BINDING OF 1,N^6-ETHENOADENOSINE 5'-DIPHOSPHATE TO HEAVY MEROMYOSIN AND TO MYOSIN SUBFRAGMENT-1

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1. Introduction

The mechanism of the magnesium-dependent ATPase of myosin and its proteolytic fragments has been intensively studied in recent years due to the importance of this system to the contractile process [1,2]. These studies have used the stopped flow technique; absorbance or intrinsic protein fluorescence is monitored as a function of time in order to elucidate the details of the ATPase mechanism. We wish to consider fluorescent analogues of ADP and ATP which would give signals directly related to the substrate/ligand–protein complex being formed, but which are nonetheless relevant substrates and ligands for myosin. The 1,N^6-ethenoadenosine nucleotides appear to fulfill these requirements: ϵ-ATP is a substrate for myosin; the binding of either ϵ-ADP or ϵ-ATP to myosin results in (a) energy transfer from tryptophan residues located near the active site to the nucleotide, and (b) quenching of nucleotide fluorescence by the protein [3]. We wish to report some initial stopped flow experiments using these signals to monitor the binding of ϵ-ADP to HMM and S-1.

The previous kinetic investigations mentioned above depend on the protein active sites being identical and, in the case of HMM, non-interacting. The binding of ϵ-ADP and ADP to myosin fragments has recently been studied by means of equilibrium dialysis [4,5] and gel filtration [6] techniques with conflicting results. These data were analysed by means of Scatchard plots, which are usually interpreted in terms of identical, non-interacting sites. In view of the conflicting results it would seem more desirable to obtain the degree (or lack) of cooperativity and identicality as a result of the data analysis rather than to use these phenomena as assumptions on which the analysis is based. With this idea in mind, we also wish to report in this communication equilibrium dialysis studies of the binding of ϵ-ADP to myosin subfragments. These results are compared with the kinetic experiments.

Abbreviations: ATPase, Adenosine triphosphatase; HMM, heavy meromyosin; S-1, myosin subfragment-1; ϵ-ADP and ϵ-ATP, 1,N^6-ethenoadenosine 5'-di- and -triphosphate; ADP and ATP, adenosine 5'-di- and -triphosphate.

2. Materials and methods

Sodium salt of ϵ-ADP was purchased from Sigma Chemical, St. Louis, MO, and was used without further purification. HMM and S-1 were prepared from rabbit muscle essentially as described in the literature [7,8]. S-1 concentrations were calculated on the basis of mol. wt. of 115 000 (except as noted) and $E^{1\%}_{280} = 8.0\ cm^{-1}$ and HMM on the basis of 340 000 and 6.47. Digitized stopped flow traces were obtained using a Durrum D-110 interfaced with a DEC PDP-8/I computer. The equilibrium dialysis experiments were done using multicavity lucite blocks (Lab Apparatus Co, Cleveland, OH) with 0.7 ml of solution on each side of the membrane. After agitating for 20 h, the concentrations of ligand on each side of the membranes were measured by comparing the fluorescence of the dialyzed solutions with the fluorescence of standard solutions containing known amounts of ϵ-ADP. The

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measurements were done on a non-commercial steady state ratio fluorometer, using an excitation wavelength of 320 nm and a 400 nm cutoff filter (Corning 3-74). All experiments were done at 4°C in 0.1 M KCl, 50 mM Tris, 5 mM $MgCl_2$, pH = 8.0.

3. Results

3.1. *Kinetics*

The overall binding of ϵ-ADP to HMM or S-1 may be described by

$$S + L \underset{k_d}{\overset{k_a}{\rightleftharpoons}} S{\cdot}L \qquad (1)$$

S represents an HMM or S-1 active site, L the ligand, and S·L the protein–ligand complex. k_a and k_d are the second and first order rate constants, respectively, and are related to the intrinsic (site) binding constant, K_B, by $K_B = k_a/k_d$. A simple way to obtain the rate constants without prior knowledge of K_B is to do the experiments under pseudo-first-order conditions. If the protein is used in large excess, the observed fluorescence change may be described by,

$$F(t) = F_\infty - F_o \exp(-\lambda t) \qquad (2)$$

$$\text{where } \lambda = k_a S_F + k_d \qquad (3)$$

S_F is the (approximately constant) concentration of uncomplexed protein sites. Stopped flow traces resulting from mixing ligand with protein were fitted to eqn. (2) by least squares to obtain values of λ. k_d was measured independently by premixing ϵ-ADP and protein in one drive syringe, and doing the experiment with a large excess of ADP, which is non-fluorescent, in the second syringe. Fig.1 shows examples of traces used in calculating the rate constants. The values of k_a, k_d, and K_B calculated from these experiments are given in table 1.

3.2. *HMM equilibrium dialysis*

The assumption of identical, non-interacting sites, implicit in the above, may be checked by analysing the HMM equilibrium dialysis data as follows. The total concentration of ϵ-ADP on the protein side, L_T, may be written as,

$$L_T = L_F + K_1 E_F L_F + 2K_1 K_2 E_F L_F^2 \qquad (4)$$

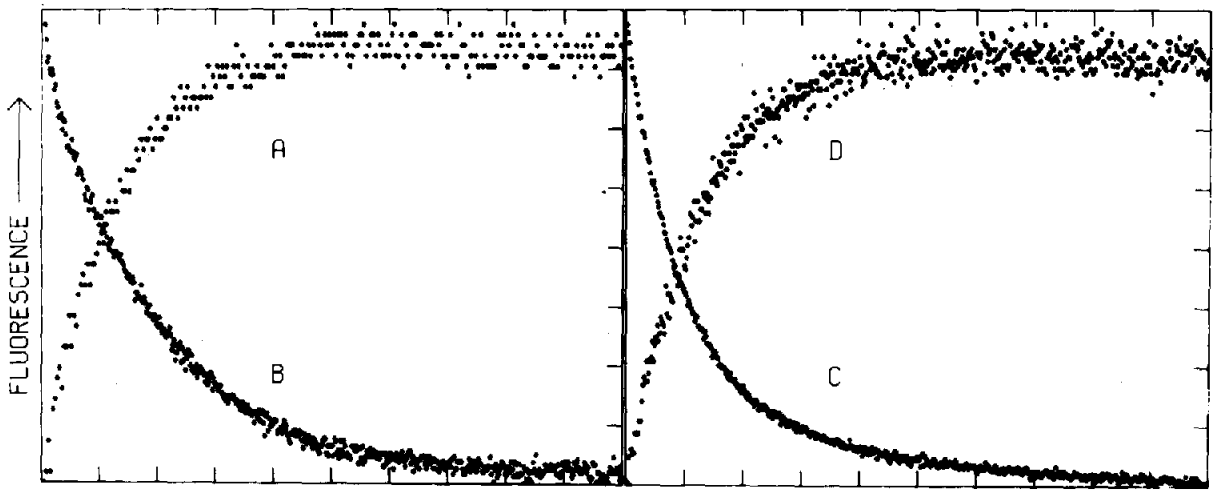

Fig.1. Digitized stopped flow traces of the association of ϵ-ADP with the proteins (A and B) and the displacement of ϵ-ADP from the proteins by ADP (C and D). Cuvette concentrations, time scales, and excitation wavelengths used are, (A) 25 μM S-1 plus 3 μM ϵ-ADP, 200 msec/div, 287 nm; (B) 35 μM HMM sites plus 3 μM ϵ-ADP, 100 msec/div, 315 nm; (C) 7 μM S-1 and 10 μM ϵ-ADP plus 0.2 mM ADP, 3 sec/div, 287 nm; (D) 5 μM HMM sites and 5 μM ϵ-ADP plus 0.2 mM ADP, 3 sec/div, 315 nm. Ligand fluorescence above 400 nm was observed.

Table 1
Kinetic and equilibrium constants for the binding of ε-ADP to HMM and S-1. The variability in the rate constants over several protein preparations is about 15%

	k_a ($M^{-1}\cdot sec^{-1}$)	k_d (sec^{-1})	$K_B^{(kin)}$ (M^{-1})	K_1 (M^{-1})	K_2 (M^{-1})	$K_B^{(eq)}$ (M^{-1})
HMM	1.6×10^5	0.26	6.1×10^5	$(1.3 \pm 0.4) \times 10^6$	$(3.8 \pm 1.9) \times 10^5$	$(6.5 \pm 0.7) \times 10^5$
S-1	2.0×10^5	0.27	7.4×10^5	$(1.6 \pm 0.5) \times 10^6$	$(4.5 \pm 2.1) \times 10^5$	$(8.0 \pm 0.8) \times 10^5$

K_1 and K_2 are the stoichiometric (association) equilibrium constants for the formation of 1-1 and 2-1 ligand-protein complexes; L_F and E_F are the concentrations of free ligand and unbound protein, respectively. The total concentration of HMM is,

$$E_T = E_F + K_1 E_F L_F + K_1 K_2 E_F L_F^2 \quad (5)$$

The dialysis data were fitted to eqn. (4) with K_1 and K_2 as *independently* adjustable parameters using a non-linear least square program [10]. L_T and L_F were experimentally measured; E_F was calculated from eqn. (5) using the current values of K_1 and K_2 at each iteration and the experimentally determined values of E_T. If the HMM sites are identical and non-interacting then [11].

$$K_1 = 4 K_2 \quad (6)$$

Thus by fitting independently for K_1 and K_2 one can not only obtain the equilibrium constants for the binding of ε-ADP to HMM, but also check on whether the sites are in fact independent and identical by seeing if the values of K_1 and K_2 obtained are related according to eqn. (6). The results, given in table 1, show that the binding constants do differ by a factor of four. Fig.2 shows the dialysis data together with the fitted curve.

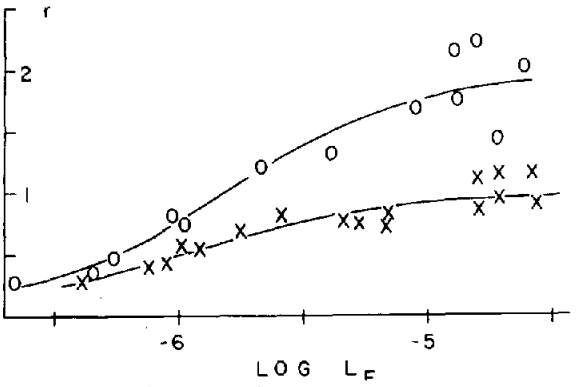

Fig.2. Equilibrium dialysis data, in which r, moles of bound ligand per mole of protein, is plotted against log free ligand concentration, is shown. The HMM data, denoted by O, shows that a maximum of two moles of ligand are bound per mole of protein whereas the S-1 data, denoted by X, shows a maximum of one mole of ligand bound per mole of protein.

3.3. *S-1 equilibrium dialysis*

We can independently check on whether or not the two binding sites are identical by doing equilibrium dialysis experiments on S-1. This is desirable since it is at least theoretically possible that positive site–site cooperativity (i.e., binding of a molecule to one HMM site enhancing the affinity of the second site for the ligand) may be fortuitously cancelled by an intrinsic difference in the affinities of the two sites for the ligand [6]. What we wish to do is to test to see whether the S-1 solutions consist of a homogeneous population of active sites, or whether they consist of two populations differing in their affinities for the ligand. A simple way of doing this is to use 230 000 as the mol. wt. of S-1 instead of 115 000. We thus take two S-1 molecules as the molecular entity. This is a legitimate procedure since the factor of four in eqn. (6) is strictly statistical in nature; it is based on the fact that the first ligand molecule has two sites to choose from per molecular entity while the second has only one. The molecular details of how the sites are actually connected have no direct bearing on the factor. The equilibrium dialysis data, in which the concentration of the pseudo S-1 moiety is half that of the actual S-1 concentration, may now be fitted to eqns. (4) and (5) in exactly the same way as the HMM data. The bogus K_1 and K_2 values are given in table 1. The dialysis data and fitted curve as shown in fig.2.

4. Discussion

The equilibrium results show rather conclusively that the active sites are identical and independent, at least as far as ϵ-ADP is concerned. Since in the S-1 case the sites are not physically connected, site–site interaction is not possible. The factor of four obtained for these data demonstrates that the sites are truly identical with respect to their affinities for ϵ-ADP. The fact that the intrinsic binding constant of S-1 is the same as that of HMM, to within experimental error, shows that the affinity of the site for the ligand is not altered upon digestion from the rod; thus it is reasonable to infer that the two HMM sites are identical. The factor of four obtained for the HMM data shows that the sites are non-interacting as well as identical.

The kinetic experiments based on energy transfer give the same results as those based on quenching. In addition, the binding constants obtained from the stopped flow data are in agreement with the constants obtained from the equilibrium dialysis experiments. We therefore conclude that both of the signals used in the kinetic experiments monitor the same process, and that this process is the overall binding of ϵ-ADP to the myosin fragments.

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