Comparison of the Calcium Sensitivity of Actomyosin from Native and L_2-Deficient Myosin†

Suzanne M. Pemrick‡

ABSTRACT: Evidence is presented that the L_2 light chain of rabbit skeletal myosin stabilizes a particular conformation which enhances actin interaction as A-M·ADP·P* complexes, thereby increasing the calcium affinity of troponin. This ability of L_2 to alter the apparent affinity of troponin for calcium required actin to myosin ratios that favored cooperative interactions between actomyosin complexes and actin molecules. L_2-deficient myosin (1 mol of L_2/mol) was prepared by treatment with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) and the hydrolytic activity restored by sulfhydryl regeneration. A comparison of acto-native myosin and acto-L_2-deficient myosin indicated: (1) at a 5- to 12-fold excess of regulated actin over myosin "heads", the free calcium concentration at half-maximal activation was decreased (an increase in calcium sensitivity) by increasing the concentration of myosin "heads" and was insensitive to partial removal of L_2; (2) at a 1:1, 0.6:1, or 2.5:1 ratio of actin to myosin "heads" partial removal of L_2 decreased calcium sensitivity and actin interaction; (3) partial removal of L_2 altered actin interaction only in the presence of calcium, at MgATP concentrations where M·ADP·P* was the predominant myosin species; (4) calcium sensitivity could be increased by addition of native myosin to acto-L_2-deficient myosin; (5) reassociation of L_2 to L_2-deficient myosin restored both calcium sensitivity and actin interaction to native levels.

The known regulatory proteins of muscle are inhibitory in nature. In vertebrate striated muscle, myosin is prevented from binding to actin when calcium-free troponin shifts tropomyosin to a "blocking" position (Haselgrove, 1972; Huxley, 1972; Wakabashi et al., 1975). In molluscs and some primitive invertebrates, the inhibitory protein is a light chain of the myosin molecule (Kendrick-Jones et al., 1972; Szent-Gyorgyi et al., 1973) and in some contractile systems both actin and myosin-linked inhibitory mechanisms are operative (Lehman and Szent-Gyorgyi, 1975). In all muscle types calcium releases inhibition by binding to these regulatory proteins to activate actin or myosin and initiate contraction.

Calcium, however, is not the only activator for this process. In skeletal muscle, myosin, within a suitable range of conformational states, can form complexes with actin which activate the thin filament in the absence of calcium. This occurs at low MgATP concentrations, when myosin is nucleotide-free (Bremel and Weber, 1972), or when myosin has been treated with *N*-ethylmaleimide at low ionic strength (Pemrick and Weber, 1976). Both complexes increase the calcium affinity of troponin (Bremel and Weber, 1972; Weber and Bremel, 1971). Therefore, myosin not only responds to the activated state of the thin filament, but myosin can also alter the regulatory properties of the thin filament in an "actin-linked" contractile system.

The experiments described in the present report show that the L_2 light chain of myosin, in a troponin–tropomyosin regulatory system, enhances the ability of myosin to interact with the thin filament in the presence of calcium, and beyond a threshold ratio of myosin to actin decreases the calcium concentration required to activate the thin filament.

A preliminary report of some of these findings has been presented (Pemrick, 1976b, 1977).

Materials and Methods

Nbs_2[1] was purchased from Eastman Organic Chemicals, Rochester, N.Y.; DTT, ATP, and $PhCH_2SO_2F$ from Sigma, St. Louis, Mo.; creatine phosphate from Calbiochem, LaJolla, Calif., and creatine phosphokinase and α-chymotrypsin from Worthington, Freehold, N.J.

Myosin was prepared from minced psoas muscle of rabbits according to standard extraction procedures (Portzehl et al., 1950; Bremel and Weber, 1975). Actomyosin was removed by several precipitations at 0.28 ionic strength. Actin was extracted from acetone powder by a modification (Tsuboi, 1968) of the procedure of Straub (1942) and purified (Spudich and Watts, 1971). An extract of troponin and tropomyosin was obtained from the muscle residue remaining after myosin extraction (Ebashi, 1964). Residual actin was removed by polymerization and centrifugation (240 000*g*, 1.5 h). Regulated actin was reconstituted by combining purified actin and the troponin–tropomyosin extract at a 2:1 weight ratio in 0.1 M KCl, 2 mM $MgCl_2$, pH 7.5. Excess extract was removed (100 000*g*, 3 h) and the regulated actin pellet solubilized in 50 mM Tris-Cl (pH 8, 4 °C).

L_2-deficient myosin was prepared by treating myosin with Nbs_2 and EDTA by the method of Gazith et al. (1970) as described by Weeds and Lowey (1971). The reaction was terminated by a tenfold dilution with ice-cold water and the ex-

† From the Department of Biochemistry and the Department of Medicine, Division of Cardiology, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029. *Received September 16, 1976; revised manuscript received June 7, 1977.*

‡ Present address: Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn, N.Y. 11203. S.M.P. was a Fellow of the New York Heart Association.

[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB is used in the figures); NEM, *N*-ethylmaleimide; HMM, heavy meromyosin, chymotryptic digestion product of myosin; S1, subfragment 1; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris-Cl, 2-amino-2-hydroxymethyl-1,3-propanediol chloride; $NaDodSO_4$, sodium dodecyl sulfate; M, head of myosin, HMM or S1, containing 1 actin binding site and 1 ATP binding site; A, actin; M·ADP·P*, class of myosin intermediates during hydrolysis of MgATP; no attempt is made in this study to distinguish between M**ADP·P, M*ADP·P, etc., described by Bagshaw and colleagues (1974).

tracted L_2 light chain removed immediately by centrifugation (19 000*g*, 20 min) and lyophilized. The myosin pellet was washed and dissolved in a final concentration of 0.5 M KCl, and 10 mM Tris-Cl, 10 mM EDTA, 10 mM DTT, pH 8.5 (4 °C). Dialysis for 3 days with three dialysate changes against 100 volumes of the same buffer containing 5 mM DTT was sufficient to remove the Nbs_2. The Nbs_2-myosin was then dialyzed overnight against 0.6 M KCl, 10 mM imidazole chloride, 1 mM DTT, pH 7.4 ("myosin buffer"), clarified, subjected to one dilution-precipitation step, and redissolved in "myosin buffer". Prior to reassociation of L_2 to L_2-deficient myosin or HMM, lyophilized L_2 extract was homogenized in a minimum volume of 10 mM Tris-Cl, 2 mM DTT (pH 8.0, 25 °C), dialyzed exhaustively against the same buffer, and clarified at 16 000*g* (15 min). Reassociation of L_2 to Nbs_2-myosin required equilibration (48 h at 0 °C) of equivalent moles of myosin and L_2 in 0.6 M KCl, 5 mM imidazole chloride (pH 7), 1 mM $MgCl_2$, 1 mM DTT, followed by three dilution-precipitation steps, and solubilization of the final pellet in "myosin buffer".

The ATPase activity was assayed in a total volume of 2.0 mL with the following media: (1) *KEDTA-ATPase,* 0.6 M KCl, 2 mM EDTA, 5 mM ATP, 15 mM Tris-Cl (pH 8, 25 °C), and 0.05 to 0.15 mg of myosin for 30 to 60 s; (2) *CaATPase,* 0.5 M KCl, 2.5 mM ATP, 2.5 mM $CaCl_2$, 25 mM Tris-Cl (pH 7.6, 25 °C), and 0.4 mg of myosin; (3) *MgATPase in the presence or absence of actin,* 20 mM imidazole chloride (pH 7, 25 °C), 1.0 mM $MgCl_2$, 30 mM KCl, 1-2 mM EGTA/CaEGTA buffer, 0.3 mg of actomyosin or 1.0 mg of myosin and 0.6 mM MgATP to start the reaction. This MgATP concentration was necessary for another series of experiments that was performed simultaneously. The assay time was adjusted so that not more than 20% of the ATP was hydrolyzed. The actin-activated ATPase activity was corrected for that of myosin alone as a function of the free calcium or MgATP concentration. Following protein precipitation with trichloroacetic acid, inorganic phosphate liberation was measured according to Taussky and Shorr (1952) and expressed as nmol of $P_i\ s^{-1}$ (nmol of myosin site)$^{-1}$ ("head"). When the ATPase activity was measured as a function of increasing ATP concentrations, creatine phosphokinase (2 mg/mL) and creatine phosphate (5 mM) were present in the medium. The reaction was started by the addition of myosin or actomyosin and terminated by *p*-mercuribenzoic acid. Following protein precipitation by $ZnSO_4$ and subsequent neutralization by $Ba(OH)_2$ (Somogyi, 1945), the liberation of creatine was measured according to Eggleton et al. (1943). All colorimetric assays were read on a Gilford Stasar II rapid-sampling spectrophotometer.

Actomyosin was preformed in 0.6 M KCl by *first* mixing regulated actin (3 mg for most experiments), followed by addition of myosin to a final volume of 3.0 mL. This sequence is necessary to optimize availability of actin monomers to myosin sites, thereby achieving greater precision in the ATPase measurements. The mole amount of actin and myosin mixed together should not be varied for a given ratio of actin:myosin "heads" when comparing actin interaction at optimal Ca^{2+} concentrations.

Free calcium concentration was regulated by varying the ratio of EGTA/CaEGTA at a constant ionic strength, assuming a K_d of 0.19 μM at pH 7 for Ca^{2+} and EGTA as estimated from Schwarzenbach's constant (Chaberek and Martell, 1959). The free Ca^{2+} concentration was calculated from the formula presented in Bremel and Weber (1975) which corrects for CaATP.

Calcium sensitivity is defined in this study as the free Ca^{2+} concentration required for half-maximal activation of the actin-activated ATPase activity. The percent relaxation is expressed as

$$\left(1 - \frac{\text{ATPase activity at 0.01 } \mu\text{M } Ca^{2+}}{\text{the maximal ATPase activity}}\right)100$$

Although both expressions are components of calcium sensitivity, unless stated otherwise, "calcium sensitivity" will be restricted to the former definition.

Protein concentration was determined according to Lowry's method (1951) standardized against bovine serum albumin and myosin; the latter protein was calibrated by an $E_{280\ nm}^{1\%}$ of 5.43 (Gellert and Englander, 1963). The concentration of regulated actin was read from the myosin curve. A molecular weight of 500 000 was assumed for both native and L_2-deficient myosin. For regulated actin a molecular weight of 67 000 was calculated by assuming one troponin-tropomyosin complex (mol wt 150 000) for every seven actin monomers (mol wt 45 000/monomer) (Bremel and Weber, 1972).

HMM was prepared simultaneously from native and Nbs_2-myosin as described for native myosin (Weeds and Taylor, 1975). Digestion with chymotrypsin at high ionic strength and 4 mM $MgCl_2$ was terminated after 2.5 or 5 min with $PhCH_2SO_2F$. Myosin rods and undigested myosin were precipitated by dilution (H_2O) and removed (centrifugation). Native and Nbs_2-HMM were concentrated from their respective supernatants at 55% saturation to ammonium sulfate. This procedure decreased the amount of low-molecular-weight components on $NaDodSO_4$ gels. Following dialysis (1:1000) against 10 mM Tris-Cl, pH 8.5 (4 °C), 0.1 mM DTT, an aliquot of both HMM preparations was combined with L_2 (1:1 or 2:1 molar ratio of L_2 to HMM "heads") and equilibrated for 48 h, at 4 °C.

$NaDodSO_4$ disc electrophoresis was performed according to a modified procedure of Laemmli (1970). Gel samples were prepared in 1% $NaDodSO_4$ and β-mercaptoethanol, immersed in boiling water for 5 min, incubated at 37 °C for 2 h, and dialyzed against the upper reservoir buffer containing 10% glycerol and a trace of bromophenol blue. The gels were run at 0.5 mA/tube until the tracking dye entered the separating gel and then at 1 to 1.5 mA/tube for 7 h.

Results and Discussion

Nbs_2 treatment of myosin at high ionic strength released approximately 1 of the 2 mol of L_2 light chain per mol of myosin without an appreciable alteration in the relative amount of either the L_1 or L_3 light chain (Figure 1).

Following prolonged dialysis with DTT, the K-, Ca-, and MgATPase activities were regenerated completely, which is indicative that the functional sulfhydryl groups of myosin were not irreversibly modified by exposure to the sulfhydryl blocking reagent (Table I).

When compared at approximately a 1:1 ratio of actin to myosin "heads" (sites), the calcium sensitivity of acto-Nbs_2-myosin differed from acto-native myosin in that, (1) a higher concentration of free Ca^{2+} was required for half-maximal activation and (2) maximal activation of the ATPase activity was reduced. In the experiment in Figure 2, removal of 50% of the L_2 light chain increased the free Ca^{2+} concentration required for half-maximal activation from 0.15 to 0.85 μM and maximal activation decreased from 1.8 to 1.1 s^{-1} (Figure 2A). Similar results were obtained from six batches of myosin (Figure 2, Table II, Figure 3A). When the data were normalized to 100% activation, the percent activation at 0.01 μM Ca^{2+} (100 − percent activation = percent relaxation) varied by less than 10% in four out of six preparations.

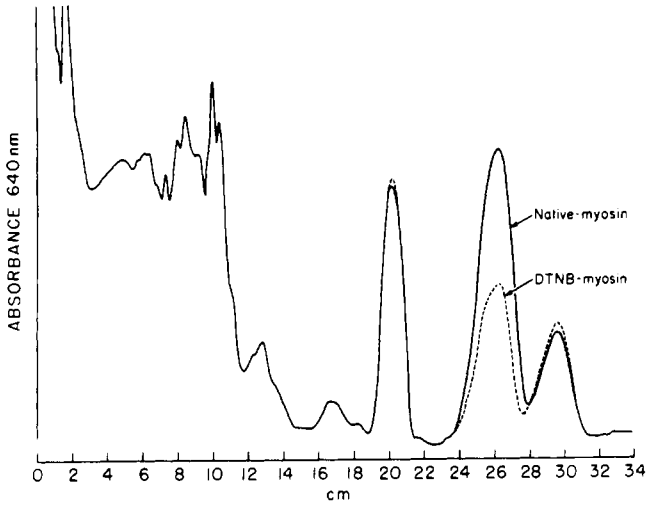

FIGURE 1: Densitometric scans obtained by $NaDodSO_4$ disc electrophoresis of native and Nbs_2-myosin; 125-μg samples in 0.2% $NaDodSO_4$ and β-mercaptoethanol, 3% stacking gels (1 cm), 15% separating gels (9 cm). Gels were stained in Coomassie brilliant blue R.

TABLE I: ATPase Activity of Myosin (s^{-1} $site^{-1}$)

Substrate	Native myosin	Nbs_2-myosin	L_2 + Nbs_2-myosin[a]
K_2ATP	18.1 ± 1.2[b]	18.1 ± 0.42[b]	15.0
CaATP	1.66[c]	1.67[c]	1.55
MgATP + EGTA[d,e]			
1.3 μM MgATP	0.052	0.055	
10 μM MgATP	0.077	0.078	
50 μM MgATP	0.085	0.09	
100 μM MgATP	0.09	0.087	
1 mM MgATP	0.086	0.085	0.061

[a] Reassociation of the L_2 light chain to Nbs_2-myosin (see Figure 4B). [b] Mean and standard error for the four preparations in Table II (1:1 A:M). [c] Mean of two preparations. [d] The MgATPase activity of native myosin is activated 1.5- to 2-fold from 0.01 to 10 μM Ca^{2+} (Pemrick, 1976a). [e] Concentration of myosin sites at 0.6 μM as in Figures 2, 3, 4, and 6.

At approximately 20 μM Ca^{2+}, the actin-activated ATPase activity of native myosin was *inhibited* 12 to 40% (Figures 2 and 3B). This inhibitory response at 20 μM Ca^{2+}, although variable in degree, occurred also with: (1) nonregulated actin, where, for native myosin, it was more sensitive to the free Mg^{2+} concentration; (2) L_2-deficient myosin and S-1 preparations which appeared to lack undegraded L_2 (Pemrick, 1976a). This phenomenon will not be examined further at this time, since it is neither *directly* related to the presence of thin filament regulatory proteins, nor to the full complement of L_2.

The effect of actin and myosin concentration on calcium sensitivity was compared for native and L_2-deficient myosin (Table II). The results obtained from one experiment are presented in Figure 3A-D. When actin was in 12-fold excess to myosin "heads" (either native or L_2 deficient), half-maximal activation was attained between 0.8 and 0.9 μM (Figure 3D). This Ca^{2+} concentration is approximately fivefold higher than that required for half-maximal activation when the actin and the native myosin concentrations were equivalent (Figures 2 and 3A, vs. Figure 3D). A 2.5-fold increase in the concentration of total myosin "heads", from 1 per 12 to 1 per 5 regulated-actin monomers, increased calcium sensitivity 1.5- (Table II) to 2-fold (from 0.8 to 0.9 μM Ca^{2+} in Figure 3D to 0.42 to 0.45 μM Ca^{2+} in Figure 3C). At a 12:1 and 5:1 ratio of A:M, *regulated* actin was in considerable excess over myosin and cooperative interactions between actomyosin complexes (MA or A-M·ADP·P*) and regulated actin were minimal. Under these conditions, calcium sensitivity was increased by increasing the concentration of myosin "heads" and was insensitive to partial removal of the L_2 light chain (Table II).

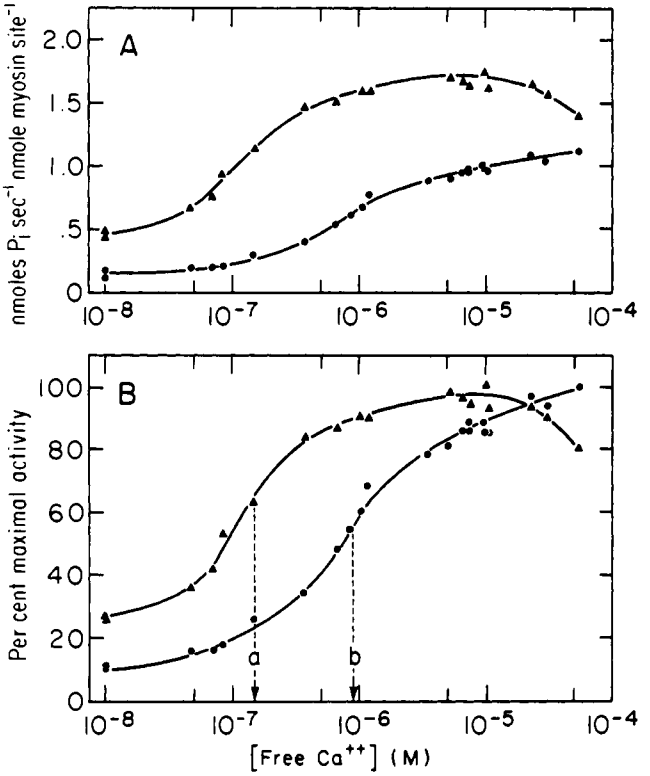

FIGURE 2: The actin-activated MgATPase activity of (▲) native (2 mol of L_2/mol) and (●) Nbs_2-myosin (1 mol of L_2/mol) as a function of the free-ionized Ca^{2+} concentration. (A) Activity expressed as nmol of P_i s^{-1} nmol of myosin $site^{-1}$. (B) Same as A, data normalized to the maximal ATPase activity. Conditions described under Materials and Methods. Total myosin sites, 0.6 μM; regulated actin, 0.75 μM. The arrows on the abscissa indicate the Ca^{2+} concentration of half-maximal activation for "a" native myosin and "b" Nbs_2-myosin.

The inhibitory effect of 50% removal of the L_2 light chain on calcium sensitivity required concentrations and ratios of actin and myosin in which more than 20% of the actin monomers were capable of interacting with myosin "heads". Loss of L_2 decreased calcium sensitivity ($p \leq 0.005$, one-tailed paired difference test) and maximal activation at approximately a 1:1 (1.25:1), 0.6:1, and 2.5:1 ratio of A:M (Figure 3A,B). Ca^{2+} sensitivity for both native and L_2-deficient myosin was optimal at a 2.5:1 ratio of A:M, regardless of whether this ratio was obtained by varying the actin or myosin concentration. The Ca^{2+} concentration at half-maximal activation was 0.086 μM for acto-native myosin and 2.5- to 4-fold greater (0.2-0.4 μM) for acto-L_2-deficient myosin (Figure 3B). Calcium sensitivity decreased slightly at the 1:1 or 0.6:1 ratio of A:M, but the inhibitory effect (4.5- to 5-fold) of partial removal of L_2 was most apparent under these conditions (Figure 3A, Figure 2, Table II). Although for a particular ratio of A:M, the actin-activated ATPase activity was quite variable from one batch of contractile proteins to another, this had little effect on calcium sensitivity (Table II). The source of the variability in actin interaction was most probably the thin-filament preparations. One batch of native or L_2-deficient myosin when assayed with two thin-filament preparations varied in both ATPase and percent relaxation (averaged in Table II).

TABLE II: Experimental Consistency in Calcium Sensitivity. Comparison of Native and L_2-Deficient Myosin.[a]

Ratio actin:myosin "heads"	Myosin type	MgATPase + reg actin[c]		Calcium sensitivity	
		0.01 μM Ca^{2+}	Max Act.	Ca^{2+} at $\frac{1}{2}$ Max Act. (μM)	Relaxation (%)
1.25:1[b]	Native	0.33 ± 0.05	1.6 ± 0.08	0.18 ± 0.04	79 ± 2.1
	Nbs_2	0.12 ± 0.01	1.0 ± 0.15	0.82 ± 0.05	87 ± 1.5
0.6:1	Native	0.65 ± 0.08	2.6 ± 0.34	0.16 ± 0.04	74 ± 6.3
	Nbs_2	0.29 ± 0.09	1.0 ± 0.03	0.83 ± 0.04	69 ± 10.7
2.5:1	Native	0.56 ± 0.02	1.9 ± 0.18	0.086 ± 0.001	74 ± 4.0
	Nbs_2	0.37 ± 0.06	1.5 ± 0.11	0.23 ± 0.01	76 ± 2.6
5:1	Native	0.54 ± 0.12	3.0 ± 0.95	0.50 ± 0.06	80 ± 3.2
	Nbs_2	0.25 ± 0.13	1.3 ± 0.18	0.53 ± 0.05	82 ± 10.1
12:1	Native	0.93 ± 0.38	3.1 ± 0.64	0.78 ± 0.12	73 ± 8.7
	Nbs_2	0.55 ± 0.11	2.2 ± 0.22	0.87 ± 0.03	76 ± 3.5

[a] Mean and standard error for three separate experiments on two batches of native myosin, three preparations of L_2-deficient myosin and thin filaments. 1:1 ratio of A:M includes data from an additional experiment. [b] For simplicity in the text, a 1.25:1 ratio of A:M was termed a 1:1 ratio. [c] nmol of P_i s^{-1} $site^{-1}$.

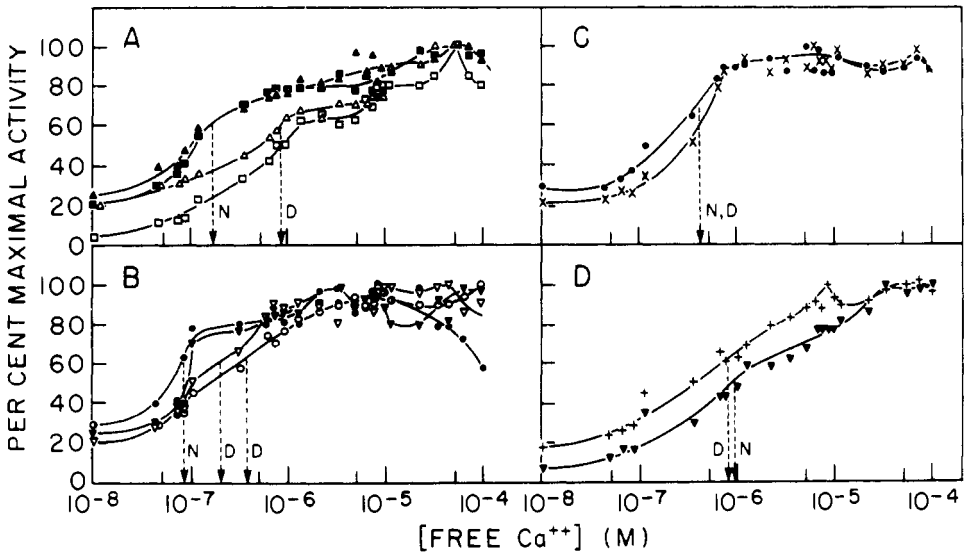

FIGURE 3: Effect of actin and myosin concentration on calcium sensitivity. (A) 0.6 μM native (▲), or Nbs_2-myosin (△) heads; 0.75 μM regulated actin; 0.6 μM native (■), or Nbs_2-myosin (□) heads; 0.37 μM regulated actin; (B) 0.6 μM native (▼), or Nbs_2-myosin (▽) heads; 1.5 μM regulated actin; 0.3 μM native (●), or Nbs_2-myosin (○) heads; 0.75 μM regulated actin; (C) 0.15 μM native (×), or Nbs_2-myosin (●) heads; 0.75 μM regulated actin; (D) 0.06 μM native (▼), or Nbs_2-myosin (+) heads; 0.75 μM regulated actin; 100% on "y" axis = (A) (▲) 1.5, (△) 0.88 s^{-1} nmol of myosin "head" $^{-1}$, (■) 3.2, (□) 1.0 s^{-1} nmol of $actin^{-1}$; (B) (▼) 2.2, (▽) 1.3, (●) 1.8, (○) 1.0; (C) (×) 2.6, (●) 1.2; (D) (▼) 1.8, (+) 2.5 s^{-1} nmol of myosin $site^{-1}$. The arrows on the abscissa indicate the Ca^{2+} concentration at half-maximal activation for N, native, and D, L_2-deficient (Nbs_2-), myosin. Other conditions as described under Materials and Methods.

Figure 3 also indicated that at a *constant concentration* of regulated actin, calcium-sensitivity increased 4–5-fold by (1) increasing native "heads" from 1 per 5 to approximately 1 per 2.5 actins or by (2) replacing L_2-deficient myosin with native myosin at approximately equivalent concentrations of actin and total myosin "heads". This suggested that the state of the thin filament (induced by the apparent Ca^{2+} affinity of troponin) was similar for a 1:1 ratio of acto-L_2-deficient myosin and a 5:1 ratio of acto-native myosin. It was hypothesized that the inhibitory effect of 50% removal of L_2 could be reversed by addition of native myosin. This was confirmed in the experiment in Figure 4A. A small amount of native myosin (3.0 μM "heads") was mixed at high ionic strength with the 1:1 ratio of acto-L_2-deficient myosin (12.0 μM Nbs_2-myosin "heads") prior to addition to the assay medium of low ionic strength. The free Ca^{2+} concentration at half-maximal activation decreased 2.5-fold from 0.88 μM for the acto-L_2-deficient myosin to 0.33 μM for the mixture of acto-L_2-deficient and native myosin. In the homologous system, however, calcium sensitivity was unchanged by decreasing the ratio of actin:myosin from 1:1 to 0.6:1 (Figure 3A).

To summarize, the experiments in Figures 3 and 4A suggest that regulated actin responds to the concentration of myosin "heads": at or below 1 "head" per 5 actins, regulated actin is insensitive to the L_2 light chain (Figure 3C,D); above this concentration, regulated actin distinguishes between "native" and L_2-deficient "heads" (Figures 3A,B and 4A). In the latter case, it is not possible to determine from the data, whether this represents a response of the thin filament to the type of myosin "head" or a response of the thin filament to altered levels of actin interaction.

Involvement of the L_2 light chain in modulating calcium sensitivity is further substantiated in the following experiment. The L_2 light chain was reassociated to Nbs_2-myosin (Figure 5) without modifying the hydrolytic site (Table I). Calcium sensitivity and maximal activation of a 1:1 ratio of actin: reassociated myosin "heads" were identical to values obtained for acto-native myosin under similar conditions (Figure 4B vs.

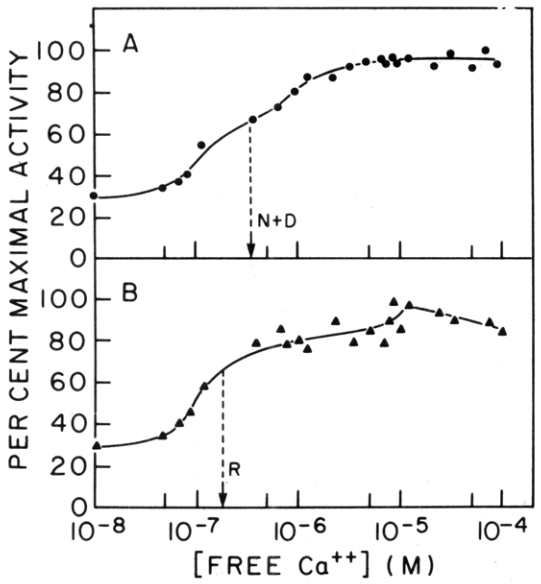

FIGURE 4: The ability of (A) native or (B) "reassociated" myosin (L_2 + Nbs_2–myosin) to enhance calcium sensitivity. (A) 3 μM native myosin "heads" added at 0.6 M KCl to 12 μM L_2-deficient "heads", 14 μM regulated actin. Final concentrations in the ATPase assay: 0.15 μM native, and 0.6 μM L_2-deficient "heads"; 0.75 μM regulated actin. (B) 1:1 ratio of "reassociated" actomyosin (0.6 μM myosin "heads"). Other conditions are described under Materials and Methods. 100% on "y" axis = 1.0 (A) and 2.1 (B) s^{-1} $site^{-1}$. Arrows on the abscissa indicate Ca^{2+} at half-maximal activation for: N + D, acto-native + Nbs_2–myosin; R, "reassociated" actomyosin. *Note similarity to Figures 2 and 3A especially for Figure 4B.*

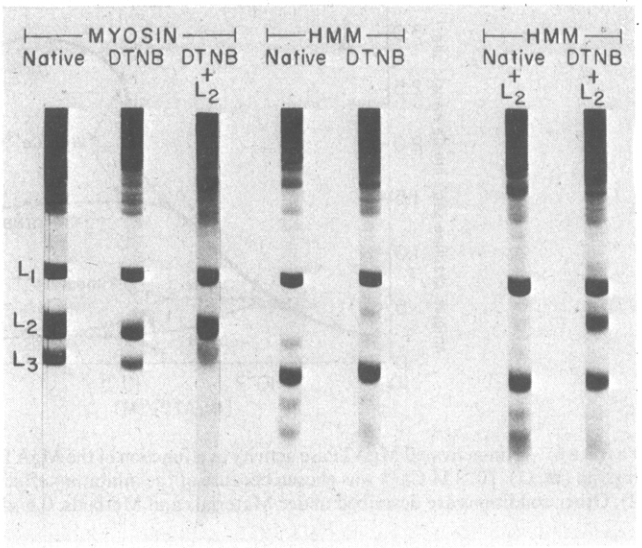

FIGURE 5: $NaDodSO_4$ disc gel electrophoresis of various populations of myosin and HMM. Native myosin, 110 μg; Nbs_2–myosin, 103 μg; L_2 + Nbs_2–myosin, 110 μg. Native HMM, Nbs_2–HMM, L_2 + native HMM, L_2 + Nbs_2–HMM, 76 μg. Native and Nbs_2–HMM prepared from native and Nbs_2–myosin, respectively. Following equilibration of HMM and L_2, HMM was reprecipitated at 55% saturation to $(NH_4)_2SO_4$ prior to gel electrophoresis; following equilibration of Nbs_2–myosin and L_2, "reassociated" myosin was subjected to three dilution–precipitation steps.

Figure 3A). This demonstrated that the inhibitory effect of 50% removal of L_2 on calcium sensitivity was reversible.

HMM was prepared from native and Nbs_2–myosin by chymotryptic digestion (Weeds and Taylor, 1975) followed by ammonium sulfate precipitation. Under these conditions the hydrolytic site was preserved (not shown). When subjected to $NaDodSO_4$ disc electrophoresis, all preparations of HMM retained less than Nbs_2–myosin of a low-molecular-weight component resembling L_2 (Figure 5). Reassociation of L_2 has, to date, been only partially successful (Figure 5). Final values of 1.75 to 1.8 mol of L_2/mol of myosin and 1 mol of L_2/mol of Nbs_2–HMM were estimated from densitometric scans (not shown). In the case of myosin, reassociation was sufficient to shift Ca^{2+} sensitivity to native levels at a 1:1 ratio of A:M (Figure 4B).

In order to determine which *general step* (MA or A–M·ADP·P*) in the actin-activated ATPase cycle was affected by partial removal of L_2, ATPase activity was compared as a function of increasing MgATP concentration (Figure 6A,B). At very low MgATP, the concentration of nucleotide-free myosin bound to actin (MA) was sufficient to destroy relaxation (see also Bremel and Weber, 1972). The lowest concentration of MgATP at which some relaxation was observed is a qualitative index of the affinity of nucleotide-free myosin for actin. This parameter was not altered after removing 50% of L_2, nor following reassociation of L_2 to Nbs_2–myosin. A small percentage of MA complexes could have persisted at millimolar ATP, but were not of sufficient concentration to activate the thin filament *in the absence of calcium* and reduce substantially the percent relaxation (Pemrick and Weber, 1976).

It can be concluded that from 50 μM to 1 mM MgATP both native and L_2-deficient myosin were saturated with substrate, since in the absence of actin the MgATPase activity plateaued at a similar value (Table I). Partial removal of L_2 decreased actin·interaction only *in the presence of calcium,* above 50 μM MgATP where M·ADP·P* is the predominant *myosin* species[1] (Figure 6A). In addition, this decrease in actin interaction could be reversed by reassociation of L_2 to L_2-deficient myosin (Figures 6B and 4B). The simplest explanation for these results is that *removal of 50% of L_2 has decreased the steady-state concentration of A-M·ADP·P* complexes.*[2] Since myosin and not actomyosin was treated with Nbs_2 to release L_2, it is a reasonable assumption that the species altered by partial removal of L_2 was a M·ADP·P* intermediate.

Conclusions

These results are compatible with the hypothesis that the L_2 light chain stabilizes a particular conformation of native myosin which enhances actin interaction, and increases calcium sensitivity. This function for L_2 has been inferred by comparing L_2-deficient and native myosin. It is concluded that alterations in myosin following Nbs_2 treatment were the direct result of loss of the light chain and not the indirect result of sulfhydryl modification. Except for the presence of EDTA, the conditions for Nbs_2 treatment (high salt and 0 °C) optimized modification of the SH_1 class of sulfhydryls (Sekine and Kielley, 1964). It is not known whether the sulfhydryl-protecting effect of EDTA at 25 °C (Watterson et al., 1975) prevails at 0 °C. The present conditions differed from those under which the "calcium-sensitive" sulfhydryls were alkylated (Weber and Bremel, 1971; Daniel and Hartshorne, 1972; 1973). Furthermore, treatment with DTT restored the native properties of the hydrolytic site (Table I). A possible exception has been observed in the pre-steady-state, at ambient free calcium concentrations (Pemrick, 1974). The steady-state kinetics of substrate saturation were hyperbolic only for L_2-deficient myosin (personal observations). For native myosin,

[2] A decrease in the actin-activated ATPase activity is presumably associated with a decrease in the steady-state concentration of A–M·ADP·P* complexes, since by analogy from the regulated acto-S1 system an increase in the number of A–M·ADP·P* complexes would *potentiate* the system (Bremel et al., 1972). The ability of MA complexes to potentiate the *actomyosin* system has been shown (Pemrick and Weber, 1976).

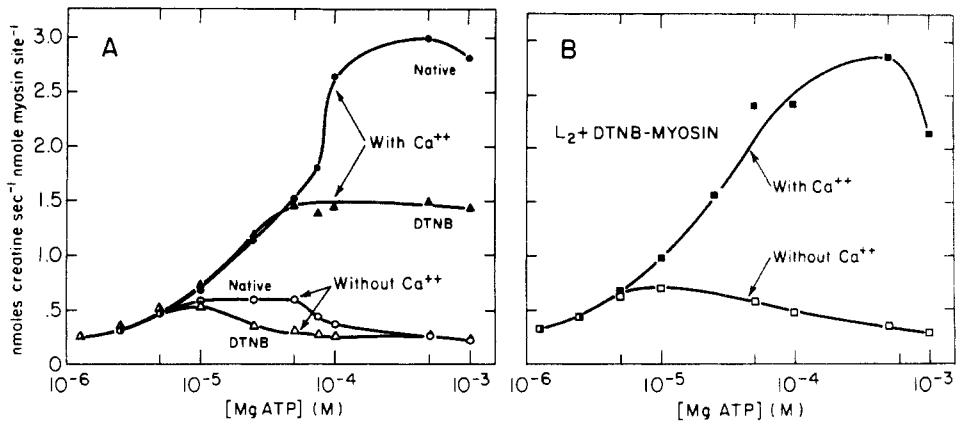

FIGURE 6: Actin-activated MgATPase activity as a function of the MgATP concentration. (A) Native (●, ○), and Nbs_2-myosin (▲, △). (B) Reassociated myosin (■, □). 10^{-5} M Ca^{2+} was chosen because of the inhibitory effect of higher Ca^{2+} concentrations on actin interaction for native myosin (Figure 2). Other conditions are described under Materials and Methods. 0.6 μM myosin sites, 0.75 μM regulated actin.

the steady-state pattern was similar to that reported by Schliselfeld (1976a) and Nihei and Filipenko (1975) for column-purified myosin. Finally, reassociation of L_2 to Nbs_2-myosin restored calcium sensitivity and actin interaction to near native levels (Figures 4B and 6B).

The predominant *species* of *myosin* which was affected by partial removal of L_2 appeared to be an M·ADP·P* intermediate[1] and this resulted in a *decrease in the steady-state concentration of A-M·ADP·P* complexes.*[2] There is no evidence that the altered complex was an "ATP-resistant" complex between myosin and actin (see Pemrick and Weber, 1976). This complex may be produced experimentally by NEM treatment, in which case, both the calcium concentration at half-maximal activation and the percent relaxation were *reduced* substantially (Weber and Bremel, 1971). Under equivalent conditions, removal of 50% of L_2 resulted repeatedly in a four to fivefold *increase* in the free Ca^{2+} concentration required for half-maximal activation with only a 6–8% increase in the percent relaxation (four out of five batches, Table II; Figure 3A). The following were indications that the number of MA complexes were equivalent for acto-native and acto-L_2-deficient myosin: (1) no change in the minimal ATP concentration at which calcium sensitivity became apparent; (2) no change in the ATPase activity in the absence of calcium from 0.5 to 1.0 millimolar ATP (Figure 6).

There is also no evidence that M·ADP·P* or A-M·ADP·P* was calcium sensitive. In the presence of regulated actin, the parameters of calcium sensitivity were altered indirectly. Presumably, the calcium affinity of troponin was increased in the presence of native myosin by an increase in the number of A-M·ADP·P* complexes similar to that observed in the presence of MA complexes (Bremel and Weber, 1972). Although calcium binding to the thin filament was not measured in this study, the shift of half-maximal actin activation to higher calcium concentrations was occurring within the range where (at 1 mM $MgCl_2$) troponin binds calcium (Potter and Gergely, 1975). From 0.01 to 5 μM Ca^{2+}, at 1 mM $MgCl_2$, there is negligible calcium binding to myosin (Bremel and Weber, 1975). Actin interaction vs. Ca^{2+} concentration was fitted to the Hill equation (not shown). At a 0.6:1, 1:1, and 2.5:1 ratio of actin to myosin "heads", for both native and L_2-deficient myosin, two slopes were apparent around the midpoint. This has been interpreted to be a specific property of regulatory systems (Cook and Koshland, 1970) rather than an artifact of the Hill equation. In the present study, below 0.5 V_{max}, the Hill plot was concave upward; above 0.5 V_{max} the Hill coefficient was less than 1.0. From the Hill plots, it was not possible to detect a substantial alteration in the type of cooperativity between acto-native and acto-L_2-deficient myosin. Therefore, it is suggested that the L_2 light chain modulates thin-filament regulation by increasing the calcium affinity of troponin, rather than by altering appreciably the nature of the cooperative response to calcium.

At higher free Ca^{2+} concentrations, there is evidence that L_2 binds calcium at physiological concentrations of magnesium (Morimoto and Harrington, 1974) and is responsible for a calcium-sensitive inhibition of actin binding (Margossian et al., 1975). This is probably related to the observation that the actin-activated ATPase activity of native myosin is inhibited at 20 μM Ca^{2+} and low free Mg^{2+} (Bremel and Weber, 1975) and to a lesser extent for acto-Nbs_2-myosin (Pemrick, 1976a).

Werber and Oplatka (1974) also reported that partial removal of the L_2 light chain decreased maximal calcium activation. In this instance, Nbs_2 treatment and/or partial removal of L_2 altered both the actin-binding site and the hydrolytic site, since the KEDTA-ATPase activity decreased while retaining their native Ca- and MgATPase activities. This suggests that the functional sulfhydryl, SH_2, may have been modified (Reissler et al., 1974).

In the present study considerable emphasis was placed on the ratio of actin to myosin, because for regulated actin this determines the cooperativity of the system (Bremel et al., 1972; Bremel and Weber, 1972). Schliselfeld (1976b) observed that concentration rather than ratio was important. These two reports are not contradictory, since the range of protein concentrations where Schliselfeld observed a concentration dependence was two- to tenfold lower than that of the present study. However, the ability of L_2 to enhance thin-filament cooperativity (Figure 3) was dependent on a threshold concentration of actomyosin complexes (presumably active complexes). In this laboratory a critical concentration of these complexes was achieved between a 5:1 and 2.5:1 ratio of A:M, but this need not be universal. It is suggested that the experiment in Figure 3 be used as a standard to predict the ratio of A:M required to observe this effect. Whatever ratio of A:M produces half-maximal activation at 0.18 μM Ca^{2+}, for native myosin, will be suitable to show the inhibitory effect of partial removal of L_2.

The ability of a slight excess of actin to enhance Ca^{2+} sensitivity is probably the result of filament constraints in the actomyosin system. This is the only explanation that can be offered, at present, for the observation that optimal calcium sensitivity occurred at a 2.5:1, rather than a 1:1 or 0.6:1 ratio

of actin to myosin (Figure 3, Table II). Beyond a 2.5:1 ratio, the increased availability of actin monomers was probably insufficient to offset the diminished cooperativity between actomyosin complexes and the thin filament. This phenomenon is not a crucial factor in the present hypothesis because (1) both acto-native and acto-L_2-deficient myosin share to a similar extent this anomaly and (2) the inhibitory effect of partial removal of L_2 on Ca^{2+} sensitivity is retained at a 2.5:1 ratio of actin to myosin (Figure 3).

There are several advantages to the actomyosin system. Firstly, it is becoming apparent that many soluble subfragments are being prepared from myosin. Chymotryptic digestion of myosin produced fewer nicks in the heavy chain (Yagi and Otani, 1974) than digestion by papain (Stone and Perry, 1973). In the presence of divalent cations, papain digestion resulted in an L_2-rich S1 (Margossian et al., 1975), whereas chymotryptic digestion produced HMM from both soluble and insoluble myosin (Weeds and Taylor, 1975; Bagshaw, 1977). In the present study, this preparation of HMM was L_2 deficient (approximately 1 mol of L_2/mol of HMM).

Secondly, variability in actin interaction from one preparation to another is not limited to the insoluble actomyosin system (Table II). Wagner and Weeds (1977) reported as much as a 28% difference in V_{max} from one preparation of L_2S1 to another, and as little as a 38% difference in V_{max} between the two S1 isoenzymes, L_1S1 and L_2S1.

Finally, the ratio of A:M "heads" is more important than the concentration of free regulated actin in determining the extent of actin interaction. Therefore, it is not necessary to work at high concentrations or ratios of M:A in order to demonstrate potentiation (Bremel et al., 1972). In the latter case, conditions are optimal for observing potentiation by ATP-resistant acto-S1 complexes (see Pemrick and Weber, 1976). The present study predicts that potentiation by active complexes will be proportional to the amount of L_2 light chain.

The ability of L_2 to enhance actin interaction at low free Ca^{2+} concentrations suggests that this light chain may play a role in regulating the resting tension of the system. Attention is, therefore, drawn to a recent observation (Moss et al., 1976) that the short-range elastic component (Hill, 1968) of resting heart and skeletal muscle resides in the cross bridges, and that the free Ca^{2+} concentration of living muscle during rest may be around 0.1 μM. By comparison, the present study suggests a mechanism by which the L_2 light chain of skeletal myosin enhances actin interaction between 0.05 and 0.5 μM free calcium.

References

Bagshaw, C. R. (1977), *Biochemistry 16,* 59.

Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., and Trentham, D. R. (1974), *Biochem. J. 141,* 351.

Bremel, R. D., Murray, J. M., and Weber, A. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 267.

Bremel, R. D., and Weber, A. (1972), *Nature (London), New Biol. 238,* 97.

Bremel, R. D., and Weber, A. (1975), *Biochim. Biophys. Acta 376,* 366.

Chaberek, S., and Martell, A. E. (1959), Organic Sequestering Agents, New York, N.Y., Wiley.

Cook, R. A., and Koshland, D. E., Jr. (1970), *Biochemistry 9,* 3337.

Daniel, J. L., and Hartshorne, D. J. (1972), *Biochim. Biophys. Acta 278,* 567.

Daniel, J. L., and Hartshorne, D. J. (1973), *Biochem. Biophys. Res. Commun. 51,* 125.

Ebashi, S., and Ebashi, F. (1964), *J. Biochem. (Tokyo) 55,* 604.

Eggleton, P., Elsden, S. R., and Gough, N. (1943), *Biochem. J. 37,* 526.

Gazith, J., Himmelfarb, S., and Harrington, W. F. (1970), *J. Biol. Chem. 245,* 15.

Gellert, M., and Englander, S. W. (1963), *Biochemistry 2,* 39.

Haselgrove, J. C. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 341.

Hill, D. K. (1968), *J. Physiol. (London) 199,* 637.

Huxley, H. E. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 361.

Kendrick-Jones, J., Szentkiralyi, E. M., and Szent-Gyorgyi, A. G. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 47.

Laemmli, U. K. (1970), *Nature (London) 227,* 680.

Lehman, W., and Szent-Gyorgyi, A. G., (1975), *J. Gen. Physiol. 66,* 1.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Margossian, S. S., and Lowey, S. (1973), *J. Mol. Biol. 74,* 301.

Margossian, S. S., Lowey, S., and Barshop, B. (1975), *Nature (London) 258,* 163.

Morimoto, K., and Harrington, W. F. (1974), *J. Mol. Biol. 88,* 693.

Moss, R. L., Sollins, M. R., and Julian, F. J. (1976), *Nature (London) 260,* 619.

Nihei, T., and Filipenko, C. A. (1975), *Can. J. Biochem. 53,* 1282.

Pemrick, S. M. (1974), *Biochem. Biophys. Res. Commun. 57,* 596.

Pemrick, S. M. (1976a), *Biophys. J. 16,* 70a.

Pemrick, S. M. (1976b), *Circulation, Suppl. II 54,* 113.

Pemrick, S. M. (1977), *Biophys. J. 17,* 8a.

Pemrick, S., and Weber, A. (1976), *Biochemistry 15,* 5193.

Portzehl, H., Schramm, G., and Weber, H. H. (1950), *Z. Naturforsch., B 5,* 61.

Potter, J. D., and Gergely, J. (1975), *J. Biol. Chem. 250,* 4628.

Reisler, E., Burke, M., and Harrington, W. F. (1974), *Biochemistry 13,* 2014.

Schliselfeld, L. H. (1976a), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1974a.

Schliselfeld, L. H. (1976b), *Biochim. Biophys. Acta 445,* 234.

Sekine, T., and Kielley, W. W. (1964), *Biochim. Biophys. Acta 81,* 336.

Somogyi, M. (1945), *J. Biol. Chem. 160,* 69.

Spudich, J. A., and Watts, S. (1971), *J. Biol. Chem. 246,* 4866.

Stone, D., and Perry, S. V. (1973), *Biochem. J. 131,* 127.

Straub, F. B. (1942), *Stud. Inst. Med. Chem. Univ. Szeged. 2,* 3.

Szent-Gyorgyi, A. G., Szentikalyi, E. M., and Kendrick-Jones, J. (1973), *J. Mol. Biol. 74,* 179.

Taussky, H. M., and Shorr, E. (1952), *J. Biol. Chem. 202,* 675.

Tsuboi, K. K. (1968), *Biochim. Biophys. Acta 160,* 420.

Wagner, P. D., and Weeds, A. G. (1977), *J. Mol. Biol. 109,* 455.

Wakayabashi, T., Huxley, H. E., Amos, L. A., and Klug, A. (1975), *J. Mol. Biol. 93,* 477.

Watterson, G., Schaub, M. C., Locher, R., Depierri, S., and Kutzer, M. (1975), *Eur. J. Biochem. 56,* 79.
Weber, A., and Bremel, R. D. (1971), in Contractility of Muscle Cells and Related Processes, Podolsky, R. J., Ed., Englewood Cliffs, N.J., Prentice-Hall, p 37.
Weeds, A. G., and Lowey, S. (1971), *J. Mol. Biol. 61,* 701.
Weeds, A. G., and Taylor, R. S. (1975), *Nature (London) 257,* 54.
Werber, M. M., and Oplatka, A. (1974), *Biochem. Biophys. Res. Commun. 57,* 823.
Yagi, K., and Otani, F. (1974), *J. Biochem. (Tokyo) 76,* 365.

Light-Induced Phosphorylation of Rhodopsin in Cattle Photoreceptor Membranes: Substrate Activation and Inactivation†

J. Hugh McDowell*,‡ and Hermann Kühn

ABSTRACT: The ability of rhodopsin in cattle rod outer segment (ROS) membranes to be phosphorylated in the presence of $AT^{32}P$ is induced by light, and thereafter slowly decreases in the dark. This light activation and dark inactivation are shown to be properties of the membrane-bound rhodopsin and to be independent of the presence of the extractable kinase. The dark inactivation is reversible under appropriate conditions; ROS membranes, which had been thoroughly bleached in the absence of ATP and allowed to lose their phosphorylation ability by a subsequent prolonged incubation in the dark, could be "reactivated" for phosphorylation if they were reilluminated for long times with white light in the presence of $AT^{32}P$. This apparent reactivation is shown to be due to "recycling" of the substrate, rhodopsin. A likely pathway of recycling is regeneration of rhodopsin from opsin and *11-cis*-retinal produced by photoisomerization of *all-trans*-retinal; subsequent bleaching then makes it available again as a substrate for phosphorylation. Conditions which minimize recycling, namely, short illumination with orange light, also suppressed "reactivation". If ROS membranes containing various mixtures of rhodopsin and opsin were illuminated in the presence of $AT^{32}P$ and kinase under conditions of minimum recycling, the phosphate incorporation corresponded exactly to the amount of freshly bleached rhodopsin and was independent of the amount of opsin present, suggesting that only the freshly bleached rhodopsin was phosphorylated. All the data presented are consistent with the model that the kinase activity is not affected by light, and that the substrate, rhodopsin, is changed by light such that it can be phosphorylated by the kinase, but this ability to be phosphorylated slowly decays. Thus, cattle opsin at long times after bleaching is not a substrate for the kinase, similar to unbleached rhodopsin.

Rhodopsin, the visual pigment located in the photoreceptor membranes of the vertebrate retina, is phosphorylated in a slow reaction consequent to photon capture (Kühn and Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973). On bleaching, the γ-phosphate group of ATP is enzymatically transferred to serine and threonine residues of rhodopsin in the presence of Mg^{2+} and a soluble protein kinase present in ROS. While light is necessary to initiate the reaction, the phosphorylation itself is a dark reaction. Although the reaction appears to be too slow to be involved in visual excitation (half time 1–2 min; Kühn and Bader, 1976), it is a possible candidate for a role in light/dark adaptation.

The light stimulation of the phosphorylation reaction has been well established; however, the mechanism of this stimulation is not clear. Several investigators using cattle ROS have proposed that the kinase is always active, independent of light, and that rhodopsin is converted into a substrate for this kinase only on bleaching (Kühn et al., 1973; Frank et al., 1973; Weller et al., 1975; Frank and Buzney, 1975); i.e., the *substrate is activated.* However, Bownds et al. (1972) have presented evidence obtained with frog ROS which suggests that bleaching a small number of rhodopsin molecules results in the phosphorylation of many unbleached rhodopsin molecules. Therefore, the role of light would not be simply the conversion of rhodopsin into a substrate for the kinase. Also, Frank and Buzney (1975) showed that illumination of cattle "opsin" preparations containing only small amounts of residual rhodopsin results in the same phosphate incorporation as illumination of previously unbleached rhodopsin preparations. These data might be interpreted as evidence for *activation of the kinase* mediated by bleaching of the rhodopsin. Nevertheless, Frank and Buzney (1975) concluded from other experiments that the kinase was not light activated, though they could not explain how the opsin could then be phosphorylated as a consequence of illumination if the kinase were not light activated. The present work presents specific experiments aimed at determining whether the light stimulation of the phosphorylation reaction is due to light activation of the kinase, or to light activation of the substrate, rhodopsin.

Methods

Preparation of Cattle and Frog Rod Outer Segments. Retinas were dissected from cattle eyes which had been enucleated shortly after slaughter and placed in the dark on ice for a few hours before use. The preparation of ROS[1] followed a

† From the Institut für Neurobiologie der Kernforschungsanlage Jülich, D-5170 Jülich, West Germany. *Received January 3, 1977.*
‡ Supported by a grant from SFB 160 "Biologische Membranen" of Deutsche Forschungsgemeinschaft, and by a travel fellowship from Alexander von Humboldt Stiftung. Present address: Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, Ill. 62901.

[1] Abbreviations used: ROS, rod outer segments; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; cGMP, guanosine 3′,5′-monophosphate; sem, standard error of the mean; DTT, dithiothreitol.