An Investigation of Heavy Meromyosin–ADP Binding Equilibria by Proton Release Measurements†

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ABSTRACT: The interaction of magnesium-ADP with skeletal muscle heavy meromyosin has been studied by measuring the accompanying release of protons. Total pH changes of the order of 0.03 were involved, and measurements were performed with a discrimination of some ten-thousandths of a pH unit. At pH 8.0 and 25 °C about 0.5 mol of protons per mol of heavy meromyosin is released at saturation. A stoichiometry of binding close to 2 mol of ADP per mol of protein was found, with a binding constant, obtained from the proton release titration curve (pH 8.0, 25 °C), of 2×10^5 M^{-1}. At 5 °C the release of protons per mole is slightly greater, and the binding constant is somewhat increased, reflecting a negative enthalpy of binding. Similar proton release behavior is observed in the presence of manganous ions in place of magnesium. The liberation of protons is thus unrelated to the temperature-dependent isomerization of myosin in the presence of substrate. Alkylation of a reactive thiol group (SH_1) does not change the proton liberation at pH 8.0. From the pH dependence of proton release, the association constant of heavy meromyosin with magnesium-ADP at other pH values can be inferred and shows an appreciable rise as the pH increases. The pH–proton release profile also allows the pK of the ionizing groups perturbed by the ligand to be deduced. At least two groups ionizing above pH 7 and one below are involved. Their pK's in the unperturbed state are assigned as 8.5, 9.3, and about 6.6, respectively; they are displaced in the complex to about 8.0, 9.1, and 6.3. A relation to the pH–activity profile of myosin ATPase is indicated. The pH–proton release profile is somewhat changed when the SH_1 group is alkylated. Measurements with potassium-ADP, in the absence of magnesium, show that at pH 8.0 there is no proton release but rather a sizeable proton absorption (about 0.5 mol of protons per mol of heavy meromyosin). The association constant derived from the titration curves (pH 8.0, 25 °C) is 3×10^4 M^{-1}.

The interaction of ADP with myosin or its active fragments is of importance because, depending on the temperature, this is the ligand associated with the myosin during the steady state of ATP hydrolysis (Taylor, 1973, and references cited therein). The nature of its interaction with myosin bears on the ATPase mechanism, the structure of the active centers, the question of whether there is cooperativity between the myosin heads and possibly also on the mode of self-association of myosin in filaments. The "early burst" of ATP hydrolysis, arising from the first turnover cycle when the substrate encounters the myosin, differs in magnitude depending on whether it is measured by release of protons or of orthophosphate; this discrepancy has been resolved (Bagshaw et al., 1974; Chock and Eisenberg, 1974; Koretz and Taylor, 1975) by the observation that protons are also released on interaction with an unhydrolyzable substrate analogue. If, as is generally to be expected, the binding of a substrate or its analogue to an enzyme is pH dependent, proton release or absorption will occur, and this phenomenon therefore provides a rather general way of studying such equilibria. We have used proton release to study the ADP–heavy meromyosin system, and from the results have derived a stoichiometric and thermodynamic description of the interaction. The effects of temperature, of pH, of cations, and of the state of the reactive thiol groups have been examined. Inferences about the side chains involved in the interaction may be drawn.

Materials and Methods

Myosin from rabbit skeletal muscle was prepared by the method of Perry (1955), and heavy meromyosin by tryptic hydrolysis, following Lowey and Cohen (1962). To avoid interference from the activities of contaminating hydrolytic enzymes, such as myokinase, it was found essential to purify the heavy meromyosin by ion-exchange chromatography (Offer et al., 1973). The ADP was obtained as the disodium salt from Sigma and was screened for purity by paper chromatography or high-resolution ^{31}P nuclear magnetic resonance. The purity was not lower than 96%. (We are indebted to Dr. C. W. Hilbers for the latter measurements.) Concentrations of protein and ligand were determined spectrophotometrically. For heavy meromyosin, $E_{1\,cm}^{1\%}$ (280 nm) was taken to be 6.5 (Young et al., 1964), and the molecular weight as 3.65×10^5.

Alkylation of the reactive thiol group, SH_1, was performed by adding iodoacetamide (8 × molar excess over protein) to the heavy meromyosin at 15 mg/mL in 0.1 M KCl–5 mM $MgCl_2$–10 mM Tris[1] (pH 8.0), and allowing the reaction to proceed at 4 °C for 48 h. A peptide analysis of subfragment 1, modified with iodoacetamide under the same conditions, indicated that of 2 mol of iodoacetamide incorporated per mol of protein, 0.7 mol was present in the SH_1 peptide (J. Sleep, personal communication). The calcium and EDTA ATPase activities were 2.5 and 0.1 times, respectively, those of the unmodified heavy meromyosin. All protein samples contained the myokinase inhibitor, P^1,P^5-di(adenosine 5′-pentaphosphate), at 1 μM, which was added before titration. The solvent was 0.1 M potassium chloride with 5 mM magnesium (or manganous) chloride, or 10 mM EDTA.

Proton release measurements were performed with an apparatus previously described (van Os et al., 1972). Titrant is

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

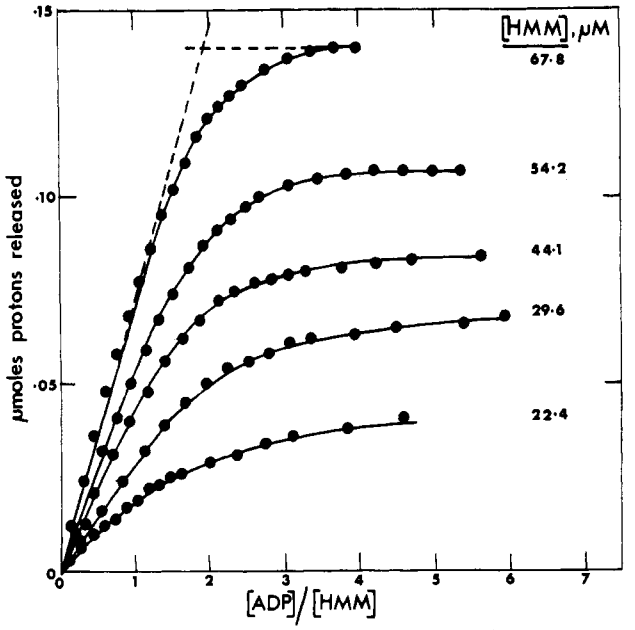

FIGURE 1: Proton release from heavy meromyosin on titration with ADP at pH 8.0, 25 °C, in the presence of magnesium. The concentrations of protein are as indicated. The broken lines give the concentration of active ADP binding sites. The solvent was 0.1 M potassium chloride–5 mM magnesium chloride.

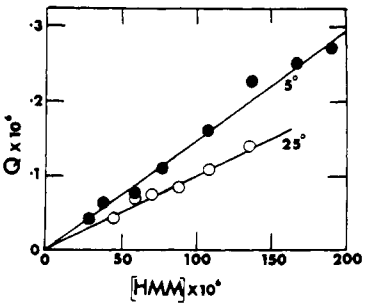

FIGURE 2: Relation between heavy meromyosin concentration and total release of protons on saturation of binding sites with ADP, at 5 and 25 °C.

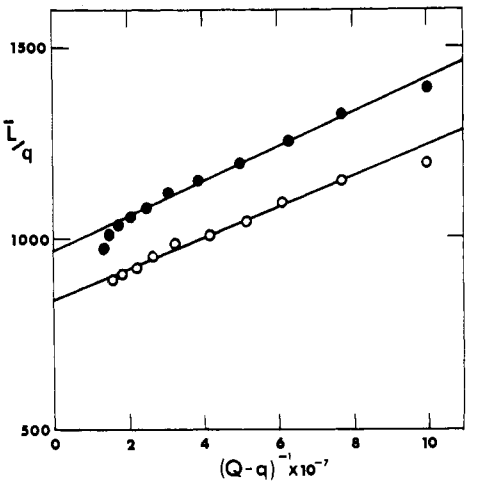

FIGURE 3: Linearized relation between proton release and ligand concentration, following equation in text; L is ligand concentration, q moles of protons released, and Q moles of protons released at saturation. The slope gives the binding constant, K_d. The plots refer to total protein concentrations of 67.8 μM (open circles) and 54.2 μM (filled circles), at pH 8.0, 25 °C.

added from a motor-driven microburet (Metrohm, E457) to ca. 4 mL of protein solution in a thermostated glass vessel by way of a fine capillary. The volumetric precision is 0.2% on 10 μL. The pH is measured with a Radiometer glass and calomel electrode pair. The ionic strength of the titrant is the same as that of the protein. The titration is carried out in an argon atmosphere, pH changes are measured with a digital voltmeter, recording the pH meter output of 100 mV per pH unit with a resolution of 10 μV. A precision of some ten-thousandths in pH is achieved. The pH change generated by the addition of a known quantity of alkali (10 μL of 0.01 N NaOH) to the protein solution at the pH of the ADP addition was used as a calibration value for the conversion of pH increments into moles of protons released. The addition of ADP does not cause a perceptible change in the buffer capacity of the solution.

Results

Typical proton release plots at 25 °C and pH 8 in the presence of magnesium ions are shown in Figure 1 for a series of protein concentrations. It is clear that at the higher protein concentrations saturation of the binding sites occurs when the ADP concentration only slightly exceeds that of myosin heads, further addition of titrant giving no more proton release. When the heavy meromyosin was not chromatographically purified, this constancy of pH was not observed and there was instead a time-dependent increase in proton release, due evidently to hydrolytic processes catalysed by contaminating enzymes, probably mainly myokinase. The curves of Figure 1 fulfill the qualitative expectations for a single strong binding process. For all but the lowest protein concentrations, the plots are linear in the region in which the molar concentration of binding sites is large compared with that of ligand. The intercept (Figure 1) gives the concentration of functional heads in respect of ligand binding. Values between 1.6 and 1.9 mol of active heads per mol of heavy meromysin were always obtained. Figure 2 shows the linearity of the response in terms of proton release at saturation with protein concentration. The slope leads to a value of 0.26 mol of protons liberated per mol of sites at pH 8.

The binding constant may be readily obtained from the region of the titration curve in which the concentrations of the two reactants are comparable. It is easily shown that, if the total molar concentration of myosin heads (active and inactive) is $\bar{P}$ and that of ligand is $\bar{L}$, the number of moles of protons released, q, may be related to the moles released at saturation Q (the plateau of the titration curve) by the equation

$$\frac{\bar{L}}{q} = \frac{K_d}{Q-q} + \frac{\bar{P}}{Q}$$

Then a plot of $\bar{L}/q$ against $(Q-q)^{-1}$ gives the dissociation constant K_d, as well as the concentration of functional active centers. Such plots for two different protein concentrations are shown in Figure 3 and lead to a value of 5×10^{-6} M for the dissociation constant.

The binding constant can also be obtained from the profile of proton release as a function of protein concentration at any fixed ratio of total ligand to protein, R. Calculated plots of q against P for all titration curves at $R = 1.0$ are shown in Figure 4 as are the experimental points, which are compatible with the value of the binding constant already obtained.

At 5 °C, at which the ATPase mechanism is controlled by the rate of product release, proton liberation similar to that at 25 °C occurs, though the magnitude is somewhat larger, and the curvature of the plots gives a dissociation constant of about

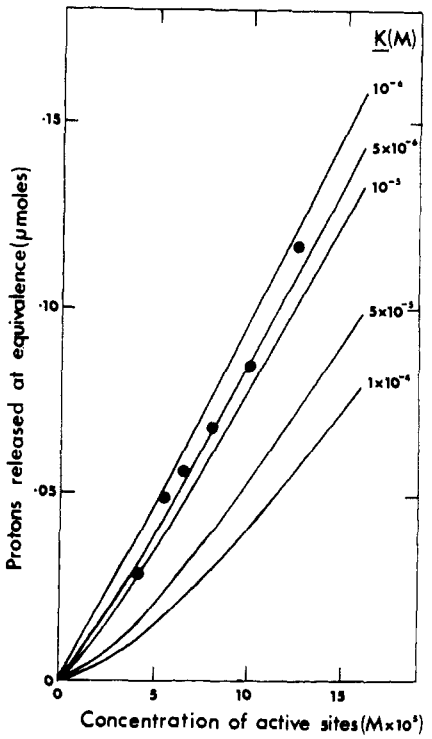

FIGURE 4: Relation between protons released at fixed ligand–protein molar ratio (unity) and protein concentration. The curves are calculated for two identical sites per molecule of heavy meromyosin, with dissociation constant as indicated. Points represent experimental values at pH 8.0 and 25 °C.

1×10^{-6} M. The interaction is thus associated with a negative enthalpy change of the order of -10 kcal mol^{-1}, assuming no change in the nature of the binding equilibrium. Whereas the magnesium–ATPase activity of myosin gives a linear Arrhenius plot (Levy et al., 1962), suggesting that this assumption is valid, it has been reported (Hozumi and Tawada, 1975) that in the case of manganese-stimulated ATPase a discontinuity occurs near 10 °C. Because of this possibility of a change in the identity of the rate-limiting step in the kinetics at this transition point, when manganese is substituted for magnesium, proton release experiments were also carried out in the presence of 5 mM manganous ion. The results showed that there is no qualitative change in the proton release titration curves.

A discontinuity in the ATPase Arrhenius plot also reportedly occurs in myosin treated with an alkylating agent to block a reactive thiol group (Levy et al., 1962), which is intimately involved in the binding of substrate (Burke et al., 1973; Reisler et al., 1974). Proton release experiments were performed on heavy meromyosin modified with iodoacetamide and at pH 8 at 5 °C give no evidence of any appreciable effect of this modification. The binding constant also remained substantially unchanged.

The pK of the groups responsible for proton release can be determined from the pH dependence of the effect. Figure 5 shows the profile of Q as a function of pH, proton release being measured for addition of a saturating excess of ADP. For a change on binding in the pK of a single ionizing group, the shape of the curve is determined by the shift in pK; for a simple unperturbed ionization, following the Henderson–Hasselbalch law, the pK values in the free and liganded states, pK and pK' say, can be evaluated from the height and width of the proton release profile. Thus at pH corresponding to maximal Q,

$$pK = pH_{max} + \log(1 + Q)/(1 - Q)$$

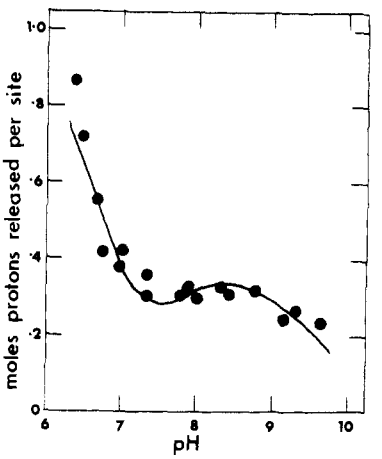

FIGURE 5: Dependence of proton release from heavy meromyosin on pH. The ordinate refers to protons released per mole of protein on saturation with excess ADP. The line is calculated for three ionizing groups with pK 6.6, 8.4_5, and 9.4 (unperturbed) and pK' 6.3, 8.0, and 9.1 (in ADP–protein complex).

and

$$pK' = pH_{max} + \log(1 - Q)/(1 + Q)$$

An inspection of Fig. 5 shows that the curve cannot be fitted by a single pK shift. In addition to the rise below neutrality, the curve at higher pH is obviously compound. It can be fitted in terms of two ionizing groups with pK 8.4_5 and 9.4, respectively (unliganded state) and pK' 8.0 and 9.1. The segment at lower pH is more difficult to analyze quantitatively because the maximum, which occurs in the pH range of insolubility of the protein, could not be determined. A satisfactory fit is obtained with pK = 6.6 and pK' = 6.3 (calculated line in Figure 5). The alkylated protein shows perceptibly different pH profiles, with somewhat decreased proton release below about pH 8 and increased release above. The elimination of the reactive thiol groups thus apparently leads to some perturbation of other ionizing groups, but this group is evidently not itself directly involved in the proton release process. These results, however, may be subject to error because of the diminished stability of the protein, which seems to denature more rapidly after modification.

It is possible by taking advantage of the theory of linked equilibria (Wyman, 1964) to determine the equilibrium constants for the ligand–protein association at any pH in the range covered by the saturation experiments (Figure 5) from the measured value at pH 8.0. For the binding of a ligand species i to the protein the general relation

$$\left(\frac{\partial \ln K}{\partial \ln a_i}\right)_{a_{j \neq i}} = \Delta\bar{\nu}_i$$

obtains, where K is the apparent dissociation constant, a_i the activity of the ligand species in question, and $\Delta\bar{\nu}_i$ the incremental degree of saturation of the binding sites. The partial derivative is referred to constant activity of all other solute species. Laskowski and Finkenstadt (1972) have examined how this relation may be explicitly applied to proton release equilibria. It is readily shown that

$$\log K_2 = \log K_1 + \int_{pH_1}^{pH_2} Q\,d(pH)$$

where K_1 and K_2 are the values of the equilibrium constant at pH_1 and pH_2. The reference pH (pH_1) is in the present case 8.0 and values of K_2 may be obtained by numerical integration of the area under the curve in Figure 5. The result of such a

calculation is that the affinity for ADP increases slowly with increasing pH, the dissociation constant reaching 1.25×10^{-6} M at about 9.7, above which inactivation rapidly occurs. Conversely, at pH 7 the dissociation constant is about 8×10^{-6}, and at pH 6.2 it rises to 2.4×10^{-5} M.

Some exploratory experiments were performed on a different system, potassium-ADP, divalent cations being excluded by addition of EDTA. The unexpected result was that no proton release occurred, but rather proton absorption. Curves are shown in Figure 6. A saturating proton absorption of some 0.25 mol of protons per mol of active heads was derived. The difficulty in determining this figure precisely because of the weaker binding, which made it impossible to reach complete saturation, introduced uncertainties into the calculation of the equilibrium constant. The precision of the value extracted is somewhat in doubt but it emerges at 3×10^{-5} M at pH 8.0 and 25 °C.

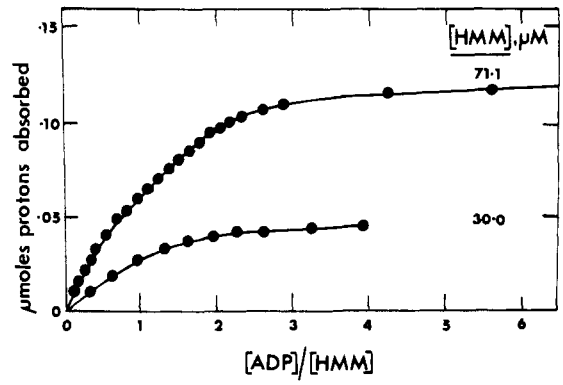

FIGURE 6: Absorption of protons on titration of heavy meromyosin with ADP in the presence of potassium ions, and absence of divalent cations at pH 8.0, 25 °C, at the indicated protein concentrations. The solvent was 0.1 M potassium chloride-10 mM EDTA.

Discussion

Based on literature values for molecular weight and specific absorptivity, our results indicate that ADP binds to heavy meromyosin at two equivalent sites on each protein molecule. The temperature-dependent difference in binding sites reported by Morita (1971) and Yoshida and Morita (1975), on the basis of optical measurements of ligand binding, is not reflected in our data.

The binding constants for magnesium-ADP derived by the treatment illustrated in Figure 3 are in reasonable agreement with literature values. Our values for the association constant of $2\text{-}10 \times 10^5$ M^{-1} at pH 8 may be compared with 1.4×10^5 (Lowey and Luck, 1969) at 5 °C and pH 7.7, 4.6×10^5 (Malik et al., 1972) at 7 °C and pH 8, and $6\text{-}7 \times 10^5$ at 25 °C and pH 7.5 (Yoshida and Morita, 1975; Sekiya and Tonomura, 1967). The enthalpy of binding was found to be negative in agreement with the results of calorimetric studies, and of a magnitude falling within the range of published calorimetric values (Yamada et al., 1973; Goodno and Swenson, 1975). The binding of ADP in the presence of potassium (0.1 M) is weaker than that of the magnesium salt: Lowey and Luck could detect no binding by equilibrium dialysis, whereas Malik et al. obtained a binding constant of 1.6×10^4 M^{-1} at 7 °C; our value at 25 °C is 3×10^4 M^{-1}. There are only minor quantitative changes in the form of the proton release profiles at 5 and 25 °C. The changes in pK of ionizing groups on binding are therefore evidently not associated with the conformational isomerization which accompanies the change in temperature (Bagshaw et al., 1974; Bagshaw and Trentham, 1974). Similarly the proton release is undisturbed, at least at pH 8, when one of the reactive thiol groups believed (Burke et al., 1973; Reisler et al., 1974) to be implicated in binding of the metal-ADP complex is blocked by alkylation. Manganese-stimulated ATPase is reported (Hozumi and Tawada, 1975) to have a nonlinear Arrhenius plot, with a discontinuity at about 10 °C, suggesting a change in identity of the rate-limiting step. This interpretation was supported by a change in the phosphate burst, indicating a transition from a mechanism that is limited by product release to one that is not. Again, the introduction of manganous ions gives no change in the magnitude of the proton release with ADP, confirming that this is not related to the temperature-dependent isomerization of the myosin.

The pH profile of the proton release (Figure 5) gives, in addition to the binding constants in otherwise barely accessible ranges, the approximate pK values of the ionizing groups perturbed by interaction with the magnesium-ADP complex. As already noted, the shape of the curve shows that more than one ionizing group is involved above pH 7. The unperturbed pK values for a satisfactory fit are 8.5 and 9.3. These could be anomalous ϵ-amino groups, the lower one at least might be an anomalous imidazole, and the upper one possibly a tyrosine or a thiol. If the latter, it is not the reactive SH_1 group; neither is there evidence of an absorbance increase that could readily be associated with a phenolic ionization. The N-terminal groups are known to be acetylated (Offer, 1965). If the reasonable assumption is made that only the doubly charged form of ADP is capable of interacting at the active center, it might be supposed that the lower part of the proton release profile would be due to the ionization of the secondary phosphate group. However, the effective pK (referred to all metal-liganded states) of magnesium ADP is given by Smith and Alberty (1956) as 5.1. This would rule out any significant contribution of the ADP to proton release in the pH range covered in our experiments. The group of unperturbed pK, probably in the vicinity of 6.6, is therefore likely to be in the active center, and might be imidazole or an anomalous carboxyl, such as has been recognized in some proteins (Susi et al., 1959; Timasheff and Rupley, 1972).

Apart from the two reactive thiol groups there is little unambiguous chemical information about side chains in the active center of myosin. There appears to be at least one lysine (Takashina, 1970), and there is fragmentary evidence in favor of a tyrosine (Shimada, 1970); the evidence regarding histidine is at best equivocal (Morales and Hotta, 1960; Stracher, 1965; Hegyi and Mühlrad, 1968).

Assuming that the perturbed groups are in or close to the active center, one might expect some correlation of their pKs with the pH-activity profile of myosin ATPase. The available data (Mommaerts and Green, 1954; Gilmour, 1960; Sekine and Kielley, 1964) are in agreement on the general features of the curve. In particular there is a sharp increase at high pH, the midpoint of which is probably at pH 9.0-9.2. The activity also rises at lower pH, the midpoint of the increase being at pH 6.5-6.7. (At still lower pH (5.6-6.2) there is an abrupt drop.) The ionization changes associated with these two rises in activity conform very closely to two of the three pK values derived from the proton release data. Sekine and Kielley (1964) find that the rise at high pH is inhibited after reaction with *N*-ethylmaleimide, suggesting that a thiol group is directly or indirectly involved. What is somewhat unexpected is the large increase in ATPase rate at high pH, at which our results indicate a significant increase in the affinity for ADP, the release of which (at 5 °C at least) presumably controls the activity. The increase in association constant may of course be linked

to a change in the association rather than the dissociation rate constant.

The wholly unexpected difference between the potassium and magnesium-ADP interactions demonstrates the extent to which the ATPase is influenced by the metal. In terms of the formulation of Harrington and co-workers (Burke et al., 1973; Reisler et al., 1974), the difference arises from the mode of attachment at a site containing both the SH_1 and SH_2 thiol groups. We do not yet know whether the proton absorption that accompanies the binding of potassium-ADP arises from a perturbation of the same groups as are involved in magnesium-ADP binding, with a pK shift in the opposite direction, or from different ionizing groups. We can also not exclude the possibility that the proton release shown in Figure 5 masks a simultaneous but smaller absorption of protons by other groups. An electrostatic free energy effect may in addition be expected to operate. If the nucleotide is bound in the form of a metal complex, that with potassium will have a greater formal negative charge and this will militate against release of protons. The potassium-ADP-heavy meromyosin system is being further studied.

Acknowledgments

The collaboration on which this work was based was made possible by a Short Term Fellowship from EMBO to D.J.M., who also acknowledges support from a Science Research Council Training Fellowship. We are indebted to Dr. H. White for discussion and access to unpublished data.

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