heavy meromyosin-HMM; ethenoadenosinetriphosphatase - εATPase; adenosinetriphosphatase-ATPase.

Gergely (6) have recently reported a transient shift in the ESR spectrum of spin-labelled myosin during the early stages of ATP hydrolysis which they interpret as resulting from a local structural transition in the globular head. Additionally, Werber et al. (7), by measuring the intrinsic fluorescence of the tryptophan residues of heavy meromyosin upon interaction with ATP, have concluded that the maximal fluorescence increase of 17% is caused by a local conformational change, associated with the enzyme-product complex.

Valuable information regarding changes in the conformation of myosin, under the influence of various reagents which modify the ATPase activity of the protein, has been ascertained by changes in energy transfer efficiency associated with the fluorescence of a macromolecular probe, 8-anilino-1-naphthalene sulfate bound to myosin (8,9). Because of the extreme sensitivity of this technique to small perturbations in the local environment of the label, it is desirable that the fluorescent probe be inserted in the region of the active site of the enzyme. Only in this way will any detectable conformational changes be relevant to tension generation in the muscle.

The possibility of specifically inserting a fluorescent chromophore at the active site of myosin has become a reality with the recent description of the synthesis of $1,N^6$-ethenoadenosine triphosphate, (εATP), a highly fluorescent analog of ATP (10,11). The biological activity of ATP appeared to be preserved, at least to a considerable extent, in εATP since it was found to be enzymatically active with hexokinase, myokinase and phosphofructokinase as well as exhibiting allosteric inhibition with phosphofructokinase (11). Prior to exploiting the excellent fluorescent properties of εATP with the myosin system, it was felt desirable to establish its effectiveness in replacing ATP as a substrate for both myosin and heavy meromyosin. Accordingly, the present investigation documents the pH dependence of εATPase for the two enzymes,

their corresponding V_{max} and K_m values contrasted with parallel measurements on the ATPase enzyme systems, the temperature dependence of V_{max}, and the activation of εATPase by Ca^{2+} and inhibition by Mg^{2+}. The results obtained are consistent with the view that εATP is an exceptionally versatile replacement for ATP in these muscle protein systems.

MATERIALS AND METHODS

ATP, as the disodium salt was obtained from either Raylo Ltd., Edmonton or Calbiochem, San Diego. εATP was synthesized by condensing chloroacetaldehyde with ATP in aqueous solution according to the procedure described by Secrist *et al.* (10,11). Purity of the εATP was established from the ultraviolet absorption spectrum of the compound, determined in aqueous buffer solution (0.025 M phosphate, pH 7.0) in a Cary 14 spectrophotometer; the 275 nm/265 nm ratio was identical with that reported in the literature (11). ATPase and εATPase activities were determined by electrometric titration, following proton liberation in the pH-stat (Radiometer TTT1, equipped with a titrator and titrigraph) during ATP or εATP hydrolysis in accordance with the equation:

$$(\varepsilon)\ ATP^{4-} + H_2O \longrightarrow HPO_4^{2-} + (\varepsilon)\ ADP^{3-} + H^+$$

The pH stat reaction vessel was stirred by passage of water from a large constant temperature bath through the jacket surrounding the vessel. Plots of proton liberation (base uptake) versus time were linear and the rates of dephosphorylation were inferred from the slopes of these zero order curves (12). Standard conditions for any one determination were: 0.5 M KCl, 10^{-3} M ATP or εATP, and 5 x 10^{-3} M $CaCl_2$ at pH 8 and 25°. Variations in these conditions are indicated. Rabbit skeletal myosin A and HMM were prepared by standard methods used in this laboratory (13,14).

RESULTS AND DISCUSSION

The dependence of myosin and HMM εATPase activities upon pH is

shown in Fig. 1. For both systems, the curves are very similar in shape to that obtained with ATP (15); however, there is an overall activation in

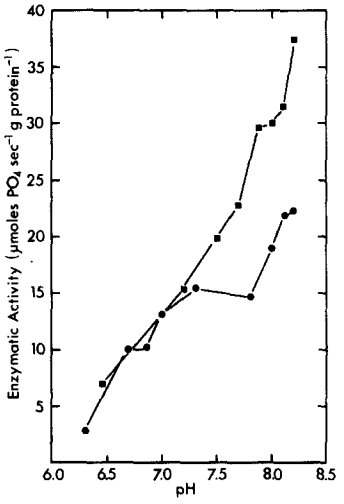

Fig. 1. pH dependence of εATPase for myosin (-●-) and HMM (-▪-), in the presence of 0.5 M KCl, 10^{-3} M εATP, 5 x 10^{-3} M $CaCl_2$, and at a temperature of 20°.

---

the rate of hydrolysis. The comparable form of the curves for both ATP and εATP suggests a similarity in the mechanism of hydrolysis of the two nucleotides, and probably involves the participation of the same groups at the active site of the enzyme in the two cases.

Figure 2 presents Lineweaver-Burk plots for both myosin and HMM εATPase and ATPase, from which the kinetic parameters V_{max} and K_m have been evaluated. These values are summarized in Table I, from which it may be noted that the K_m and V_{max} values for both myosin and HMM ATPase are in the range of other reported values (16,17). It is also significant that the K_m value for myosin εATPase is larger than that found under comparable conditions for myosin ATPase. The higher K_m value for myosin εATPase indicates that the apparent affinity of this enzyme for εATP is less than for ATP. The poorer binding may be a reflection of the principal structural difference

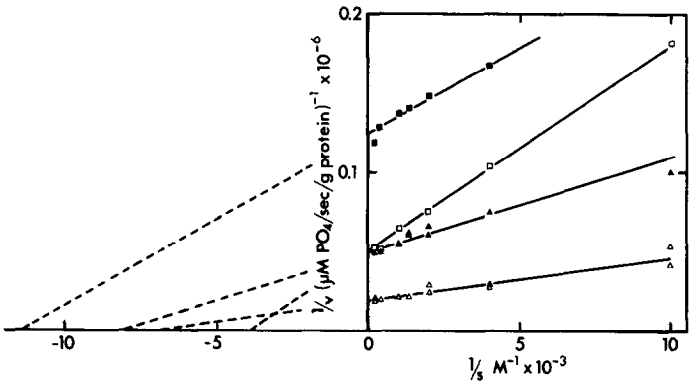

Fig. 2. Lineweaver-Burk plots of the reciprocal of the rate of hydrolysis, (1/v), as a function of the reciprocal of substrate concentration, (1/s), in the presence of 0.5 M KCl, 5 x 10^{-3} M $CaCl_2$, pH 8 at 25°C. Data are presented for myosin with: (a) ε-ATP (□) and (b) ATP (■) as well as HMM with: (a) ε-ATP (Δ) and (b) ATP (▲).

TABLE I

KINETIC DATA FOR MUSCLE PROTEINS

Enzyme	Substrate	K_m (mole ℓ^{-1})	V_{max} (μM phosphate sec^{-1} g $protein^{-1}$)
myosin	ATP	8.8 x 10^{-5}	8.1
	ε-ATP	2.6 x 10^{-4}	20.0
HMM	ATP	1.23 x 10^{-4}	20.4
	ε-ATP	1.47 x 10^{-4}	54.2

between ATP and εATP, viz., the masking of the N-1 and 6 NH_2 by the etheno bridge. At the same time one may conclude that since ATP is active in these systems, free N-1 and 6-NH_2 are not necessary for the binding of the nucleotide. The essential identity of the K_m values for both substrates

with heavy meromyosin, as opposed to their non-identity in the case of myosin, may well be a reflection of the fact that in the preparation of HMM, the nature of the active site is altered in such a way so as to be reflected in the relative binding strengths of these two substrates.

On the other hand, with both muscle protein systems, the V_{max} values for εATP are considerably higher than for ATP. For example, with myosin, at pH 8, the V_{max} for εATP is about 2.5 times the value for ATP (20 μmole phosphate sec^{-1} g $protein^{-1}$ as opposed to 8.1 μmole phosphate sec^{-1} g $protein^{-1}$). Perhaps the reason for this can be found in the lack of a free 6-NH_2 position in εATP, which in the case of ATP is postulated to interact strongly with the SH-bound intrinsic Mg^{2+} of the enzyme, and so slow down the rate of desorption of ADP (18,19) which is, of course, what V_{max} measures. With εATP, after P-O-P hydrolysis, desorption of εADP from the enzyme will tend to be more rapid and hence give rise to a larger V_{max}.

The variation of the velocity of εATP hydrolysis by myosin and HMM with temperature, at high εATP concentration, (10^{-3} M), was found to be linear in both cases, as depicted in Fig. 3. The heats of activation, ΔH_2^+

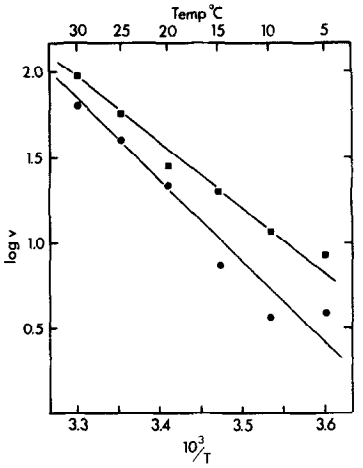

Fig. 3. Arrhenius plot of dependence of log velocity on 1/T for myosin (-●-) and HMM (-■-) in a medium containing 10^{-3} M εATP, 5 x 10^{-3} M $CaCl_2$, 0.5 M KCl at pH 8.0.

values, calculated from the slopes of these plots according to the equation: $\Delta H_2^+ = [-\frac{Rd(\ell nV_{max})}{d\frac{1}{T}}] - RT$, are 21.0 and 15.3 kcal mole^{-1} for myosin and HMM, respectively. These values compare with the figure of 12.4 kcal mole^{-1}, recorded by Ouellet, Laidler and Morales for skeletal myosin and ATP (20).

It is well known that Ca^{2+} is an activator and Mg^{2+} an inhibitor of myosin ATPase, in the presence of 0.5 M KCl. It therefore seemed logical to test the effect of these cations on myosin and HMM εATPase. The results of the experiments are portrayed in Table II, from which it will be noted

TABLE II

EFFECT OF Ca^{2+} AND Mg^{2+} ON THE εATPase ACTIVITIES OF MYOSIN AND HMM*

Metal Molarity	V_{max} (μM PO_4 sec^{-1} g protein^{-1})			
	Myosin		HMM	
	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}
10^{-6}	0.39	0.25	3.6	3.3
10^{-5}	0.41	0.21	5.4	
10^{-4}	7.7	0.18	6.6	3.3
10^{-3}	18.3	0.15	10.8	
10^{-2}	39.2	0.14	37.6	3.2

* Measurements made in a medium containing 0.5 M KCl, 0.1 M Tris, 2 x 10^{-3} M εATP at pH 8.2. Myosin studies were carried out at 25° and those on HMM at 20°C.

that Ca^{2+} produces an activation of εATPase with both muscle protein systems, in a manner reminiscent of that of myosin ATPase (12). On the other hand,

Mg^{2+} appears to be a more potent inhibitor with εATPase, maximum inhibition being achieved at much smaller Mg^{2+} concentrations than with the ATPase system (10^{-6} M with εATPase as opposed to 10^{-3} M with ATPase (12)).

In summary, the enzymic data presented indicate that the synthetic analog εATP can replace ATP as a substrate in the myosin and HMM ATPase systems. This is extremely important because it indicates that fluorescent labelling studies with this probe specifically inserted at the active site of these enzymes to monitor conformational changes, will have considerable relevance in terms of tension generation within muscle.

ACKNOWLEDGEMENTS

The authors are indebted to K. Oikawa and A. Keri for competent technical assistance. The financial assistance of the Medical Research Council of Canada, the Canadian Muscular Dystrophy Association and the Alberta Heart Foundation is gratefully acknowledged.

REFERENCES

1. Kay, C. M., Green, W. A. and Oikawa, K. Arch. Biochem. Biophys. 108, 89 (1964).
2. McCubbin, W. D., Kay, C. M. and Oikawa, K. Biopolymers 5, 236 (1967).
3. Gratzer, W. and Lowey, S. J. Biol. Chem. 244, 22 (1969).
4. Hartshorne, D. J. and Stracher, A. Biochem. 4, 1917 (1965).
5. Segal, D. M. and Harrington, W. F. Biochem. 6, 768 (1967).
6. Seidel, J. C. and Gergely, J. Biochem. Biophys. Res. Commun. 44, 826 (1971).
7. Werber, M. M., Szent-Györgi, A. G. and Fasman, G. D. Biochem. 11, 2872 (1972).
8. Cheung, H. C. Biochim. Biophys. Acta 194, 478 (1969).
9. Cheung, H. C. and Morales, M. F. Biochem. 8, 2177 (1969).
10. Secrist, J. A. III, Barrio, J. R. and Leonard, N. J. Science 175, 646 (1972).
11. Secrist, J. A. III, Barrio, J. R., Leonard, N. J. and Weber, G. Biochem. 11, 3499 (1972).
12. Brahms, J. and Kay, C. M. J. Biol. Chem. 238, 198 (1963).
13. Tonomura, Y., Appel, P. and Morales, M. F. Biochem. 5, 575 (1966).
14. Lowey, S. and Cohen, C. J. Mol. Biol. 4, 293 (1962).
15. Gilmour, D. Nature 186, 295 (1960).
16. Eisenberg, E. and Moos, C. Biochem. 9, 4106 (1970).
17. Schlisefeld, L. H. and Bárány, M. Biochem. 7, 3206 (1968).
18. Blum, J. J. Arch. Biochem. Biophys. 87, 104 (1960).
19. Hasselbach, W. Biochim. Biophys. Acta 20, 355 (1956).
20. Ouellet, L., Laider, K. J. and Morales, M. F. Arch. Biochem. Biophys. 39, 37 (1952).