Myosin Subfragment 1 Binding to Relaxed Actin Filaments and Steric Model of Relaxation†

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ABSTRACT: It is widely assumed that relaxation of vertebrate skeletal muscle is the result of steric blockage of myosin attachment by troponin–tropomyosin. Therefore, we determined to what extent actin binding of nucleotide-free myosin subfragment 1 and actin binding of subfragment 1 containing the adenosine triphosphate (ATP) analogue β,γ-imido-ATP (AMPPNP) was inhibited when troponin–tropomyosin–actin filaments were in the relaxed state. Maintaining the relaxed state requires, in addition to calcium removal, a very low ratio of subfragment 1 to actin because saturation of the actin filament with myosin reverses relaxation in the absence of calcium. We observed that actin binding of the two subfragment species was inhibited to a different extent when the actin filaments were relaxed. These data are analyzed in terms of the steric model of relaxation under two different assumptions, model 1 and model 2. According to model 1, which represents the classical case of competitive inhibition, the S-1 binding site on actin is occupied either by troponin–tropomyosin or by S-1, but never by both simultaneously. The S-1–actin binding constants are not altered by calcium removal. In terms of model 1 the binding sites for each of the two S-1 species must be different and nonoverlapping so that in the relaxed state troponin–tropomyosin blocks a larger fraction of the sites for nucleotide-free than for AMPPNP containing S-1. According to model 2, troponin–tropomyosin in the absence of calcium is bound to all S-1 binding sites at all times, but it occupies them only partially. Each binding site, therefore, can contain both tropomyosin and S-1 at the same time. With only part of the binding site available to S-1 during relaxation, the S-1–actin binding constants are lowered while the number of accessible sites is the same as in the presence of calcium. The binding constant for nucleotide-free subfragment 1 was lowered 10-fold and that for S-1-AMPPNP only 2–3-fold. In terms of model 2 that means that tropomyosin blocks about 15% of the rigor binding site and about 12% of the S-1-AMPPNP binding site; i.e., both binding sites could largely overlap.

The resting state of all muscles is characterized by a low resistance to stretch [for a review, see Weber & Murray (1973)]. Galen already recognized that this property of the resting state is essential for skeletal movement governed by antagonistic muscle pairs. After the discovery of the sliding filament mechanism of muscle contraction, X-ray diffraction measurements suggested that the high degree of extensibility can be explained by the absence of cross-bridge formation during rest [for a review, see Huxley (1969)]. Such a reduction in actin–myosin interaction can be produced by one of several control mechanisms, each of which is calcium dependent. While all vertebrate skeletal muscles seem to be subject to the same kind of regulation, there is some variety in invertebrate skeletal muscle. The activity of the actin filament is turned off in all vertebrate skeletal muscles (Ebashi et al., 1969) and in many invertebrate muscles according to Lehman & Szent-Gyorgyi (1975) whereas that of the myosin filament is turned off in mollusc muscle (Szent-Gyorgyi et al., 1973). In other invertebrate muscles both control mechanisms operate simultaneously (Lehman & Szent-Gyorgyi, 1975).

Ebashi and his colleagues (1968) showed that in vertebrate skeletal muscle the activity of the actin filament is turned off by the combined action of troponin and tropomyosin. Huxley (1972) and Haselgrove (1972) observed that the part of the X-ray diffraction pattern of intact muscle which is derived from the regulated actin (thin) filament is different in rest and in contraction. Haselgrove's (1972) calculations and those of Parry & Squire (1973) indicate that the change in pattern can be explained by a shift of tropomyosin from the periphery into the groove of the actin filament. This conclusion is supported by similar changes in the optical diffraction patterns of tropomyosin-containing actin paracrystals on removal and addition of the complex between the troponin subunits troponin I and troponin T which suppresses actin–myosin interaction in a calcium-insensitive manner (Wakabayashi et al., 1975). Similar results were obtained with troponin–tropomyosin-containing actin paracrystals in the presence and absence of calcium (Gillis & O'Brien, 1975).

Huxley (1972), Haselgrove (1972), and Parry & Squire (1973), assuming that tropomyosin and myosin are bound to the same side of the actin filament, postulated that tropomyosin when it is bound near the periphery of the actin filament occupies the myosin binding sites and thereby blocks cross-bridge formation during relaxation and rest. The side of the actin filament to which tropomyosin is bound can be deduced from the optical diffraction pattern only if there is information about the polarity of the actin filament. Seymour & O'Brien (1980) recently made measurements on troponin–tropomyosin (regulated) actin filaments whose polarity was known because the filaments were still attached to the Z band. They concluded that tropomyosin is situated on the side opposite to the myosin binding site, as determined by Moore et al. (1970). Recently, however, on the basis of new measurements with improved methods M. Amos and E. W. Taylor (personal communication) in Huxley's laboratory have reinterpreted the data of Moore and his colleagues. Thus, it is possible that both myosin and tropomyosin do bind to the same side of the actin filament as required by the Huxley–Haselgrove–Parry–Squire model of relaxation by steric interference.

This steric model of relaxation provides an elegant structural explanation of some of the cooperative behavior of the regulated actin filament. As mentioned above, according to this model relaxation occurs when troponin–tropomyosin prevails in its competition with myosin for the same actin sites. This

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is possible when calcium-free troponin bound to actin holds tropomyosin to the periphery of the actin filament. Nevertheless, myosin can be made to prevail in the absence of calcium, and consequently relaxation prevented, when the affinity of myosin for actin is sufficiently increased. In muscle this occurs during rigor when ATP has been exhausted. Thus, Haselgrove (1972) observed that tropomyosin is displaced into the groove in rigor muscle from which calcium had been extracted. This displacement of tropomyosin by myosin forming rigor complexes can explain why relaxation of myofibrils is possible only at high and not at low ATP concentrations [for a review, see Weber & Murray (1973)]. It has been shown in earlier experiments (Bremel & Weber, 1972) that in the absence of calcium ATPase is less and less inhibited with increasing accumulation of rigor complexes. Rigor complexes accumulated either because of an increase in the lifetime of the rigor complexes with decreasing ATP concentrations (cf. Taylor, 1979) or because of an increase in the concentration of nucleotide-free S-1 (single myosin heads) or both. At very high S-1 concentrations, vastly in excess over actin, relaxation was completely abolished in the absence of calcium, so that actin activation of S-1 ATPase was as high in the absence as in the presence of calcium.

In studies designed to further probe the validity of the steric model, we determined to what extent actin binding of nucleotide-free S-1 (rigor complex) and S-1 AMPPNP is inhibited when the troponin–tropomyosin filament is relaxed. We compared the extent of inhibition of binding with the extent of inhibition of actin activation of ATPase activity since it is assumed that inhibition of actin binding of S-1 ADP·P is responsible for the inhibition of actin activation of ATPase activity. Since during contraction rigor complexes are presumably formed from complexes of S-1 ADP·P with actin and since rigor complexes in turn are presumably transformed to ATP S-1–actin complexes before myosin bridges detach in the cross-bridge cycle, one may expect that all of these S-1–actin complexes are formed with the same actin site. Therefore, one may expect that during relaxation formation of all of these complexes is equally blocked by tropomyosin. For S-1 ATP or S-1 ADP·P we substituted the S-1 complex with the ATP analogue β,γ-imido-ATP (AMPPNP) and we evaluated S-1 ADP·P binding to actin by actin-activated ATPase activity which, at these actin concentrations, is supposed to be limited by the equilibrium constant of the actin–S-1 ADP·P complex (Taylor, 1979).

Contrary to expectations, calcium removal did not affect actin binding of the two S-1 species to the same extent. In terms of the steric model, that means that the binding sites for nucleotide-free S-1 and S-1 AMPPNP do not exactly superimpose. If we treat the binding changes on calcium removal in terms of the classical model of competitive inhibition of S-1 by tropomyosin, the two binding sites cannot even overlap significantly, but are separate and distinct. If, alternatively, we assume partial blocking of all S-1 binding sites by tropomyosin during relaxation, then the site for rigor complex formation and the binding site for S-1 AMPPNP can have a high degree of overlap.

Methods

Protein Preparations. All proteins were derived from the hind leg and back muscle of rabbits. Myosin subfragment 1 (S-1) was prepared according to Lowey et al. (1969) by proteolytic digestion of myosin at 25 °C, pH 7.0, in 0.2 M KCl, for 20 min using a ratio of papain to myosin of about 1:200. The reaction was terminated by iodoacetate (1 mM, pH 6.3). After removal of insoluble proteins (rods and undigested myosin) from the 10-fold diluted solution (1 mM dithiothreitol = DTT) S-1 was collected by ammonium sulfate precipitation. The discrete fraction which precipitates between 50 and 55% saturation was judged by $NaDodSO_4$ gel electrophoresis and ATPase assay to be more intact than the remainder and was used for these experiments. Ammonium sulfate was removed by dialysis against pH 7 buffer containing 0.2 mM DTT (protein concentration 20–30 mg/mL), and the S-1 was centrifuged at 100000*g* for 30 min immediately before use to remove any aggregated material.

Actin was prepared from acetone powder by a 30-min extraction at low ionic strength, polymerized with 0.1 M KCl–2 mM MgCl, and then centrifuged in the presence of 0.8 M KCl–10 mM Tris, pH 8.5. These conditions, modified somewhat from Spudich & Watt (1971), were found to consistently remove all associated proteins in one step and yield actin which was homogeneous on overloaded $NaDodSO_4$ gels except for an occasional α-actinin band visible at heavy loading. The actin was depolymerized by dialysis against 10 mM Tris, pH 8.4, 0.2 mM $CaCl_2$, 0.2 mM DTT, and 0.50 mM ATP, centrifuged clear, and repolymerized again before use.

Troponin and tropomyosin were prepared as a complex by extraction at low ionic strength for 30 min of a lyophilized Ebashi powder (Ebashi & Ebashi 1964), adding KCl and $MgCl_2$ to polymerize the contaminating actin, followed by centrifugation at 100000*g* for 1 h. The supernatant contained the tropomyosin–troponin complex. *To prepare regulated thin filaments*, G-actin was mixed with twice its weight of this extract and sufficient KCl and $MgCl_2$ to give final concentrations of 0.1 M and 2 mM, respectively. After the actin was allowed to polymerize for 30–45 min at 15 °C, the thin filaments were pelleted at 100000*g*. It is important for the retention of high cofactor activity that the next step, resuspension of the actin pellets, be carried out as gently as possible. We found it useful to rinse the pellets thoroughly with water and then allow them to sit, covered with a small layer of buffer (10 mM imidazole, pH 7.0, and 1 mM $MgCl_2$) overnight at 4 °C. During this period the pellets swell and soften to a consistency which allows dispersion by fairly gentle homogenization, especially if taken up into 2 mM $MgCl_2$.

Protein concentrations were measured with the Lowry reaction (1951), using bovine serum albumin as standard and calculating the concentrations of myosin and actin from tables calibrated by Kjeldahl.

Protein Modifications. Myosin was labeled before proteolysis with either [^{3}H]iodoacetamide (IAA) or in the later experiments with *N*-[^{3}H]ethylmaleimide ([^{3}H]NEM). In the former case, myosin at 75–100 μM was incubated with 400 μM [^{3}H]iodoacetamide at pH 7.4–7.6, 0 °C, in 0.6 M KCl overnight. The mixture was then diluted 20-fold with 0.5 mM DTT and the labeled myosin collected by centrifugation. The pellets were redissolved in 0.6 M KCl and used to prepare S-1 as described. [^{3}H]NEM labeling was carried out in 0.6 M KCl at pH 7.5–7.8 with 0.5 mM EDTA present and terminated with excess DTT after 3–5 min. Myosin was used at a concentration of 50 μM and [^{3}H]NEM at 200 μM. The incorporation of label into the S-1 fraction of myosin varied from 0.13 to 0.76 mol of [^{3}H]NEM/mol of protein, under apparently identical reaction conditions.

In control experiments with native S-1, labeled actin was used. F-actin was labeled for 3 h with [^{3}H]NEM, 0.6 mM in excess over DTT (usually 0.1 mM), in a medium containing 10 mM Tris, pH 8.2, 2 mM MgCl, 0.5 mM ATP, 1.0 mM MgCl, and 0.1 M KCl. Incorporation of label varied from 0.7 to 1.0 mol/mol of actin. Previous experiments in this

laboratory (Detmers et al., 1980) and by Elzinga & Collins (1975) have shown that Cys-373 incorporates more than 90% of the label under these conditions.

AMPPNP was obtained from Boehringer and was routinely checked for purity before use. Contamination with ADP + ATP varied from 1 to 3 mol/mol of AMPPNP.

Titration of Acto-S1 with AMPPNP. Dissociation of acto-S-1 by AMPPNP was measured by separating the actin-bound from the free S-1 by a brief high-speed centrifugation. After considerable experimentation the following protocol was adopted. Actin plus the required salts were mixed with as dilute a solution of S-1 as was consistent with the desired final concentration. It is remarkably difficult to obtain a homogeneous mixture of acto-S-1 (without such violent mixing as to denature the proteins), and only by repeatedly sampling aliquots from the top, middle, and bottom of the mixing vessel for radioactivity could we be sure that adequate mixing was obtained. It was extremely difficult to obtain a homogenous final mixture if one started with concentrated protein solutions. Various concentrations of AMPPNP together with compensating amounts of KCl to maintain the ionic strength constant were added to the aliquots of this mixture. The final conditions were 10 mM Tris, pH 7.4 or 7.0, 2.5 mM or 5 mM $MgCl_2$, and 60 mM KCl, 15 °C, total volume 0.7 mL. The solutions were centrifuged for 15–20 min (unless noted otherwise) at 50 000 rpm in a Beckman 65 rotor or at 40 000 rpm in a 40 rotor. Control experiments established that this was sufficient to completely pellet the sedimentable actin (about 90% of the total actin present) but an insignificant fraction of the free S-1. For experiments in which dissociation by ATP was measured, sufficient creatine phosphate + creatine kinase to maintain the ATP at its initial concentration were added immediately before centrifugation. Immediately after centrifugation the tubes were swirled gently to resuspend sedimented S-1 and decanted. Radioactivity in aliquots of the supernatant was determined by liquid scintillation counting. With each experiment a set of control tubes was run containing no AMPPNP to measure the amount of S-1 incapable of binding to actin, and a tube containing no S-1 to determine the fraction of actin which was not sedimentable. In the experiments using native S-1, which were done at higher protein concentrations, the protein remaining in the supernatant after centrifugation was determined by the Lowry method. In the experiments with labeled actin, the amount of actin in the supernatant was determined by radioactivity measurements.

ATPase Assays. The quality of S-1 was assessed by K^+-activated ATP hydrolysis at pH 8.0, 5 mM ATP, 5 mM EDTA, and 0.6 M KCl (inorganic phosphate was determined according to Taussky & Schorr (1953)); rates of 16–20 s at 25 °C were considered satisfactory. Acto-S-1 ATPases were measured in 10 mM imidazole hydrochloride, pH 7.0, 1 mM $MgCl_2$ in excess over MgATP, 1–2 mM MgATP, 1 mM EGTA or Ca-EGTA, and 0.1 mM $CaCl_2$ as required. Actin was mixed with S-1, the assays were started by the addition of MgATP and terminated with trichloroacetic acid, and inorganic phosphate was determined as above.

Calculations. The percentage dissociation caused by a given AMPPNP concentration was calculated by taking the ratio of the supernatant counts after centrifugation in the presence of AMPPNP to the total counts (after first correcting both numbers for the radioactivity found to be incapable of sedimenting with actin even in the absence of nucleotide. This was about 10–15% of the total counts, but a lower fraction of the total S-1 due to variations in labeling and a higher rate of denaturation of labeled S-1 compared to native S-1). The calculation of the apparent dissociation constants is described under Results.

Regression lines for the double-reciprocal plots, least-squares estimates of the binding constants, and sampling errors were computed by a weighted linear regression of K_5 on 1/[AMPPNP]. Weighting coefficients were calculated by application of the principles outlined in Wilkinson (1961).

Results

We determined how relaxation affects myosin binding to regulated actin by measuring the dissociation of acto-S-1 induced by increasing concentrations of AMPPNP. Actin and S-1 associate in the absence of nucleotide to form the very stable "rigor" complex which has a binding constant of about 2×10^7 M^{-1} (Marston & Weber, 1975; Highsmith, 1977; Margossian & Lowey, 1978). Binding of nucleotide to S-1 greatly reduces the affinity of S-1 for actin [for review, cf. Taylor (1979)]. Whereas the reaction with ATP must produce a number of intermediary complexes, binding of its stable analogue AMPPNP results only in the formation of S-1 AMPPNP and of the ternary complex acto-S-1 AMPPNP. If conditions are chosen so that the concentration of free S-1, not associated with either actin or AMPPNP, is a negligible fraction of the total S-1 (AMPPNP above 10 μM), the equilibria between the various components are described by the following reactions (A = free actin; S = S-1; N = AMPPNP)

$$AS + N \overset{K_1}{\leftrightarrow} ASN \overset{K_2}{\leftrightarrow} A + SN$$

and the corresponding equations:

$$\frac{[ASN]}{[AS][N]} = K_1 \quad (1)$$

$$\frac{[ASN]}{[A][SN]} = K_2 \quad (2)$$

When, under our experimental conditions, the AMPPNP-induced release of S-1 from acto-S-1 is measured two quantities are obtained, free S-1 AMPPNP (SN) and the total actin-bound S-1 (acto-S-1 (AS) + acto-S-1 AMPPNP (ASN)). Free and bound S-1 are related by

$$\frac{[AS + ASN]}{[A][SN]} = \frac{K_2}{K_1[N]} + K_2 = K_5 \quad (3)$$

The ordinate intercept of a plot of K_5 vs. 1/[AMPPNP] indicates the value of the binding constant of S-1 AMPPNP to actin, one of the two numbers we wanted to determine. Changes in the association constant for the rigor complex, the second number of interest, can also be calculated from these data, as will be described.

This kind of analysis has been carried out previously with pure acto-S-1 by Greene & Eisenberg (1978), who obtained the binding constants of AMPPNP to acto-S-1 and of the nucleotide containing S-1 to actin. Like these investigators, we measured the released S-1 in the supernatant after spinning down actin filaments by high-speed centrifugation. However, since we were interested in S-1 binding to relaxed actin, we needed to vary our conditions from those of the previous workers not only by using reconstituted regulated actin filaments but also by arranging for a very large excess of actin over S-1 so as to avoid any reversal of relaxation due to the accumulation of rigor complexes. Bremel & Weber (1972) showed that rigor complexes in sufficiently high concentration completely abolish relaxation and that they still diminish re-

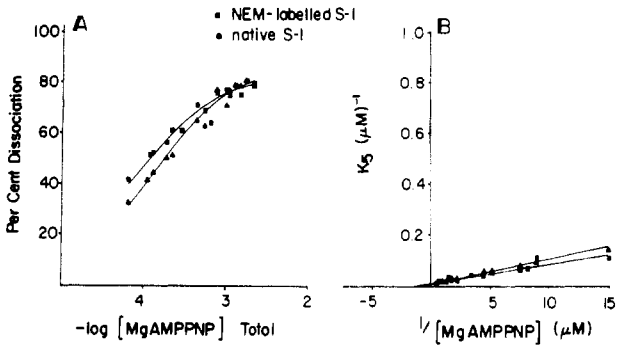

FIGURE 1: Dissociation of S-1 from pure actin with increasing concentrations of MgAMPPNP. Comparison between [^{3}H]NEM-labeled and unmodified S-1: 14 μM of [^{14}C]NEM actin (after correction for 29% unpolymerizable actin) labeled with 1 NEM/actin, 14 μM S-1; 13% [^{3}H]NEM-S-1 (assuming 1 NEM/S-1), 87% unmodified S-1; 10% of the native and 40% of the labeled S-1 was denatured, i.e., incapable of forming rigor complexes with actin. Other conditions as in Figure 2B. Squares labeled S-1; triangles, unmodified S-1. This S-1 was rejected for an experiment on the effect of relaxation.

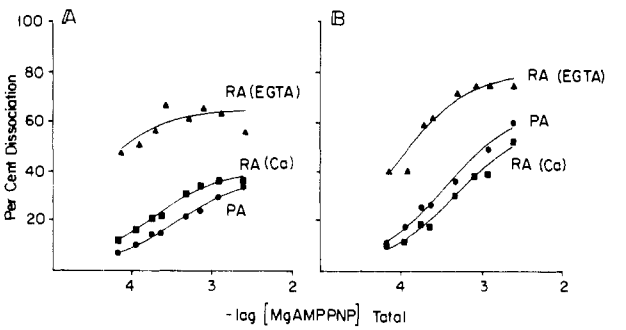

FIGURE 2: Dissociation of S-1 from regulated relaxed and nonrelaxed actin in comparison to pure actin. A and B are two different experiments, with different protein preparations. Native actin, 21 μM (A) and 30 μM (B), [^{3}H]NEM-labeled S-1, 0.7 (A) and 0.8 (B) μM; 10 mM imidazole, pH 7.4 (A) and 7.0 (B), 2.5 mM (A) and 5.0 mM (B) $MgCl_2$, ionic strength 63 and 68 mM, 15 °C; centrifugation for 60 min (A) and 20 min (B). Denatured S-1 10% (A) and 20% (B). Squares, (1.0 mM CaEGTA + 0.1 mM $CaCl_2$) and triangles (1.0 mM EGTA), regulated actin; circles, pure actin (1.0 mM EGTA).

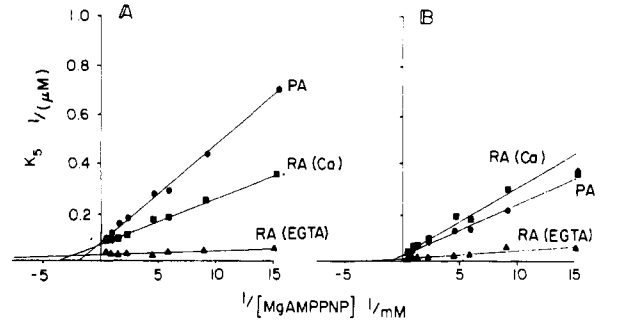

FIGURE 3: Graphical determination of binding constants for ternary complexes between S-1, actin, and MgAMPPNP from the data in Figure 2A,B.

laxation somewhat when S-1 is present in one-tenth of the actin concentration. Only at very high actin excess over S-1 do rigor complexes not interfere with relaxation (Murray et al., 1980). We used a 20- to 40-fold excess of actin over S-1. Since unavoidably each actin preparation contains some unpolymerizable actin (in addition to the native monomeric actin in equilibrium with the polymer), it was necessary to distinguish the unbound S-1 by a radioactive marker. We used either iodo[^{14}C]acetamide or *N*-[^{3}H]ethylmaleimide (NEM) under conditions where they bind preferentially to SH_1 of S-1 (Sekine & Kielley, 1964).

It is well-known that modifications of myosin SH groups affect the molecule's biological properties. Although the binding constants for rigor complex formation are not altered by the binding of iodoacetamide (Marston & Weber, 1975), the actin-activated ATPase activity is lowered (Silverman et al., 1972). Furthermore, *N*-ethylmaleimide attached to groups not yet identified can either prevent ATP binding or uncouple the ATP binding site from its interaction with the actin binding site (Pemrick & Weber, 1976). Therefore, it seemed advisable to check whether NEM-labeled S-1 was dissociated from actin by AMPPNP to a different extent than was native S-1. In order to measure dissociated native S-1 by a Lowry assay, we used a high S-1 concentration, just slightly below that of actin, and actin whose response is not affected by the accumulation of S-1–actin complexes, i.e., pure actin. We compared native and labeled S-1 ([^{3}H]NEM) in the same assay, which contained 14% modified and 86% native S-1 (Figure 1). The concentration of total (native + modified) supernatant S-1 was obtained from a Lowry assay and that of modified supernatant S-1 alone by its tritium content. The main difference between native and modified S-1 was usually a considerable increase in the fraction of denatured S-1, completely incapable of forming rigor complexes with actin, even in the presence of a large excess of actin (20–50 μM). This denatured fraction was 10% of the total in the experiment of Figure 2A, while it was 20% in that of Figure 2B. We subtracted this denatured fraction from the total and free S-1. In addition, some S-1 preparations contained a small amount of S-1 that formed rigor complexes with a considerably reduced affinity so that this S-1 is bound to actin only when the concentration of free actin was high, 10 μM or more. This fraction contaminates our measurements. It is seen (Figure 1) that S-1 modification resulted in a relatively small change of the association constants in spite of the large fraction of denatured S-1 in this control, a much larger fraction than was present in any of the preparations used for an experiment. It was found that the difference between labeled and native S-1 was smaller than the random variations between different preparations of either unmodified (not shown here) or labeled protein (compare Figure 2, A and B). Furthermore, our binding constants with pure actin and labeled S-1 (Figure 2A) are quite comparable to those of others obtained with native S-1 (Greene & Eisenberg, 1978). Thus, we conclude that our mild NEM modification has not affected the actin binding properties of S-1 to an extent that would alter the results of these experiments.

Figure 2A,B represents two different experiments showing AMPPNP titrations of S-1 bound to pure actin and to regulated actin. In both experiments, AMPPNP dissociated S-1 much more readily from relaxed actin than from calcium-saturated regulated actin, whereas there was little difference between S-1 bound to the latter and to pure actin. Figure 3A,B represents the same experiments except that K_5 (see above) was plotted against the inverse of the AMPPNP concentration, so that the ordinate intercept gives the binding constant of S-1 AMPPNP to actin (K_2) and the abscissa intercept the binding constant of AMPPNP to acto-S-1 (K_1). It is seen that the apparent binding constant of S-1 AMPPNP to regulated actin was reduced 2- to 3-fold when the actin was relaxed and that, in addition, the apparent affinity of AMPPNP was increased 3–4-fold for S-1 bound to relaxed actin as compared to S-1 bound to regulated actin in the presence of calcium. Although the lower quality of the proteins in experiment B (20% denatured S-1 as compared to 10% in experiment A) manifested itself in lower binding constants, the relative difference between S-1 binding to relaxed and nonrelaxed actin was similar. It

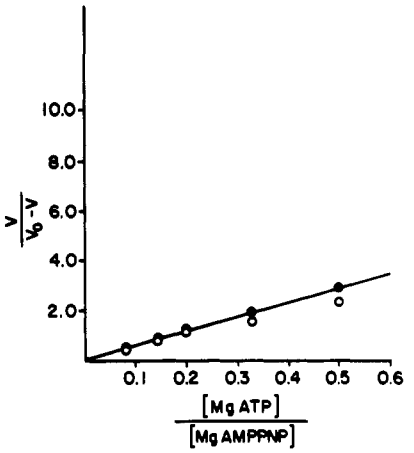

FIGURE 4: Effect of calcium on the competitive inhibition of S-1 MgATPase activity by MgAMPPNP: 3.5 μM S-1, 200 μM MgATP, increasing MgAMPPNP, 2.0 mM $MgCl_2$, 10 mM imidazole, pH 7.0, 50 mM KCl, 40 μg/mL each of lactic dehydrogenase and pyruvate kinase, 150 μM NADH, 1.0 mM phosphoenolpyruvate. Absorption at 340 nm was measured.

should be noted that the binding constants of AMPPNP for S-1 bound to pure actin, 2–2.5 and 1–1.5 10^3 M, respectively, are comparable to those reported by others using native S-1 (Greene & Eisenberg, 1978).

To obtain the constant for rigor complex formation one must consider the reactions (A = free actin, S = S-1, N = AMPPNP)

$$A + S \overset{K_3}{\leftrightarrow} AS$$

$$S + N \overset{K_4}{\leftrightarrow} SN$$

leading to

$$\frac{[AS]}{[A][S]} = K_3 \quad (4)$$

$$\frac{[SN]}{[S][N]} = K_4 \quad (5)$$

Substituting eq 4 and 5 into eq 3 gives

$$K_3/K_4 = K_2/K_1 \quad (6)$$

Provided that the concentration of free actin and K_4 are insensitive to the free calcium ion concentration, the change in K_2/K_1 defines the reduction in the constant for rigor complex formation due to calcium removal from regulated actin. We assessed the calcium sensitivity of AMPPNP binding to free S-1 by comparing the competitive AMPPNP inhibition of the MgATPase of S-1 in the presence and absence of calcium. Figure 4 shows that it is not significantly affected by the free calcium ion concentration. There is no direct method to assess the concentration of free actin, [A], i.e., S-1 binding sites not occupied by tropomyosin. Therefore, the data will be analyzed (see Discussion) under the assumption, first, that the concentration of free actin sites changes on calcium removal (model 1) and, second, that it remains constant (model 2). For model 2 the rigor constant was reduced 8- and 11-fold, respectively, for experiment A and B of Figure 2, when S-1 was bound to relaxed as compared to nonrelaxed regulated actin.

The interaction of regulated acto-S-1 with ATP in the presence and absence of calcium is described in Table I. The quality of the regulated actin in both experiments of Figure 2 may be judged by the extent of inhibition of ATPase activity on calcium removal, 97% and 90% for the preparations used

Table I: ATPase Activities

	protein concentrations (μM)			ATPase rate (s^{-1})		
	actin	S-1				
expt	regulated	native	NEM	Ca	EGTA	% relaxation
2A	7	15		22	0.6	97
	7		15	12	0.7	94
	0.8		15	28[a]	2.0	93
2B	3	10		12	1.1	90
	3		16	16	4.1	74
6	3		14	16	4.1	74
	0.36		14	32[a]	12.2	62
7	3.9		3.8	1.4	0.11	92
	1.3		3.8	2.2[a]	0.64	70

[a] The increase in ATPase activity in the presence of calcium with increasing S-1/actin ratio can be explained by potentiation (Bremel et al., 1972; Weber & Murray, 1973).

in experiments A and B, respectively, when the actin was tested with native S-1. With labeled S-1 relaxation was less, 94% and 74%, respectively. Since rigor complex formation is the only known mechanism by which S-1 has been shown to reduce relaxation, we assume that a small fraction of the modified S-1 was partially denatured, so that it formed ATP-resistant rigor complexes, i.e., rigor complexes that were no longer dissociated by ATP. According to Pemrick & Weber (1976), such myosin and HMM can be created by NEM treatment, presumably due to modification of SH groups (which groups are involved is not known), and we suggest that oxidation of SH groups can also produce such S-1. The extent to which rigor complexes inhibit relaxation depends on the extent of actin filament saturation with rigor complexes (Bremel & Weber, 1972). In agreement, the data of Table I show a reduction of relaxation, sensitive to the ratio S-1/actin, even when that ratio was increased without significantly affecting the concentration of free S-1, i.e., by lowering the actin concentration from one-fifth to one-fiftieth of the S-1 present (experiment 6, Table I) under conditions of stoichiometric rigor complex formation. Since in all our binding experiments actin was present in an excess over S-1 calculated to be large enough to rule out any interference by rigor complexes, it seems correct to compare the binding data with the ATPase measurements that are least influenced by rigor complexes, i.e., the ATPase activities with native S-1. For native S-1, calcium removal caused a 30-fold and a 10-fold reduction of actin-activated ATPase activity, respectively, for experiments A and B.

Table II shows the analysis of five experiments in terms of model 2 with different preparations of proteins, in the sequence they were performed over the period of 1 year. Experiments 4 and 5 are technically superior to experiments 1–3. Many other experiments were rejected because of low actin binding or low actin activated ATPase activity. The last three columns compare the effect of calcium removal on actin binding of S-1 AMPPNP (in terms of model 2), on rigor complex formation, and on actin-activated ATPase activity. In four out of five experiments (omitting experiment number 3)[1] calcium removal resulted in an apparent 6- to 11-fold decrease of nucleotide-free

[1] Experiment 3 cannot be considered to invalidate our results. It differs from the other experiments insofar as the abscissa intercepts (K_1) in the presence and absence of calcium are rather close together. However, experiment 3 has the least number of points, which, furthermore, scatter considerably. If the lines are drawn to give the permissible maximal difference between the data points in the presence and absence of calcium, the data are in general agreement with the other four experiments, as indicated in the legend of Table II.

Table II: Change in Equilibrium Constants on Calcium Removal in Terms of Model 2[a]

	RAS + N ⟷ RASN $K_1 \times 10^{+3}$ M^{-1}		RA + SN ⟷ RASN $K_2 \times 10^{+5}$ M^{-1}		$\frac{\text{Ca-RA + S} \longleftrightarrow \text{Ca-RAS}}{\text{EGTA-RA + S} \longleftrightarrow \text{EGTA-RAS}}$		
expt	Ca	EGTA	Ca	EGTA	$\frac{K_3(\text{Ca})^b}{K_3(\text{EGTA})}$	$\frac{K_2(\text{Ca})}{K_2(\text{EGTA})}$	ATPase Ca/EGTA
1	3.4	11.1	0.4	0.22	5.9	1.8	
2	0.67	1.6	1.3	0.59	5.6	2.2	4
3[c]	10.2	13.5	2.0	0.91	2.9	2.2	20
4[d]	3.6	14.2	0.67	0.24	11.1	2.7	37
5[d]	0.91	2.6	0.25	0.09	8.3	2.8	11

[a] RA = regulated actin; S = S-1; N = AMPPNP; RAS = rigor complex with regulated actin; SN = S-1 AMPPNP; RASN = ternary complex between regulated actin, S-1 and AMPPNP. [b] $K_3(\text{Ca})/K_3(\text{EGTA}) = [K_2/K_1(\text{Ca})]/[K_2/K_1(\text{EGTA})]$. [c] Experiment with largest scatter and smallest number of points; largest possible spread of K_1(Ca) vs. K_1(EGTA) was 8 and 21 $\times 10^3$ M^{-1}, respectively, with the corresponding K_2 values of 1.8 and 1.0 $\times 10^5$ M^{-1} which gives $K_3(\text{Ca})/K_3(\text{EGTA}) = 4.7$. [d] Experiments 4 and 5 are experiments A and B of Figures 2 and 3.

S-1 binding to actin (K_3) and a 2–2.5-fold decrease in S-1 AMPPNP binding to actin (K_2) and in an inhibition of ATPase activity usually greater than that of rigor complex formation.

Discussion

The reduction of S-1 binding to actin caused by calcium removal from troponin is of course a cooperative event, as are most other phenomena associated with the relaxed state. The possible structural basis for the cooperativity was described by Huxley, Haselgrove, and Parry and Squire in what is now generally called the steric model of relaxation. It is assumed that the tropomyosin strand (all tropomyosin molecules are bound to each other at their overlapping ends, thus forming one long strand) completely blocks the myosin binding sites during relaxation, and that the strand before contraction moves toward the groove, completely freeing the myosin binding sites. In confirmation, tropomyosin movement from the periphery to the groove has been found associated with excitation of muscle, presumably as the result of calcium binding to troponin. When Ca^{2+} is bound to troponin C, troponin I is dissociated from its actin binding site through which it presumably anchored tropomyosin to the periphery, thus enabling the released tropomyosin to take a position on the actin filament in accordance with the actin affinity of the tropomyosin segments (Stewart, 1975). Whereas during relaxation the troponin–tropomyosin complex prevails in the competition for the myosin binding sites on actin, in the absence of ATP myosin prevails, so that during rigor tropomyosin moves toward the groove in the absence of calcium, and myosin is bound to actin. The change in dominance is the result of the much greater actin affinity of nucleotide-free myosin as compared to nucleotide-containing myosin. The steric model is elegant in that it utilizes all major structural features of the troponin–tropomyosin strand, the observed tropomyosin movements, and the troponin I–actin binding properties. Furthermore, with only one more plausible assumption concerning the flexibility of the tropomyosin strand (Murray & Weber, 1980), the steric model can provide the structural basis for the cooperativity of myosin binding to relaxed actin filaments (Hill et al., 1980), reversal of relaxation leading to contraction in the absence of calcium, and the high degree of cooperativity of the calcium response of the regulated filament (Murray & Weber, 1980).

Therefore, we shall discuss the implications of this binding study in terms of the steric model although other explanations based on unknown molecular features of the regulated actin filament cannot be ruled out. For explanation of how steric interference by tropomyosin may partially inhibit S-1 and S-1 AMPPNP binding when the actin filaments are relaxed, two different possibilities may be considered: either complete blockage of some of the myosin binding sites (model 1) or partial blockage of all myosin binding sites (model 2). It is not possible to distinguish between these alternatives by any kind of binding experiment, although one can do so when two molecules in solution bind to a protein. In the latter case partial overlapping of binding sites can be distinguished from exclusive binding of either molecule to the identical site by the occurrence of ternary complex formation. Tropomyosin, however, is bound to the actin filament even when it does not at all interfere with myosin binding. A decision between the alternative of partial blocking of all sites or complete blocking of some of the sites requires knowledge of the exact position of each tropomyosin segment on the actin filament, which cannot be obtained at this time. Therefore, we shall discuss each possibility, i.e., models 1 and 2 in turn.

For model 1 (a case of classical competitive inhibition with a fixed concentration of the inhibitor), all molecular binding constants remain unchanged during the transition from the nonrelaxed to the relaxed state of the actin filament. Myosin binding is reduced in accordance with the decrease in the number of accessible myosin binding sites due to complete blockage by tropomyosin. That means the change in both intercepts, at the ordinate and the abscissa, must be determined by the change in [A], the number of myosin binding sites not blocked by tropomyosin. Rearranging eq 3

$$\frac{[\text{AS} + \text{ASN}]}{[\text{SN}]} = [\text{A}]\left(\frac{K_2}{K_1[\text{N}]} + K_2\right)$$

shows the ordinate intercept determined by $[A]K_2$. (The appropriate values can be obtained from the graphs of Figure 3 by multiplying the ordinate by the concentration of total actin.[2]) On the other hand, the abscissa intercept when [AS + ASN] = 0 is given by $-1/[\text{N}] = K_1[\text{A}]/[\text{A}]$ or, substituting eq 4 and 5 for eq 1, by $-1/[\text{N}] = K_2K_4/K_3\ [\text{A}]/[\text{A}]$. That means changes in the abscissa intercept can be attributed to changes in [A] only if [A] derived from eq 2 has a different numerical value from [A] derived from eq 4 (see Appendix). In other words, the binding sites for S-1 must be distinct from the binding sites for S-1 AMPPNP. Assuming that to be so, the difference in the ratio of the abscissa intercept over the ordinate intercept in the presence and absence of calcium allows one to calculate the change in the number of binding sites available to S-1 due to the removal of calcium. In experiment 4 (Table II; Figure 3A), the number was reduced

[2] In Figure 3 and Table II the data were treated according to model 2: no change in the concentration of accessible myosin binding sites [A] during the transition to the relaxed state and, because of the large excess of actin over S-1, the concentration of free myosin binding sites [A] was assumed to be equal to the total number of binding sites, ie., the total actin concentration.

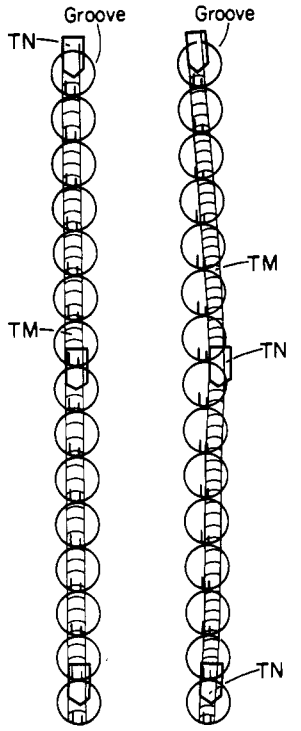

FIGURE 5: Steric model of relaxation, model 1, assuming that S-1 binding sites are either fully blocked or completely uncovered. Only one of the two strands in an actin filament is depicted. The groove edge is marked by arrow. TM = tropomyosin, TN = troponin. Myosin binding sites indicated by vertical lines: longer line = binding site for S-1-AMPPNP, shorter line = binding site for nucleotide-free S-1. When one troponin spontaneously (in the absence of calcium) dissociates from actin, the tropomyosin strand forms a large bend toward the groove [for tropomyosin rigidity, cf. Phillips et al., (1979)], uncovering 3 rigor binding sites and 11 of the more peripheral binding sites for S-1 AMPPNP. For each 11 uncovered S-1 AMPPNP binding sites there are 22–28 fully covered sites, as illustrated by the actin strand to the left.

by factors of 11 and 2.7, respectively.

How does one envision, first, that tropomyosin fully blocks some myosin binding sites and others not at all, and, second, that it blocks more rigor than S-1 AMPPNP binding sites? Since tropomyosin is neither floppy (it forms crystals) (Cohen et al., 1972) nor completely rigid (Phillips et al., 1979), one may visualize it forming occasional bends even during relaxation, for instance when, as depicted in Figure 5, a troponin molecule has been dissociated (from actin but not from tropomyosin) during an occasional random event. Such bends may fully uncover most of the binding sites in the area of the bend whereas the main fraction of the tropomyosin strand which remains in the peripheral position fully blocks the S-1 binding sites.

Next, one must deal with the problem how 3 times more S-1 AMPPNP binding sites could be uncovered than binding sites for nucleotide-free S-1. Obviously the binding sites (the sites where most of the binding energy is released) of the two S-1 species must be small and must be distinct from each other. If the S-1 AMPPNP sites are further from the groove and nearer the periphery of the actin filament than the S-1 sites (rigor sites), one can construct a situation where bending of tropomyosin toward the groove uncovers more S-1 AMPPNP than rigor sites. If we equate inhibition of ATPase activity during relaxation with inhibition of S-1 ADP·P binding, we must postulate a third distinct binding site for S-1 ADP·P which is placed even nearer the groove than the rigor sites, in order to account for the greater inhibition of S-1ADP·P binding compared to the inhibition of rigor complex formation.

As just mentioned, differential uncovering of two or three distinct binding sites is possible only if tropomyosin bends exclusively toward the groove and never toward the periphery (as depicted in Figure 5). If tropomyosin could move equally well to either side of the myosin binding area, the number of accessible sites would be equal for all three S-1 species. This requirement for an asymmetrical tropomyosin shift is not difficult to model. Only three assumptions are necessary. First, as in Parry's & Squire's model (1973), tropomyosin must fit snugly on the actin surface at all times. Second, unlike Parry's and Squire's model, the tropomyosin strand is of fixed length, i.e., it does not lengthen or shorten when it changes position. Third, the myosin binding sites must be at such an angle from the groove that the tropomyosin strand is too short for tropomyosin to move to the area peripheral from the myosin binding site (cf. Parry & Squire, 1973). A tropomyosin strand of fixed length could then bend only toward the groove, as depicted in Figure 5.

The alternative possibility of explaining the reduction in myosin binding to relaxed actin filaments on the basis of the steric model of relaxation is partial blocking of all myosin binding sites by tropomyosin (model 2). In this case, it is assumed that on calcium removal the number of the accessible binding sites is not altered because there is enough binding area left for myosin to bind to actin even while tropomyosin occupies part of the myosin binding site. The reduction in binding area available to myosin results in an apparent decrease in the binding constants. For model 2 a decrease in the ordinate intercept signifies a lowering of the binding constant for S-1 AMPPNP to actin, whereby the value of the binding constant can be read directly from the ordinate of Figure 3. The ratio of the abscissa intercepts with and without calcium multiplied by the inverse ratio of the ordinate intercepts with and without calcium indicates for model 2 the ratio of the association constants for rigor complex formation with and without calcium. Removal of calcium lowered the actin binding constants for S-1 and S-1 AMPPNP to a different extent.

In order to evaluate how much tropomyosin intruded into the myosin binding site, one needs to know how much the affinity of myosin for actin was lowered. That requires knowledge of the absolute values of the binding constants since the affinity is proportional to the logarithm of the binding constant. While our experiments provide those values for S-1 AMPPNP, we must obtain the constants for rigor complex formation from the literature. If, as above, we assume that inhibition of ATPase activity reflects inhibition of S-1 ADP·P binding to actin, we must make an assumption for the actin binding constant for S-1 ADP·P, such as assigning it the lowest possible value, i.e., the value for $1/K_M$ for S-1 in the presence of calcium under conditions of no potentiation. Adopting, on this basis, 10^7 M^{-1} and 10^4 M^{-1} (Bremel et al., 1972), respectively, as constants for rigor complex formation and S-1 ADP·P binding in the presence of calcium but without potentiation, we calculate for experiment 4 (Table II) that tropomyosin blockage reduced the affinity of S-1 for actin [(log 10^7 − log (10^7/11))/log 10^7] by 15% and the affinities of S-1 AMPPNP and S-1 ADP·P for actin by 12% and 39%, respectively. If one wants to explain lesser degrees of relaxation with this model, one may assume that some binding sites are completely exposed, possibly because some troponin molecules no longer bind to actin or bind with low affinity.

The attractive feature of the second model is that it allows for large partially overlapping binding surfaces for the three S-1 species rather than the three completely distinct very small

binding areas of the first model.

Since both models are based on the same data, they both lead to the conclusion that the conformation of S-1 AMPPNP must be significantly different from that of S-1 ADP·P. This conclusion depends on the assumption that inhibition of ATPase activity reflects inhibition of S-1 ADP·P binding to actin. However, there are no kinetic data which make this assumption a fact. On biochemical grounds alone it is quite possible that S-1 ADP·P binding decreases only 2–3-fold like that of S-1 AMPPNP and that the much greater inhibition of ATP turnover is due to a block in the further processing of the actin–S-1 ADP·P complex. Since the actin–S-1 ADP·P complex presumably is converted to the rigor complex during ATPase activity (cf. Taylor, 1979), a tropomyosin block of the rigor binding site in model 1, making it impossible for S-1 to shift over to the rigor binding sites, would inhibit ATPase activity. For model 2 one may speculate that an actin amino acid residue essential for catalytic activity is buried in the fraction of the binding site covered by tropomyosin. In fact, Eisenberg and his colleagues (Chalovich et al., 1980) have recently reported data suggesting to them that calcium removal reduces actin binding of S-1 ADP·P only 2-fold. On the other hand, such a small change in the binding of S-1 ADP·P during relaxation is incompatible with the present interpretation of the X-ray diffraction pattern of muscle during the relaxed state (Huxley, 1969).

In conclusion, we have collected new data on the interaction between S-1 and relaxed actin and have interpreted them entirely in terms of the steric model of Huxley, Haselgrove, Parry, and Squire. This does not imply that this model must be correct: all observations could be just as well explained by any other model, for instance by postulating different conformational states of actin depending on the calcium content of troponin. However, the steric model which accounts for the observed shifts in tropomyosin position is more elegant because it relies on the known structural features of the troponin–tropomyosin actin filament. The data can be fitted to the steric model in two different ways: in relaxed actin, tropomyosin either fully blocks some of the myosin binding sites and leaves others completely free, or tropomyosin partially blocks all of the sites. It is not possible at this time to distinguish experimentally between the two models.

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Appendix

Derivation of the expressions for model 1 that gives the fraction of S-1 rigor and S-1 AMPPNP binding sites not blocked by troponin–tropomyosin in the absence of calcium is as follows: we call the specific binding sites for S-1 AMPPNP (SN) A_1 and those for nucleotide-free S-1 (S) A_2. It is assumed that the binding constant of each S-1 species for its specific binding site is greater by a factor of 1000 than that for the binding site of the other S-1 species. Thus $K_3/K_3' = 10^3$ when $[A_2S]/([A_2][S]) = K_3$ and $[A_1S]/([A_1][S]) = K_3'$, and $K_2/K_2' = 10^3$ when $[A_1SN]/([A_1][SN]) = K_2$ and $[A_2SN]/([A_2][SN]) = K_2'$. That means when AMPPNP dissociates from A_1SN S-1 is transferred to the rigor binding sites, A_2, and when AMPPNP binds to A_2S S-1 is transferred to the binding site for S-1 AMPPNP, A_1. The possible species of bound S-1 are $A_1SN + A_2SN + A_2S + A_1S$. In the presence of calcium, A_2NS and A_1S may be neglected since they are less than 0.1% of $A_1NS + A_2S$ under conditions where S-1 can freely transfer from one binding site to the other. In the absence of calcium, S-1 can always transfer from A_2 to A_1 when AMPPNP binds to A_2S (Figure 5), but it cannot transfer from A_1 to A_2 on dissociation of AMPPNP from the ternary complex on those actin molecules where A_2 is blocked by troponin–tropomyosin. Thus, in the absence of calcium, the following species of bound S-1 must be considered: $A_2S + A_1S + A_1SN$.

$$\frac{[A_1SN + A_1S + A_2S]}{[SN]} = [A_1]K_2 + \frac{[A_1]K_3'}{K_4[N]} + \frac{[A_2]K_3}{K_4[N]}$$

The ordinate intercept is $[A_{1(EGTA)}]K_2$ and the abscissa intercept is

$$-\frac{1}{[N_{(EGTA)}]} = \frac{[A_{1(EGTA)}]K_2K_4}{[A_{1(EGTA)}]K_3' + [A_{2(EGTA)}]K_3}$$

In the presence of calcium $[A_1] = [A_2]$ and since $[A]/[S\text{-}1] = 20–30$, $[A_1] = [A_2] = [A_{total}]$ and, therefore, the ordinate intercept is $[A_{total}]K_2$ and the abscissa intercept K_2K_4/K_3. The ratio of intercepts in the presence and absence of calcium is for the ordinate intercepts

$$\frac{\text{ordinate intercept (Ca)}}{\text{ordinate intercept (EGTA)}} = \frac{[A_{total}]}{[A_{1(EGTA)}]}$$

(column 6 Table II) and for the abscissca intercepts

$$[N_{(EGTA)}]/[N_{(Ca)}] = \frac{K_3'}{K_3} + \frac{[A_{2(EGTA)}]}{[A_{1(EGTA)}]}$$

Since K_3'/K_3 is less than 10^{-3}, it may be neglected, and we arrive at

$$\frac{[A_{total}]}{[A_{2(EGTA)}]} = \frac{[N_{(Ca)}] \times \text{ordinate intercept (Ca)}}{[N_{(EGTA)}] \times \text{ordinate intercept (EGTA)}}$$

(column 5, Table II).

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Environmental Effects on Formation and Photoreaction of the M_{412} Photoproduct of Bacteriorhodopsin: Implications for the Mechanism of Proton Pumping†

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ABSTRACT: Photochemical studies of the effects of temperature, pH, and dehydration on the formation and back photoreaction of the M_{412} intermediate in the photocycle of light-adapted bacteriorhodopsin (bR_{570}) are carried out. Continuous illumination experiments in the range between −40 and −90 °C indicate that at low temperatures branching occurs at the stage of the L_{550} intermediate in which a back reaction to the parent pigment competes with the formation of M_{412}. At low temperatures the yield of M_{412} is markedly increased at high pH. The effect is attributed to the catalytic action of a protein group of $pK \simeq 10$ on the rate of the $L_{550} \rightarrow M_{412}$ process. Our results, taken together with previous evidence for deprotonation of a tyrosine during the $L_{550} \rightarrow M_{412}$ transition, suggest that the formation of a tyrosinate ion is a *prerequisite* for deprotonation of the Schiff base. A model is proposed in which both the Schiff base and the tyrosine translocate their protons to two acceptor groups, A_1 and A_2, accessible to the outside of the cell through a segment of a proton wire. The model accounts for the observation that up to two photons may be pumped per cycle. The proton-pump mechanism is analyzed in terms of a generalized kinetic scheme for pumping. In contrast to current models for proton pumping which are based on a (primary) light-induced accessibility change of the chromophore (class I models), we introduce a new class (II) of models based exclusively on pK changes. We suggest that in bR_{570} the Schiff base and the tyrosine are accessible to protons on the *outside* surface of the membrane. An analysis of the back photoreaction from M_{412} tends to favor class II models over previous class I models.

In this paper we consider aspects of the molecular mechanism of proton pumping in bacteriorhodopsin, the single protein in the purple membrane of *Halobacterium halobium* [see Stoeckenius et al. (1979) for a comprehensive review]. The chromophore of the light-adapted form of bacteriorhodopsin (bR_{570}) is *all-trans*-retinal bound to the ϵ-amino group of a lysine in the protein in the form of a protonated Schiff base. Absorption of a photon by bR_{570} initiates a photocycle (Figure 1; see Ottolenghi (1980) for a recent review) during the course of which protons are transported from the cytoplasmic side to the outside of the cell.

The $L_{550} \rightarrow M_{412}$ transition appears to play an important role in the proton-pumping mechanism. First, the ejection of protons to the outside of the membrane occurs on a time scale comparable to the rate of M_{412} formation (Lozier et al., 1976;

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