Relationship of Structure to Function in Myosin. II. Salt Denaturation and Recombination Experiments*

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ABSTRACT: Complete dissociation of light chains from myosin by concentrated salt solutions, pH 7, 4°, is accompanied by irreversible inactivation of Ca–adenosine triphosphatase (ATPase), presumably from reactions of critical sulfhydryl groups and/or other side-chain groups under denaturing conditions. However, after rapid LiCl–citrate fractionation with thiol protection, fully reconstituted myosin retains 70% of control ATPase and contains 1.6 adenosine diphosphate (ADP) binding sites ($\Delta F° = 6.9$ kcal/mole); Ca–ATPase and ADP binding vary with the ratio of light to heavy chains, and neither property is found in heavy-chain core or light chains alone. The dissociation of about 3% light component on treatment of myosin in 4.7 M NH_4Cl–0.001 M dithiothreitol does not significantly affect Ca–ATPase, and recombination experiments on the residual myosin yield results comparable with those obtained on native myosin. These data would support the conclusion that myosin comprises two enzymatically active protomers, each containing one heavy chain and one essential light chain; an additional light chain (fast electrophoretic band) may be tentatively considered a distinct protein closely associated with myosin proper. An interaction between essential light and heavy chains is implicated in Ca–ATPase and ADP binding, and it has been suggested that nucleotide may bridge essential light and heavy chains within each protomer.

The dissociation of rabbit skeletal myosin into heavy-chain core (420,000 mol wt) and 2.7 light chains (av mol wt 20,200) at pH 11 and in 2 M guanidine is accompanied by irreversible inactivation of Ca–ATPase (Gershman *et al.*, 1966, 1969; Dreizen *et al.*, 1967), and on heat denaturation values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are comparable for light-chain dissociation and ATPase inactivation (Gershman *et al.*, 1968, 1969). However, 10–15% ATPase is recovered after dissociation of myosin in 2 M KCl, 0.01 M ATP, and 0.1 M Na_2CO_3 (pH 11.0), and recombination experiments indicate that recovery of ATPase is dependent on the presence of light and heavy chains (Dreizen and Gershman, 1969a; Gershman *et al.*, 1969). These findings would indicate than an interaction between light and heavy chains may be involved in myosin ATPase. Frederiksen and Holtzer (1968) reached a similar conclusion from other evidence that has, however, been criticized on major aspects (Gershman *et al.*, 1969; Stracher, 1969; Dreizen and Gershman, 1970).[1] In any case, a low recovery of Ca–ATPase in recombination experiments and the somewhat enigmatic presence in myosin of two heavy chains and close to three light chains that are electrophoretically heterogeneous (Gershman *et al.*, 1966) preclude a definite interpretation from the alkali data alone.

It has also been shown that light chains may be selectively dissociated from myosin in concentrated salt solutions, pH 7, 4° (Gershman *et al.*, 1968; Gershman and Dreizen, 1969a,b, 1970); and this paper describes the use of salt denaturation and recombination experiments to explore (1) the possible role of light and heavy chains in ATPase activity and ADP binding, and (2) the question of essential subunits and light-chain heterogeneity. Preliminary accounts have been reported (Dreizen and Gershman, 1969a,b, 1970), and parallel experiments based on chromatographic fractionation of myosin in 4 M LiCl were reported by Strachei (1969).

Methods

Details of the purification of myosin and ultracentrifugal

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[1] The interpretation of Frederiksen and Holtzer (1968) was based on experiments in which heavy and light alkali components alone showed no ATPase activity, while myosin titrated to pH 11.0 and back-titrated to pH 7 retained 50–80% of control ATPase, the extent of activity independent of duration of alkaline treatment for up to 100 min. In our experience the purification procedure described by Frederiksen and Holtzer in itself destroys the ATPase activity of myosin. Moreover, the Frederiksen–Holtzer data are disparate with other reported evidence of time-dependent ATPase denaturation during alkaline treatment of myosin (Mommaerts and Green, 1954; Stracher, 1961; Seidel, 1967; Gaetjens *et al.*, 1968; Gershman *et al.*, 1968, 1969). More recently, we have reinvestigated the discrepancy in results and were unable to account for it by minor differences in pH or the method of alkaline treatment; however, we did find that traces of P_i in the myosin preparation may lead to spurious ATPase values, in the absence of sequential phosphate measurements during reaction of alkaline-treated myosin with ATP (Dreizen and Gershman, 1970). This kind of artifact, if present, would not be picked up by the customary procedure (used by Frederiksen and Holtzer) of basing ATPase on simply a 5-min determination of P_i; but whether or not this effect was contributory to the Frederiksen–Holtzer data is unknown.

procedures have been presented (Dreizen *et al.*, 1966; Gershman and Dreizen, 1970).

In salt denaturation experiments, sedimentation velocity was initiated promptly after addition of myosin to the appropriate salt solution; and ATPase was assayed after salt treatment for 5 min to 24 hr prior to dialysis against 0.4 M KCl–0.005 M $NaHCO_3$ (pH 7). When used, 0.001 M dithiothreitol was present during salt denaturation and renaturation, and 0.001 M EDTA was present during salt denaturation.

LiCl–citrate fractionation of myosin was done by addition of 8 M LiCl to an equivalent volume of myosin at approximately 20 mg/ml in 0.4 M KCl, 0.005 M $NaHCO_3$ or 0.1 M Tris-maleate (pH 7) followed by salting out in 60% saturated potassium citrate–4 M LiCl and centrifugation at 5000 rpm for 5 min, or 10,000 rpm for 10 min. Second-cycle precipitate was obtained by dissolving first-cycle precipitate in 4 M LiCl and repeating citrate salting out. Identically treated samples of first-cycle precipitate and supernatant were recombined in different proportions, and all samples were dialyzed simultaneously against repeated changes of 0.4 M KCl–0.005 M $NaHCO_3$ (pH 7) over 24 hr. A control sample of myosin was introduced into the final dialysate. Samples of reconstituted protein, containing light and heavy chains in different proportion, were assayed for Ca–ATPase or used for ADP binding experiments. Supernatant and precipitate fractions were dialyzed against 0.4 M KCl-0.1 M Na_2CO_3 (pH 11.0) for determination of subunit composition by sedimentation velocity, sedimentation equilibrium, or both (Gershman *et al.*, 1966, 1969).

Ca-activated ATPase was determined according to Perry (1955), on samples of protein dialyzed against 0.4 M KCl (pH 7). Assays are based on reaction of ATP and protein for 5 min prior to precipitation by trichloroacetic acid; a blank was obtained for each determination in which the order of addition of ATP and trichloroacetic acid was reversed. On occasion, assays for inorganic phosphate were obtained after reaction for 1, 3, and 5 min.

ADP binding was determined by equilibrium dialysis of denatured and reconstituted protein against ADP solutions or, in one experiment, ATP solutions that were hydrolyzed to ADP during dialysis. Na_2ADP (grade 1, from equine muscle) was used within 2 weeks of delivery from the Sigma Chemical Co. The concentration of ADP was determined from optical density at 2590 Å, assuming ϵ_{2590} of 15,400 M^{-1} cm^{-1} (Pabst Laboratories Circular OR-10, 1967). Difference spectra obtained at 20-Å intervals (or less) over the range 2300–3000 Å were consistent with the known absorption of ADP (Pabst Laboratories Circular OR-10, 1967), and the absorption spectra did not change significantly during dialysis against reconstituted protein. The binding data were analyzed according to Scatchard (1949), plotting $\nu/(ADP)_f$ *vs.* ν, where ν is moles of ADP bound per mole of protein and $(ADP)_f$ is free ADP concentration at equilibrium. For independent, noninteracting sites, $\nu/(ADP)_f = K(n - \nu)$, where n is the number of binding sites and K is the apparent association constant. The apparent free energy of binding was determined from the customary equation, $\Delta F^\circ = -RT \ln K$.

Results

Salt Denaturation of Myosin ATPase. After treatment of myosin in salt solutions in which the subunit structure remains intact at salt concentrations to saturation (KCl, NaCl, and sulfate salts), Ca–ATPase is completely or nearly completely recovered on dialysis against 0.4 M KCl. For example, treatment of myosin in concentrated NaCl solutions as long as 24 hr does not result in significant loss of Ca–ATPase on return to 0.4 M KCl (Figure 1).

In contrast, Ca–ATPase is irreversibly inactivated on treatment of myosin in salt solutions in which light chains are fully dissociated from the heavy-chain core (see Table I in Gershman and Dreizen, 1970). In general, brief salt treatment of myosin is characterized by a transition for rapid irreversible ATPase inactivation at or slightly below the transition salt concentration for light-chain dissociation, and prolonged salt treatment of myosin leads to time-dependent ATPase inactivation at salt concentrations below the transition for light-chain dissociation. For example, after 5-min treatment of myosin in KSCN solutions and dialysis against 0.4 M KCl, there is a sharp fall in Ca–ATPase at KSCN concentrations between 0.25 and 1 M KSCN (Figure 1), essentially the same range over which light component is fully dissociated from myosin. Prolonged KSCN treatment leads to progressive ATPase inactivation at concentrations below 1 M KSCN, even in the presence of EDTA or dithiothreitol (Figure 1).

Similarly, after 5-min treatment of myosin in LiCl solutions and dialysis against 0.4 M KCl, Ca–ATPase is irreversibly inactivated on increase in LiCl concentration from 3 to 4 M LiCl (Figure 1); and light chains are dissociated from myosin within the same range of LiCl concentration. In the absence of sulfhydryl protection, there is a sharp fall in Ca–ATPase at about 3.5 M LiCl, but prolonged LiCl treatment leads to progressive ATPase inactivation at concentrations as low as 1 M LiCl. Although dithiothreitol leads to some protection of myosin ATPase from LiCl denaturation, prolonged LiCl treatment still results in time-dependent ATPase inactivation (Figure 1). EDTA is less effective in protecting myosin ATPase from LiCl denaturation.

The effect of NH_4Cl treatment on myosin ATPase is of special interest in that only a fraction of the total light component is dissociated from myosin in 4.7 M NH_4Cl (Figure 2). During treatment of myosin in 4.7 M NH_4Cl and dialysis against 0.4 M KCl, there is irreversible inactivation of Ca–ATPase according to first-order kinetics, with a rate constant of 8×10^{-5} sec^{-1} (Figure 2). This effect apparently derives in part from reaction of heavy metal contaminants in the NH_4Cl reagent with critical sulfhydryl groups of myosin, since the rate of ATPase loss is greatly diminished on NH_4Cl treatment in the presence of EDTA or dithiothreitol; but the occurrence of slow ATPase inactivation despite the presence of EDTA or dithiothreitol suggests that other side-chain residues may also be involved. Extrapolation of Ca–ATPase to zero time of NH_4Cl treatment indicates essentially full recovery of control ATPase activity provided that thiol groups are protected, despite irreversible dissociation of 3% light component (Figure 2).

Recombination Experiments. ATPase activity. The partial recovery of Ca–ATPase following light-chain dissociation in 4 M LiCl and dialysis against 0.4 M KCl permitted recombination experiments to study the possible relationship between enzymatic activity and subunit composition. The different samples of protein in each recombination experiment were denatured and renatured identically and simultaneously, so

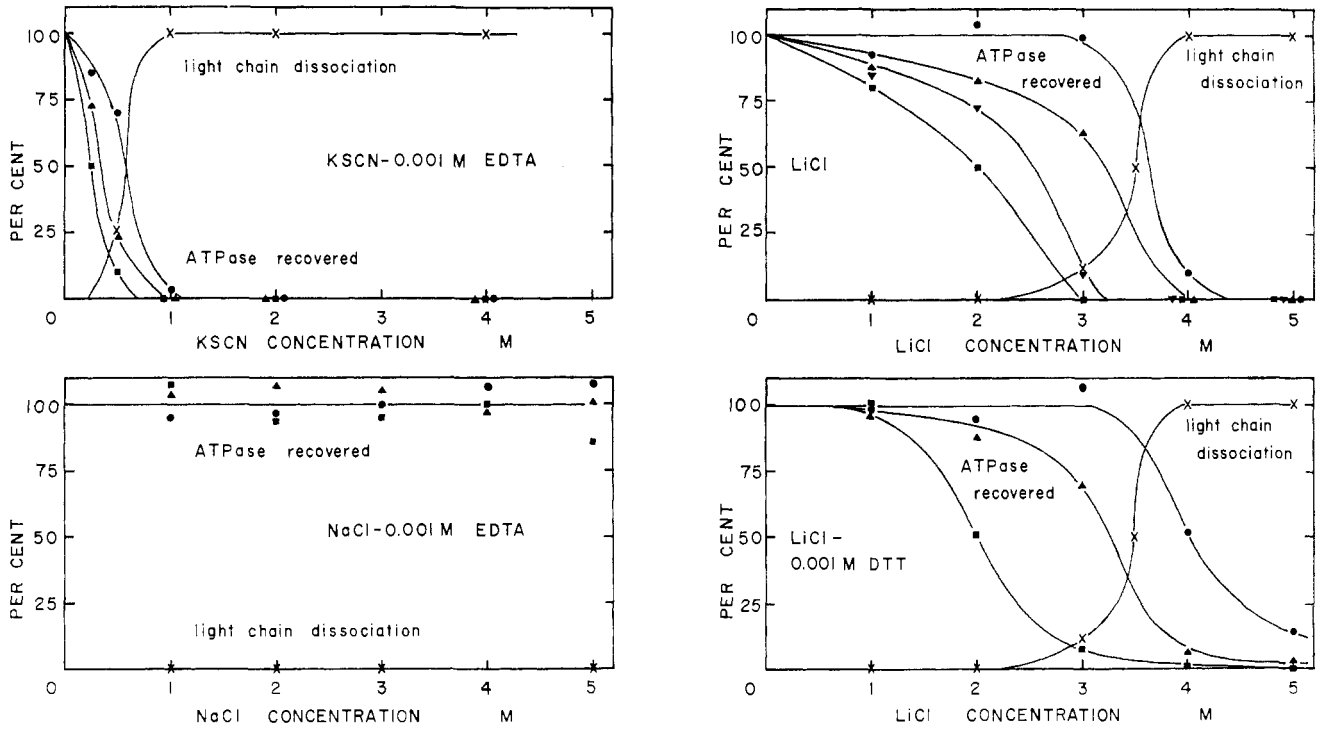

FIGURE 1: Denaturation profiles for light-chain dissociation and reversible Ca-ATPase on treatment of myosin in concentrated salt solutions, pH 7, 4°. Percentage dissociation of light component (×) from sedimentation velocity experiments initiated promptly after adjustment of salt concentration to indicated value. Ca-ATPase was determined on samples treated in specified salt solution for 5 min (●), 2 hr (▲), 8 hr (▼), and 24 hr (■), respectively, prior to dialysis against 0.4 M KCl.

TABLE I: ATPase Activity and ADP Binding Following LiCl-Citrate Fractionation and Subunit Recombination in Varying Proportion.

	Native Myosin				NH_4Cl-$(NH_4)_2SO_4$ Precipitate of Myosin			
			ADP Binding				ADP Binding	
Fraction	Light Component[a] (%)	ATPase Act.[b] (%)	Sites	$\Delta F°$ (kcal/mole)	Light Component[a] (%)	ATPase Act.[c] (%)	Sites	$\Delta F°$ (kcal/mole)
Precipitate + 2 volumes of supernatant	18.5	75	1.68	6.9	14	70	1.53	7.0
Precipitate + 1 volume of supernatant	12.0	70	1.68	6.9	9.2	65	1.53	7.0
Precipitate + 0.5 volume of supernatant	8.0	57	0.81	6.8	6.0		1.01	6.5
Precipitate + 0.25 volume of supernatant	6.0	42	0.81	6.8	4.5	40	1.01	6.5
Precipitate	3.9	10	0		2.9	9	0	
Second cycle precipitate	1.0	0						
Supernatant (water soluble)	100	0	0		100	0	0	

[a] From sedimentation velocity and sedimentation equilibrium experiments on precipitate and supernatant fractions; other values are based on volumetric dilution. [b] Control ATPase activity 0.70 μmole of P_i/min per mg. [c] Control ATPase activity 0.45 μmole of P_i/min per mg.

that the only variable feature was the proportion of light component as determined by the arbitrary mixing ratio of supernatant and precipitate from LiCl-citrate fractionation of myosin. In recombination experiments done in the absence of EDTA or dithiothreitol, only 0.12 μmole of P_i/min per mg of ATPase is recovered in fully reconstituted myosin, but significantly less ATPase is recovered in identically treated samples reconstituted with 6 or 8% light component (Figure 3).

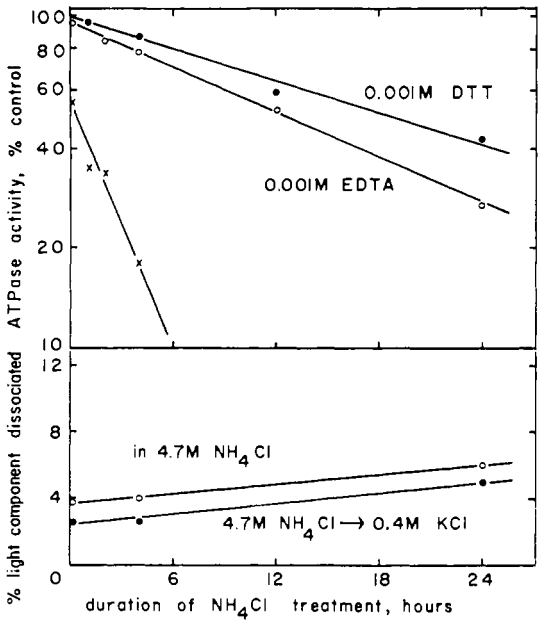

FIGURE 2: Effect on myosin of storage in 4.7 M NH_4Cl, pH 7, 4°. Upper graph: Ca-ATPase following dialysis of NH_4Cl-treated myosin to 0.4 M KCl, plotted against duration of NH_4Cl treatment in 0.001 M dithiothreitol (●), 0.001 M EDTA (○), or neither (×). Control ATPase 0.55 μM P_i/mg per min. Lower graph: proportion of dissociated light component from sedimentation velocity and/or sedimentation equilibrium of myosin in 4.7 M NH_4Cl (○) and NH_4Cl-treated myosin further dialyzed against 0.4 M KCl (●), plotted against duration of NH_4Cl treatment. Results comparable with or without EDTA or dithiothreitol.

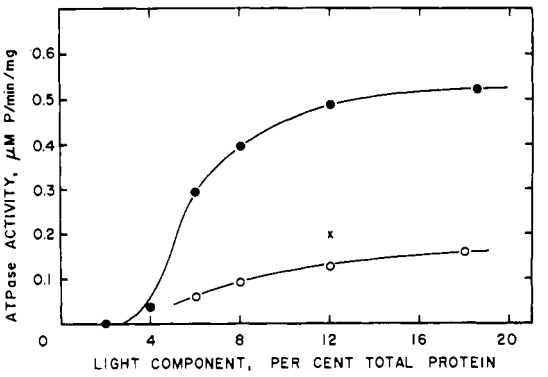

FIGURE 3: Ca-ATPase plotted against proportion of light component, from experiments based on LiCl-citrate fractionation of myosin and subunit recombination in different proportions. (●), 0.001 M dithiothreitol present throughout; (×), 0.001 M EDTA present during LiCl-citrate fractionation; (○) no dithiothreitol or EDTA.

Somewhat greater ATPase activity is recovered after LiCl-citrate fractionation of myosin in the presence of 0.001 M EDTA, but maximal recovery of ATPase is obtained after LiCl-citrate fractionation and recombination in the presence of 0.001 M dithiothreitol. With thiol protection, fully reconstituted myosin exhibits 0.49 μmole of P_i/min per mg of Ca-ATPase activity (Figure 3), that is, about 70% of control ATPase (Table I). Protein reconstituted with less than 8% light component shows considerably less recovery of Ca-ATPase, and ATPase activity is totally lost in reconstituted protein with only 2% light component. In the presence of excess light component, there is a slight increase in Ca-ATPase, and the purified light chains do not exhibit any ATPase activity.

Similar recombination experiments were done using the precipitate fraction obtained from 5-min treatment of myosin in 4.7 M NH_4Cl and salting out in 50% saturated $(NH_4)_2SO_4$-4.7 M NH_4Cl, with 0.001 M dithiothreitol present throughout. The precipitate fraction so obtained contains 9.2% light component of mol wt 20,700, that is, approximately 2.1 light chains/myosin molecule. After subsequent LiCl-citrate fractionation and recombination in varying proportion in the presence of dithiothreitol, the fully reconstituted protein (9.2% light component) retains about 65% of control ATPase; whereas samples containing fewer light chains show less ATPase activity, and the purified light chains exhibit no activity at all (Table I).

Recombination Experiments. ADP BINDING. ADP was dialyzed against reconstituted myosin following LiCl-citrate fractionation and subunit recombination in varying proportion. The dialysis solutions for these experiments contained ADP at 0.1–2.0 μM (or no) ADP, 0.4 M KCl, 0.001 M $MgCl_2$, and 0.001 M dithiothreitol (pH 7). The data indicated binding of ADP to reconstituted protein containing light and heavy chains, but no binding to identically treated heavy-chain core or light chains under the same dialysis conditions (Table I). On analysis according to Scatchard (1949), the data on fully reconstituted myosin or on myosin reconstituted with excess light component (18.5%) show a linear dependence of $\nu/(ADP)_f$ on ν, consistent with the presence of 1.7 ADP binding sites/myosin molecule, with apparent free energy, $\Delta F°$, of 6.9 kcal/mole of ADP (Figure 4, Table I). Protein reconstituted with 6–8% light component contains 0.81 ADP binding site/myosin molecule, with $\Delta F°$ = 6.8 kcal/mole of ADP.

Equilibrium dialysis experiments were also done following LiCl-citrate fractionation of the NH_4Cl-$(NH_4)_2SO_4$ precipitate fraction. Fully reconstituted protein (9.2% light component) or protein reconstituted with excess light component (14%) contains 1.5 ADP binding sites/myosin molecule, with $\Delta F°$ = 7.0 kcal/mole of ADP (Figure 4, Table I). In samples containing 4.5–6.0% light component, there is roughly 1.0 ADP binding site/myosin molecule, with $\Delta F°$ = 6.5 kcal/mole of ADP; but precipitate fraction (containing 2.9% light component) and light chains alone do not bind ADP in measurable proportion. These results are not significantly different from values obtained on LiCl-citrate fractionation of native myosin, indicating that the fraction of the total light component which is removed on NH_4Cl-$(NH_4)_2SO_4$ salting out is not essential for ADP binding to myosin.

Discussion

The denaturation profiles obtained on treatment of myosin in concentrated salt solutions, pH 7, 4°, are comparable with those obtained on treatment of myosin at alkaline pH and in guanidine solutions (Gershman *et al.*, 1966, 1969; Dreizen *et al.*, 1967), in that dissociation of myosin into light chains and heavy-chain core is accompanied in general by rapid irreversible inactivation of Ca-ATPase. The rate of ATPase

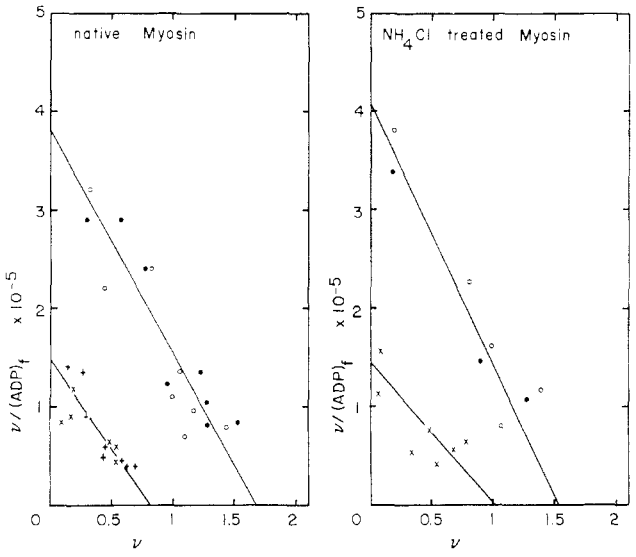

FIGURE 4: Scatchard (1949) plot on equilibrium binding of ADP following LiCl–citrate fractionation and subunit recombination in different proportions. Left, experiments on native myosin; right, experiments on precipitate fraction from rapid NH_4Cl–$(NH_4)_2SO_4$ fractionation. Symbols indicate fractions recombined: precipitate + 2 volumes supernatant (○); precipitate + 1 volume supernatant (●); precipitate + 0.5–0.75 volume of supernatant (+); and precipitate alone (×). Lines indicate least-squares regression, averaged for abscissa and ordinate as independent variables.

inactivation is somewhat lessened on salt treatment in the presence of dithiothreitol, without significant change in the extent of subunit dissociation, suggesting that ATPase inactivation is due to reactions of critical sulfhydryl groups and also other side-chain groups of the dissociated subunits under denaturing conditions. Prolonged salt treatment at molar concentrations below the transition for light-chain dissociation may be accompanied by slow irreversible inactivation of myosin ATPase (Figures 1 and 2), presumably from reactions of the same (or other) groups in myosin with the salt reagent or contaminants in the salt reagent. Irreversible denaturation of myosin ATPase should be differentiated from direct inactivation of Ca-ATPase by salt solutions (Tonomura *et al.*, 1962; Warren *et al.*, 1966), an effect that occurs in dissociating salts at molar concentrations well below the transition for light-chain dissociation and irreversible loss of ATPase, and also occurs in nondissociating salts (like NaCl) where ATPase inactivation is fully reversible.

The recombination experiments based on LiCl–citrate fractionation of myosin in 0.001 M dithiothreitol indicate recovery of 70% ATPase in fully reconstituted myosin; Ca-ATPase is lost on decrease in the ratio of light to heavy chains, and is absent in heavy-chain core and light chains alone. In experiments based on chromatographic fractionation of myosin in 4 M LiCl, Stracher (1969) described recovery of 30% ATPase in fully reconstituted myosin, and no activity in heavy or light components alone. The LiCl data clearly substantiate the recombination experiments based on alkaline–citrate fractionation of myosin in 0.01 M ATP–2 M KCl (Gershman *et al.*, 1969), and the overall data implicate some kind of interaction between light and heavy chains in the Ca-activated ATPase of myosin. The further evidence that approximately 3% light component (fast electrophoretic band) is dissociated from native myosin on short treatment in 4.7 M NH_4Cl–0.001 M dithiothreitol, without significant loss in Ca-ATPase, would indicate that only the two remaining light chains are involved in ATPase; and recombination experiments based on LiCl–citrate fractionation of the NH_4Cl–$(NH_4)_2SO_4$ precipitate fraction confirm that Ca-ATPase requires a molar ratio of light to heavy chains.

These data permit some clarification of earlier work on the structure of myosin. Myosin is split by trypsin into two fragments, light and heavy meromyosin (Szent-Györgyi, 1953; Lowey and Holtzer, 1959; Rice, 1964). Heavy meromyosin, an enzymatically active fragment (Szent-Györgyi, 1953), contains the light chains and two-thirds of the heavy-chain core (Gershman *et al.*, 1966; Dreizen *et al.*, 1967). Heavy meromyosin, or myosin directly, may be degraded by trypsin (Mueller and Perry, 1963) or papain (Kominz *et al.*, 1965) into a smaller particle, subfragment 1, that retains ATPase activity. That each myosin molecule yields two particles of subfragment 1 was suggested on stoichiometric grounds (Stracher and Dreizen, 1966; Nauss and Gergely, 1967), and shown directly on electron microscopy of myosin (Slayter and Lowey, 1967). Ultracentrifugal experiments indicate that subfragment 1 contains remnants of one heavy chain, 86,000 in weight, and one light chain, 18,000 in weight, in approximately molar ratio (Trotta *et al.*, 1968).

The present data would support the conclusion that myosin proper contains two enzymatically active protomers, each comprising one heavy chain and one essential light chain; an additional light chain (NH_4Cl-dissociated light component) may be tentatively considered a distinct protein closely associated with myosin proper. According to this interpretation, the two essential light chains are sampled from a population of three or more electrophoretically different components, and the nonessential light chain comprises a single (fast) electrophoretic component (Gershman and Dreizen, 1969a,b, 1970). The interpretation is expressed with caution in that present evidence does not exclude participation by the nonessential light chain in a regulatory role in myosin ATPase or in the interaction of myosin with other myofibrillar proteins.

A plausible explanation for interaction between essential light and heavy chains in myosin ATPase is that one subunit, say the heavy chain, contains the hydrolytic site and the adjacent subunit (essential light chain) stabilizes the unique heavy-chain conformation required for enzymatic activity. Alternatively, residues from heavy chain and essential light chain may both be located at or near the hydrolytic site. Although the recombination experiments on ATPase do not settle this important question, the data on ADP binding provide some insight into the nature of the myosin–nucleotide interaction.

Kiely and Martonosi (1969) and Lowey and Luck (1969) have reported 1.4 and 1.8 ADP binding sites, respectively, per myosin molecule.[2] Subfragment 1 contains 1.06 ADP

[2] These results are based on use of a 500,000 value for the molecular weight of myosin. This weight-average molecular weight appears to include a minor contribution from aggregated myosin, and the monomer weight of myosin has been found to be 468,000 (Gershman *et al.*, 1969). Assuming 468,000 molecular weight, the binding data would indicate 1.3 and 1.7 ADP binding sites per myosin molecule, respectively. An

binding sites (Young, 1967), indicating symmetric binding of ADP to each myosin protomer, with some suggestion of nucleotide–nucleotide interaction. The data on ADP binding to dissociated and reconstituted myosin indicate about 1.6 ADP binding sites/myosin molecule, with or without the nonessential light chain present. The number of ADP binding sites is diminished on decrease in the ratio of light to heavy chains, without apparent binding to heavy-chain core or light chains alone; and $\Delta F°$ is 6.8 ($\pm$0.3) kcal/mole of ADP, irrespective of the ratio of light to heavy chains. These data suggest that ADP binding too involves some kind of interaction between heavy-chain core and essential light chains. The most straightforward interpretation would be that ADP bridges essential light and heavy chains within each myosin protomer, and this interpretation is supported by experiments (Dreizen and Gershman, 1969a,b), to be described in detail in the following paper, that the interaction between essential light and heavy chains is stabilized by the terminal phosphate groups of adenosine or inosine nucleotides and by chain phosphates alone. Nevertheless, the binding data (Table I) do not exclude the possibility of weak interaction (<4 kcal/mole) of ADP with essential light or heavy chains alone, and this matter is under study.

Acknowledgments

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earlier report (Young, 1967) of 2.77 ADP binding sites/myosin molecule is based on use of a 595,000 molecular weight value for myosin, and these experiments have been criticized on other grounds as well (Lowey and Luck, 1969).

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