EQUILIBRIUM DIALYSIS BINDING STUDIES OF 1,N^6-ETHENOADENOSINE DIPHOSPHATE (εADP) TO MYOSIN, HEAVY MEROMYOSIN, AND SUBFRAGMENT ONE

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SUMMARY: The binding of the fluorescent analog of adenosine diphosphate (ADP)*, 1,N^6-ethenoadenosine diphosphate (εADP) to myosin and its subfragments, heavy meromyosin (HMM) and subfragment one (S1), has been studied under analagous conditions to those previously used in comparable studies on the binding of ADP to these molecules. The results indicate that there are two binding sites for εADP on myosin and HMM, and one site on S1. The dissociation constants for all had an identical value, within experimental error, of 2.0 (± .5) x 10^{-5} M^{-1}. This is identical to the values found by Young (J. Biol. Chem., 242, 2790 (1967)) for ADP. In addition, the kinetics of hydrolysis of εATP versus ATP by S1 were studied. Values of V_{max} and K_m were 25 μM phosphate sec^{-1} (gm protein)$^{-1}$ and 5 x 10^{-5} M^{-1} for ATP, and 80 μM phosphate sec^{-1} (gm protein)$^{-1}$ and 45 x 10^{-5} M^{-1} for εATP. The results indicate that the increased V_{max} that occurs when εATP is used as a substitute for ATP is not due to either an increased binding affinity of ATP for myosin and its subfragments, nor due to a decreased binding affinity of εADP versus ADP. This in turn suggests that the increase in V_{max} may be due to an increased hydrolytic rate of εATP vs ATP in the enzyme substrate complex.

In a previous communication (1), we showed that the fluorescent analog of ATP, 1,N^6-ethenoadenosine triphosphate (εATP), would substitute for ATP as a substrate for myosin and its subfragment, heavy meromyosin (HMM). Various parameters which characterize this reaction, such as pH and temperature dependence, and the effect of the divalent cations, Ca^{2+} and Mg^{2+}, were found to be similar, thereby suggesting that the mechanism of the ATP and εATP phosphorolysis were similar.

However, the V_{max} of εATP hydrolysis versus that of ATP was about 3-fold larger, suggesting that εADP might be less tightly bound than ADP, since this could be the rate limiting step in the reaction. In addition, the

*Abbreviations: adenosine diphosphate and triphosphate—ADP and ATP; 1,N^6-ethenoadenosine diphosphate and triphosphate—εADP and εATP; heavy meromyosin—HMM; subfragment one—S1; ethenoadenosinetriphosphatase—εATPase; adenosinetriphosphatase—ATPase.

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K_m for εATP was about 3-fold less than with ATP in the case of myosin, but essentially identical in the case of HMM. This latter observation is somewhat puzzling, since it suggested there could be an alteration in the active sites when HMM is prepared from myosin.

In order to interpret the kinetic results, as well as utilize fully the potential of this fluorescent analogue in studying the mechanism of the myosin ATPase activity, we have carried out an additional study of the binding of εADP to myosin, HMM, and subfragment one (S1). The solvent conditions used were those used in the comparable studies on the binding of ADP carried out by Young (2) and Lowey and Luck (3), in order to facilitate comparison with the previously published work on ADP binding.

The results will show that the number of binding sites to myosin and the subfragments are the same with εADP as ADP, and perhaps surprisingly, that the binding constants are the same for either substrate, within experimental error.

MATERIALS AND METHODS

The disodium salt of ATP was purchased from Raylo Ltd., Edmonton. εATP was synthesized according to the procedure of Secrist et al. (4). The ATPase and εATPase activities of the S1 preparations were measured as previously described (1). The standard solvent for the kinetic assays had the following composition: 0.5 M KCl, 10^{-3} M ATP or εATP, and 5×10^{-3} M $CaCl_2$ at pH 8 and 25°. Rabbit skeletal myosin A and HMM were prepared as previously described (5,6). S1 was prepared according to the method of Lowey et al. (7). The low ionic strength (50 mM tris) fraction was chromatographed on DEAE cellulose to separate subfragments one and two. The preparation gave a single peak in the ultracentrifuge, indicating there was no contamination with subfragment 2.

The dialysis equilibrium experiments were carried out in lucite blocks, each of which contained 5 chambers. Each half chamber had the

dimensions 15.9 x 4.8 mm, giving a maximum volume of 0.9 ml. In each experiment, 0.8 ml of the myosin, HMM or S1 was loaded into one side of each chamber, and an equal volume of the solution containing the appropriate total concentration of the εADP in the other. The standard solvent used contained 0.5 M KCl, 0.1 M tris, 1 mM $MgCl_2$, pH 7.6. All experiments were carried out at 4°. Preliminary experiments indicated that the fluorescence quantum yield of εADP did not change when mixed with these proteins. Consequently, the fluorescence ratio of the protein and non-protein containing sides was used to obtain the free and bound εADP. For this purpose, the exciting wavelength was 320 nm, and the fluorescence was obtained by scanning over the peak at 418 nm using the luminescence mode of the Turner Model 210 spectrophotofluorimeter.

If x is the ratio of the fluorescence on the protein side to that on the non-protein side, then the concentration of the free ligand, c^f is given by

$$c^f = c^T/(x + 1),$$

where c^T is the total concentration of ADP initially placed on the non-protein side. The concentration of bound ligand, c^b is simply obtained as

$$c^b = (x - 1)c^f.$$

The high salt concentration renders any correction due to the Donnan equilibrium essentially negligible. Using the usual designation of ν as the ratio of the moles of bound ligand to the moles of protein, the data has been plotted according to the equation of Scatchard (8):

$$\frac{\nu}{c^f} = (n - \nu)K$$

where n = maximum number of binding sites, K is the binding constant for equivalent, non-interacting sites, and c^f is the concentration of free ligand, in this case εADP.

RESULTS AND DISCUSSION

Figure 1 presents representative data for the binding of εADP to

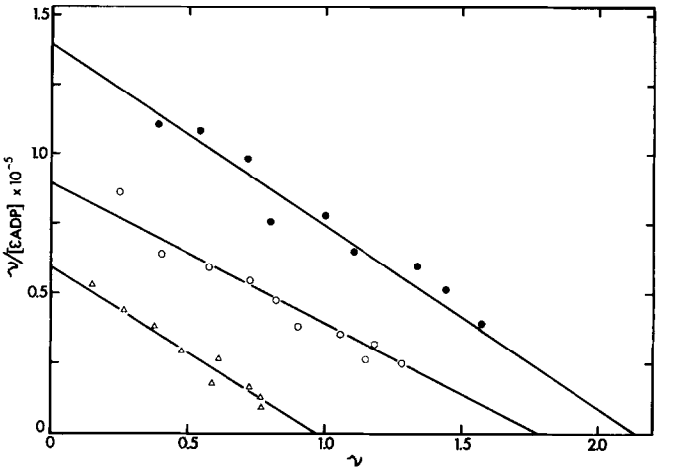

Figure 1: Scatchard plots for the binding εADP to myosin, 5.6 mg/ml (○); HMM, 5.1 mg/ml (●); S1, 4.4 mg/ml (Δ). The solvent is 0.5 M KCl, 0.1 M tris, 1 mM $MgCl_2$, pH 7.6. The temperature was 4°.

myosin, HMM, and S1. For the data shown, the values of n and $\bar{K}$, the dissociation constant, were 1.8 and 2.0 x 10^{-5} M^{-1} for myosin, 2.1 and 1.5 x 10^{-5} M^{-1} for HMM, and 1.0 and 1.6 x 10^{-5} M^{-1} for S1. The molecular weights used in the calculations were 450,000, 340,000, and 115,000 for myosin, HMM and S1, respectively.

The number of binding sites for εADP, i.e. two for myosin and HMM, and one for S1, agree with the number of sites found for myosin and HMM with ADP (2,3), and S1 with ADP (2). The data of Young (2) has been corrected to the molecular weights used here for myosin and HMM. The dissociation constant determined by Young (2) for ADP was found to be 2 ($\pm$.5) x 10^{-5} for myosin, HMM, and S1, i.e. identical for myosin and its subfragments. Within experimental error, the binding constant we have determined for εADP is the same as for the binding of ADP. Thus, a larger dissociation constant for εADP does not appear to be the reason for the higher V_{max} found for this substrate, as opposed to ADP, when used with myosin (1).

In our previous communication (1), the kinetics of hydrolysis of εATP vs ATP were not measured with S1. Using identical experimental

conditions, we find that the values of V_{max} and K_m obtained with S1 are 25 μM phosphate sec^{-1} (gm protein)$^{-1}$ and 5×10^{-5} M^{-1} for ATP, and 80 μM phosphate sec^{-1} (gm protein)$^{-1}$ and 45×10^{-5} M^{-1} for εATP.

Coupled with the previous kinetic data (1), these results again suggest that production of the subfragments of myosin, HMM and S1, is not accomplished without some alteration, on the average, of the active site, for the ratio of the values for V_{max} and K_m for εATP vs ATP differ, and the difference observed is outside experimental error.

However, in all cases the V_{max} for εATP is higher than that for ATP, while the K_m value is higher. To the extent that K_m can be viewed as a measure of the binding affinity of the substrate to the enzyme, in all cases εATP is bound less tightly than ATP. Thus, this cannot explain the increased V_{max}. Neither can it be explained by a decrease in the affinity of εADP vs ADP for the myosin and its fragments. This seems to suggest that the difference in the rates of hydrolysis occurs because of an increase in the rate of formation of $E \cdot ADP \cdot P_i$ in the intermediate step:

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

when εATP is substituted for ATP. Such a step has been postulated by a number of workers (9).

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