Cross-Linking of Actin to Myosin Subfragment 1 in the Presence of Nucleotides†

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ABSTRACT: Chemical cross-linking of actin to the 20K and 50K fragments of tryptically cleaved myosin subfragment 1 (S-1) by the zero-length cross-linking reagent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was used as a probe of the acto–S-1 interface in the presence of nucleotides. The course of the two reactions was monitored by measuring on sodium dodecyl sulfate (SDS)–polyacrylamide gels the time-dependent formation of the 20K–actin and 50K–actin cross-linked products. Both reactions were inhibited somewhat in the presence of MgADP, were slowed 3–4-fold in the presence of magnesium 5′-adenylyl imidodiphosphate (MgAMPPNP), and proceeded at least 7-fold slower with *N,N′-p*-phenylenedimaleimide (pPDM) modified S-1, as compared to the respective rates in the absence of nucleotides. However, neither the binding of the nucleotides MgADP and MgAMPPNP to S-1 nor the modification of S-1 by pPDM significantly changed the ratio of the cross-linking rates of actin to the 20K and 50K fragments. Similar to what was previously observed in the absence of nucleotides [Chen, T., Applegate, D., & Reisler, E. (1985) *Biochemistry 24*, 137–144], actin was cross-linked at an approximately 3-fold faster rate to the 20K fragment than to the 50K fragment under all reaction conditions tested. Thus, irrespective of the extent of acto–S-1 dissociation or the binding of nucleotides to acto–S-1, the 20K fragment remains the preferred cross-linking site for actin. These results show that the interaction of actin with each of the cross-linking sites on S-1 is not under selective or preferential control by nucleotides.

It is generally believed that muscle contraction involves some changes in the structure and the angle of attachment of myosin cross-bridges to actin (Huxley, 1969; Huxley & Simmons, 1971; Huxley et al., 1981, 1983). Until now, the search for such transitions has mainly involved the detection and definition of different cross-bridge orientations in muscle fibers (Goody & Holmes, 1983). A variety of studies employing electron microscopy and X-ray diffraction (Reedy, 1967; Goody et al., 1976; Marston et al., 1979; Reedy et al., 1983), as well as spectroscopic methods (Borejdo & Putnam, 1977; Thomas & Cooke, 1980; Yanagida, 1981; Borejdo et al., 1982), have demonstrated that nucleotides and nucleotide analogues affect the states of myosin cross-bridges. Recently the successful cross-linking of the actomyosin subfragment 1 (S-1)[1] complex has opened the possibility for chemical probing of the actomyosin interface (Yamamoto & Sekine, 1979; Mornet et al., 1981; Sutoh, 1983). In his elegant work, Sutoh (1983) identified two regions on the myosin head that are involved in the cross-linking to actin by carbodiimide. These sites span the 18K–20K region on the 20K tryptic fragment and the 27K–35K region on the 50K tryptic fragment as mapped from the C terminus of S-1. According to Sutoh (1982) the same N-terminal portion of actin is cross-linked to either the 20K or the 50K fragment of S-1, and the cross-linked species are 1:1 complexes of actin and S-1. Subsequent work confirmed the 1:1 stoichiometry of the cross-linked acto–S-1 complex (Greene, 1984; Heaphy & Tregear, 1984; Chen et al., 1985).

Although at present there is no evidence for simultaneous binding of two actin molecules to a single myosin head, the two actin cross-linking sites on S-1 appear to be nonequivalent. We have shown that actin is preferentially cross-linked by carbodiimide to the 20K fragment on S-1. We speculated that this portion of S-1 may contain the strong binding site for actin (Chen et al., 1985). This possibility has also been suggested by other groups (Muhlrad & Morales, 1984; Katoh & Morita, 1984). Consistent with this assertion is the stronger affinity for actin exhibited by the isolated 20K fragment as compared to that of the isolated 50K fragment (Muhlrad & Morales, 1984).

In view of the possible existence of two mutually exclusive binding sites for actin on S-1, as suggested by the cross-linking experiments, it is intriguing to examine the effect of nucleotides on the acto–S-1 binding interface. Such information is needed for structural description of actomyosin binding and its modulation by nucleotides, particularly in terms of the conformation of the attached states of myosin cross-bridges.

In this work we have examined the carbodiimide cross-linking of actin to intact S-1 and tryptically cleaved S-1 in the presence of MgADP and MgAMPPNP. The S-1·MgATP complex was replaced in our cross-linking experiments with *N,N′-p*-phenylenedimaleimide-modified S-1. We find that the rates of cross-linking the 20K and 50K fragments to actin are reduced by nucleotides and that under all reaction conditions the 20K fragment remains the preferential site of attachment to actin.

Materials and Methods

Materials. All the proteases, inhibitors, catalase, the cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide

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[1] Abbreviations: S-1, myosin subfragment 1; AMPPNP, 5′-adenylyl imidodiphosphate; DTE, dithioerythritol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; pPDM, *N,N′-p*-phenylenedimaleimide; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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(EDC), nucleotides, and other reagents were products of Sigma Chemical Co. (St. Louis, MO). *N,N'-p*-Phenylenedimaleimide (pPDM) was from Aldrich (Milwaukee, WI). All reagents used were analytical grade.

Proteins. Actin was extracted from the acetone powder by the method of Spudich & Watt (1971). Myosin was prepared from rabbit psoas muscle as previously described (Godfrey & Harrington, 1970). Subfragment 1 was prepared by chymotryptic digestion of myosin according to Weeds & Pope (1977). The concentrations of the proteins were measured at 280 nm spectrophotometrically with $A^{1\%} = 5.5$ cm^{-1} for myosin, 7.5 cm^{-1} for S-1, and 11 cm^{-1} for actin.

N,N'-p-Phenylenedimaleimide (pPDM) Modification of S-1. S-1 (2 mg/mL) in 0.1 M KCl and 50 mM Tris at pH 8.0 was reacted with pPDM (at a pPDM to S-1 molar ratio of 1.4:1) in the presence of 2 mM MgADP or MgATP for 30 min at 0 °C (Reisler, 1982). The reaction was terminated by the addition of 1 mM DTE. Modified S-1 was then precipitated by adding 2.5 volumes of saturated ammonium sulfate (4 °C) and centrifuged at 15000*g* for 30 min. The pellet was redissolved in the original buffer and passed through two Sephadex G-50 Penefsky columns (Penefsky, 1977). In the final step, the remaining small amount of unmodified S-1 was removed by centrifuging the sample in the presence of actin (actin to S-1 molar ratio of 1:5). To check the extent of modification of S-1 by pPDM, Ca^{2+} and EDTA (K^+) stimulated adenosinetriphosphatase (ATPase) activities were measured by employing the procedure of Kielley et al. (1956). Only those preparations of pPDM-modified S-1 that had ATPase activities lower than 5% of that of the unmodified S-1 were used in cross-linking experiments. In the three preparations used in the cross-linking experiments, the EDTA (K^+) stimulated ATPase activities were 0% and the Ca^{2+}-stimulated ATPase activities were all below 4% of the ATPase activities of control S-1. The concentrations of the modified protein solutions were determined by the Lowry assay (Lowry et al., 1951).

Tryptic Digestion of S-1 and pPDM-Modified S-1. S-1 or pPDM-modified S-1 (3 mg/mL) was digested by trypsin (0.3 mg/mL) in 0.1 M KCl and 50 mM Tris at pH 8.0 for 45 min at 22 °C. The digestion was terminated by the addition of soybean trypsin inhibitor to a final concentration of 0.9 mg/mL. The digested S-1 or pPDM-modified S-1 was then dialyzed into the appropriate solution for the cross-linking reaction. The modification of S-1 with pPDM did not change the tryptic digestion of this protein.

Cross-Linking of the Acto–S-1 Complex in the Presence of MgADP and MgAMPPNP. Intact S-1 and tryptically cleaved S-1 were equilibrated with actin at final concentrations of 0.5 mg/mL each and allowed to form the acto–S-1 complexes by incubation at 22 °C for 30 min in either the standard reaction solutions or the low ionic strength buffer. The standard reaction solvent contained 0.1 M NaCl, 2 mM $MgCl_2$, and 10 mM imidazole at pH 7.0 (Sutoh, 1983). The low ionic strength buffer contained 10 mM NaCl, 2 mM $MgCl_2$, and 10 mM imidazole at pH 7.0. A nucleotide (MgADP or MgAMPPNP) was added to a final concentration of 1 or 3 mM as indicated. The cross-linker EDC was then added to 1 mg/mL to initiate the cross-linking. At indicated time points, aliquots were removed and the cross-linking reaction was terminated by the addition of 10 mM β-mercaptoethanol. The samples were then denatured and examined on SDS–polyacrylamide gels.

Digestion of Cross-Linked Acto–S-1 with Elastase. Acto–S-1 was cross-linked according to the above conditions for 2 h and then centrifuged in the presence of 15 mM MgATP and 2 mM magnesium pyrophosphate at 40K for 1 h to remove un-cross-linked S-1. The supernatant contained only S-1. The pellet containing isolated cross-linked acto–S-1 species was homogenized in the standard reaction buffer and digested by elastase (1:10 w/w ratio to S-1). The concentration of S-1 in the pellet was estimated by measuring the concentration of S-1 recovered in the supernatant. At indicated time intervals, aliquots of the reaction mixture were removed and the digestion was terminated by the addition of 1 mM PMSF. These samples were then analyzed on SDS–polyacrylamide gels. A more detailed description of this procedure was presented previously (Chen et al., 1985).

Cross-Linking of pPDM-Modified S-1. The cross-linking of tryptically cleaved and pPDM-modified S-1 (2 mg/mL) to actin (2 mg/mL) was carried out by the same procedure as described for unmodified S-1, except for the higher protein concentrations employed for the modified system. The reactions were carried out in the low ionic strength buffer containing 10 mM NaCl, 2 mM $MgCl_2$, and 10 mM imidazole at pH 7.0. For the purpose of comparison, control reactions were done under identical conditions by using unmodified S-1.

Gel Electrophoresis. Gel electrophoresis was carried out according to the procedure of Laemmli (1970). In most cases, a two-phase resolving gel of 10% (upper, w/v) and 15% (lower, w/v) acrylamide was employed. Catalase was added as an internal quantitation standard.

Densitometric Measurement. The optical densities of the Coomassie blue stained protein bands were determined with a Biomed Soft Laser gel scanning densitometer equipped with an integrator and interfaced into an Apple IIe computer. To account for experimental variations, the intensities of all the protein bands in a given lane were first normalized to the intensity of the catalase band in the same lane. The differences in dye absorption were corrected by dividing the normalized intensities by the molecular weights of the respective protein bands. Justification of this procedure and more detailed discussion of the staining corrections as applied to our experimental system were presented previously (Chen et al., 1985). The apparent molecular weights of bands corresponding to cross-linked species were determined by comparing their mobilities on the SDS–polyacrylamide gels with those of protein standards of known molecular weights.

RESULTS

Chemical cross-linking of acto–S-1 complexes in the presence of nucleotides carried out in this work is a straightforward extension of our previous study on the course and the products of carbodiimide reaction with acto–S-1 (Chen et al., 1985). In that study we have shown that the cross-linking of acto–S-1 can be monitored in kinetic terms by measuring the decay of the free 20K and 50K fragments and the formation of cross-linked products. The relative contributions of each of these fragments to the overall cross-linking of S-1 to actin can then be easily discerned. In our hands this analysis was most reliable for cross-linking of preformed complexes of tryptically cleaved S-1 and actin at pH 7.0. Most of the experiments described below follow this procedure.

Cross-Linking of Acto–S-1·MgADP Complexes. Cross-linking of acto–S-1 complexes with EDC yields the same products and proceeds in a manner that is similar for reactions carried out in the presence and absence of MgADP (Figure 1). In both cases the cross-linking of intact S-1 to actin results in the formation of two electrophoretically resolved products (the 165K and 175K doublet) which were identified before as corresponding to actin cross-linked to either the 20K or the

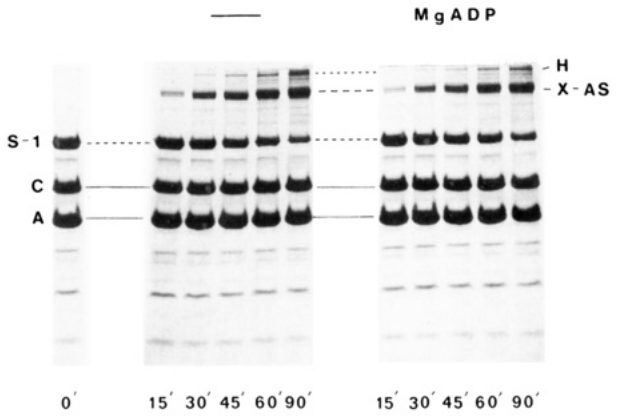

FIGURE 1: SDS–polyacrylamide gel showing the time course of cross-linking actin to intact S-1 by EDC in the absence and presence of 1 mM MgADP. The reaction was carried out in 0.1 M NaCl, 2 mM $MgCl_2$, and 10 mM imidazole at pH 7.0. Experimental details are described under Materials and Methods. Cross-linking times (in minutes) are indicated under each lane. C refers to catalase and A to actin. H refers to the high molecular weight cross-linked species, and X-AS refers to the doublet of 165K and 175K cross-linked acto–S-1 (Mornet et al., 1981; Sutoh, 1983).

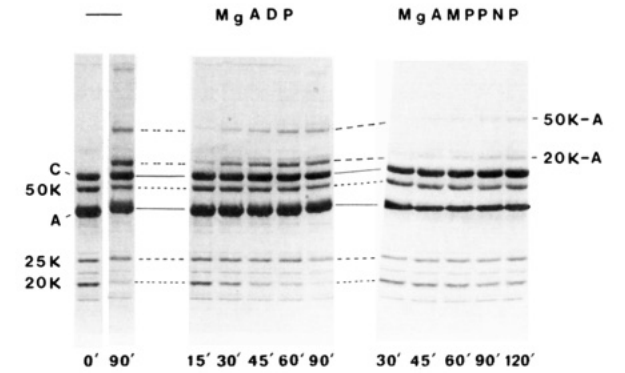

FIGURE 2: SDS–polyacrylamide gel showing the time course of cross-linking actin to tryptically cleaved S-1 in 0.1 M NaCl, 2 mM $MgCl_2$ and 10 mM imidazole at pH 7.0 in the presence of 1 mM MgADP and 1 mM MgAMPPNP. Cross-linking times (in minutes) are indicated under each lane. For reference, the second lane shows a gel of 90-min cross-linking of the acto–S-1 complex in the absence of nucleotides. C refers to catalase and A to actin. The 50K–actin and 20K–actin correspond to actin cross-linked to the 50K and 20K fragments of S-1, respectively.

50K fragments of S-1 (Sutoh, 1983). Examination of SDS gels monitoring the progress of acto–S-1 cross-linking in the presence and absence of MgADP (Figure 1) reveals only small differences in the rates of the respective reactions. As measured either by the rate of disappearance of intact S-1 or by the rate of formation of cross-linked products, MgADP inhibits the rate of cross-linking of actin to S-1 by about 30%.

In order to determine whether MgADP acts preferentially or to an equal extent on the reactions of the 20K and 50K fragments of S-1, we have examined the cross-linking of tryptically cleaved S-1 to actin. As shown in our previous work (Chen et al., 1985), such reactions can be monitored on SDS gels both in terms of the rates of disappearance of the 20K and 50K fragments and in terms of the rates of formation of the 20K–actin and 50K–actin products. In Figure 2 we show the cross-linking of tryptically cleaved S-1 to actin in the presence of MgADP and MgAMPPNP. Since the cross-linking of acto–S-1 in the absence of nucleotides was extensively documented in our previous work (Chen et al., 1985), only a representative gel of such a reaction is included in Figure 2 for comparative purposes. Clearly, the presence of nucleotides in the cross-linking reaction has no effect on the nature of the generated cross-linked products.

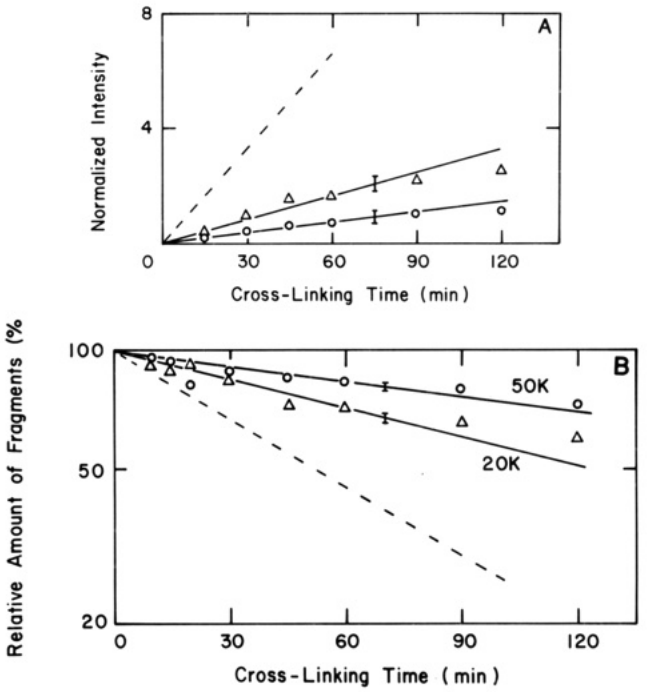

FIGURE 3: Effect of MgAMPPNP on the cross-linking reactions. (A) Time course of formation of cross-linked 20K–actin (△) and 50K–actin (○) products in the presence of 1 mM MgAMPPNP. The dashed curve indicates the formation of the 20K–actin product in the cross-linking of acto–S-1 in the absence of nucleotides. All data were derived from SDS gels similar to those shown in Figure 2. The bars indicate experimental error. (B) Time course of decay of the 20K (△) and 50K (○) fragments during EDC cross-linking of tryptically cleaved S-1 in the presence of 1 mM MgAMPPNP. The bars indicate experimental error. For comparative purposes, we also show the decay of the 20K fragment of S-1 in parallel cross-linking reactions carried out in the absence of nucleotides (dashed curve, experimental points not included).

Table I: EDC Cross-Linking of Acto–S-1: Relative Rates of Formation of Cross-Linked 20K–Actin and 50K–Actin Products[a]

reaction conditions	$k_{20\text{-}A}$	$k_{50\text{-}A}$	$k_{20\text{-}A}:k_{50\text{-}A}$
(A) Cross-Linking in 0.1 M NaCl, 2 mM $MgCl_2$, and 10 mM Imidazole at pH 7.0			
control	100	31	3.2
1 mM MgADP	83	27	3.1
3 mM MgADP	80	27	3.1
(B) Cross-Linking in 0.01 M NaCl, 2 mM $MgCl_2$, and 10 mM Imidazole at pH 7.0			
control	150	50	3.0
3 mM MgADP	142	45	3.1

[a] The data shown in Table I were obtained by normalizing the rates of formation of all cross-linked products to the rate of formation of the 20K–actin material in cross-linking of tryptically cleaved S-1 to actin in 0.1 M NaCl, 2 mM $MgCl_2$, and 10 mM imidazole at pH 7.0 and in the absence of nucleotides. All rates are determined from the initial slopes of product formation curves such as those shown in Figure 3A. Each experiment was repeated at least 3 times; the experimental error of rate determinations is ±10%.

On the basis of our previous experience (Chen et al., 1985) we elected to follow the cross-linking reactions shown in Figure 2 by measuring on SDS gels the time-dependent formation of the 20K–actin and 50K–actin products. Their accumulation is linear with time over the first 45 or 60 min of EDC cross-linking of acto–S-1 (e.g., Figure 3). Using the initial slopes of the product formation curves, such as those shown in Figure 3A, we determine the rates of cross-linking the 20K and the 50K fragments to actin, namely, $k_{20\text{-}A}$ and $k_{50\text{-}A}$. These

rates are normalized to the rate of formation of the 20K–actin product in the absence of nucleotides and are summarized in Table I. Although MgADP appears to decrease the value of $k_{20\text{-}A}$ by approximately 20% and that of $k_{50\text{-}A}$ only by about 10%, such differences are hardly significant given the 10% error margin in these experiments. The ratio of the two cross-linking rates is approximately constant for all reactions carried out in the presence of MgADP and virtually unchanged from the value observed for acto–S-1 cross-linked in the absence of MgADP ($k_{50\text{-}A}$:$k_{50\text{-}A}$ = 3:1).

To verify that the use of tryptically cleaved S-1 does not affect the outcome and the interpretation of cross-linking experiments, we also analyzed the cross-linking of intact S-1 to actin in the presence and absence of MgADP. As in the previous work (Chen et al., 1985), the cross-linked samples were digested with elastase to yield the 22K–actin and 50K–actin products as well as the free 22K and 50K peptides (Applegate & Reisler, 1983, 1984). Measurements of these proteolytic products of cross-linked acto–S-1 revealed that MgADP did not have any significant effect on the preferential cross-linking of the 22K peptide to actin. Irrespective of the presence or absence of MgADP in the cross-linking reaction, a major fraction of the 22K fragment (between 65% and 80%) is cross-linked to actin, whereas a significantly smaller fraction of the 50K fragment (between 35% and 40%) undergoes the similar reaction [see also Chen et al. (1985)].

As can be seen from Table I, cross-linking of acto–S-1 by EDC proceeds much faster under low ionic strength conditions (10 mM NaCl) than in the standard solvent system (0.1 M NaCl). However, it is unlikely that this difference in the rates of acto–S-1 cross-linking is caused by different binding affinities of actin to S-1. Indeed, the binding of MgADP to S-1 is known to affect the acto–S-1 interaction to a greater extent than ionic strength conditions (Greene, 1981), yet this nucleotide brings about comparatively smaller changes in the rates of the cross-linking reactions. It is worth noting that although the rates $k_{20\text{-}A}$ and $k_{50\text{-}A}$ are faster in 0.01 M NaCl solvent than in 0.1 M NaCl solvent, their ratio ($k_{20\text{-}A}$:$k_{50\text{-}A}$ = 3.0) is about the same under both salt conditions, either in the presence or in the absence of MgADP.

Cross-Linking of Acto–S-1·MgAMPPNP Complex. The binding of MgAMPPNP to acto–S-1 greatly reduces the rate of cross-linking of this complex by EDC (Figure 2). By monitoring the rates of disappearance of the 20K and 50K fragments (Figure 3B) as well as the rates of formation of the cross-linked products $k_{20\text{-}A}$ and $k_{50\text{-}A}$ (Figure 3A), we conclude that the reactions of both fragments are inhibited by MgAMPPNP. It appears that the cross-linking of the 20K fragment to actin is reduced somewhat more than that of the 50K peptide. Consequently, the ratio $k_{20\text{-}A}$:$k_{50\text{-}A}$ is slightly lower in the presence of MgAMPPNP (2.4) than similar ratios determined in the absence of nucleotides (Table II). Yet, considering the fact that the individual rates $k_{50\text{-}A}$ and $k_{50\text{-}A}$ are inhibited between 3- and 4-fold in the presence of MgAMPPNP, the change in the ratio of these rates is rather small. In addition, cross-linking of intact S-1 to actin in the presence of MgAMPPNP, followed by digestion of the isolated cross-linked complex with elastase, shows the same preferential reaction of the 20K fragment as observed in the presence of MgADP. Thus, neither MgADP nor MgAMPPNP can induce major changes in the pattern of acto–S-1 cross-linking with EDC.

Cross-Linking of N,N'-p-Phenylenedimaleimide-Modified S-1 (pPDM-Modified S-1) to Actin. The most interesting complex, acto–S-1·MgATP, is also the most difficult to cross-link. The low affinity of actin for S-1 in the presence of MgATP (Chalovich et al., 1983) can be partially countered by low ionic strength conditions and high protein concentrations. However, these conditions also lead to rapid hydrolysis of ATP. Consequently, either the cross-linking of acto-S-1 in the presence of MgATP is marginal or it does not proceed with the desirable protein–nucleotide complex. To circumvent this difficulty, we substituted in our experiments pPDM-modified S-1 for S-1·MgATP. According to previous evidence such modification of S-1, i.e., cross-linking of its two reactive thiols with pPDM, locks the myosin head into a conformation similar to that of the S-1·MgATP complex (Burke et al., 1976; Chalovich et al., 1983; Shriver, 1984). Thus, pPDM-modified S-1 and actin can be cross-linked in lieu of the acto–S-1·MgATP complex.

Table II: Relative Rates of Formation of Cross-Linked 20K–Actin and 50K–Actin Products in the Presence of MgAMPPNP or with pPDM-Modified S-1[a]

reaction conditions	$k_{20\text{-}A}$	$k_{50\text{-}A}$	$k_{20\text{-}A}$:$k_{50\text{-}A}$
(A) Cross-Linking in 0.1 M NaCl, 2 mM $MgCl_2$, and 10 mM Imidazole at pH 7.0			
rigor	100	31	3.2
1 mM MgAMPPNP	26	11	2.4
(B) Cross-Linking in 0.01 M NaCl, 2 mM $MgCl_2$, and 10 mM Imidazole at pH 7.0			
unmodified S-1	600	200	3.0
pPDM-modified S-1	75	33	2.3

[a] All rates were obtained by normalizing the relative rates of formation of cross-linked products from SDS gels shown in Figures 2 and 4 to the rate of formation of 20K–actin species in reactions carried out in 0.1 M NaCl and in the absence of nucleotides. The rates were derived from the initial slopes of product formation curves. (A) In reactions carried out in 0.1 M NaCl, the concentrations of actin and S-1 were fixed at 0.5 mg/mL each. (B) In the low-salt solvent, actin and S-1 were cross-linked at final concentrations of 2 mg/mL each. All experiments were done at least 3 times; the experimental error of rate determinations is ±10%.

The main advantage of the modified system is that it does not require the presence of ATP. Protein concentrations and salt conditions can then be freely adjusted to partially compensate for the weak affinity of actin for pPDM-modified S-1. Accordingly, our reactions were carried out in 10 mM NaCl solvent and at protein concentrations of 2.0 mg/mL. Control reactions of unmodified tryptically cleaved S-1 proceed under such conditions at rates about 6-fold faster than under our standard reaction conditions (Table II). At the same time the cross-linking of pPDM-modified S-1 to actin is slow and occurs at very low yields (Figures 4 and 5, Table II). The rates of cross-linking $k_{20\text{-}A}$ and $k_{50\text{-}A}$ are between 8- and 10-fold slower for the modified S-1 than for its unmodified counterpart (Table II). In spite of the large inhibition of the cross-linking reaction, the ratio of the cross-linking rates $k_{20\text{-}A}$:$k_{50\text{-}A}$ is only moderately reduced. The small variation in the value of the $k_{20\text{-}A}$:$k_{50\text{-}A}$ ratio indicates that the 20K fragment is preferentially cross-linked to actin under all reaction conditions used in this work.

Discussion

The main objective of this study was to determine the effect of nucleotides on the cross-linking of actin to the 20K and 50K tryptic fragments of S-1. The immediate interest in this issue stems from the recent indications and speculations that myosin heads contain two binding sites for actin, one on the 20K fragment and the other on the 50K fragment of S-1 (Mornet et al., 1981; Sutoh, 1983; Muhlrad & Morales, 1984; Katoh & Morita, 1984). If indeed true, such a situation would allow construction of various schemes by which nucleotides could regulate the transition between the "tight" and "weak" acto-

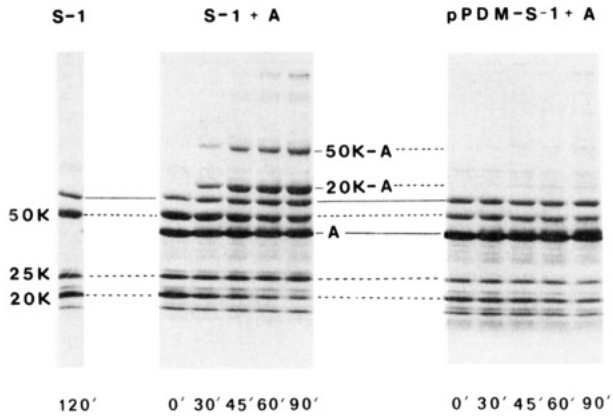

FIGURE 4: SDS–polyacrylamide gels showing the time course of cross-linking actin (2 mg/mL) to tryptically cleaved pPDM-modified S-1 (2 mg/mL) and to unmodified tryptically cleaved S-1 (2 mg/mL) in 10 mM NaCl, 2 mM $MgCl_2$, and 10 mM imidazole at pH 7.0. The first lane shows the reaction of tryptically cleaved S-1 (2 mg/mL) with EDC in the low-salt buffer and in the absence of actin. A refers to actin. The cross-linking times (in minutes) are indicated under each lane. The catalase band is above the 50K band. The 50K–actin and 20K–actin bands correspond to actin cross-linked to the 50K and 20K fragments of modified or unmodified tryptically cleaved S-1, respectively.

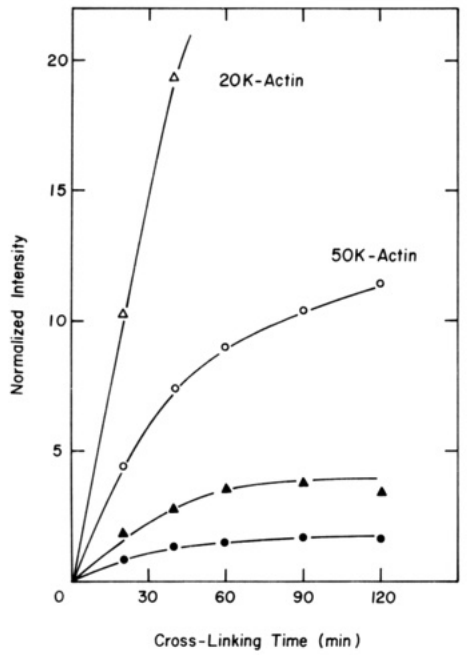

FIGURE 5: Time course of formation of cross-linked 20K–actin (△, ▲) and 50K–actin (○, ●) products in the cross-linking of actin to tryptically cleaved pPDM-modified S-1 (△, ○) and unmodified S-1 (▲, ●). The data are obtained by quantitating the corresponding protein bands in the SDS gels shown in Figure 4.

myosin complexes through perturbation of one of the actin binding sites (Goody & Holmes, 1983; Katoh & Morita, 1984).

The existence of two actin binding sites on S-1 was deduced from the important contributions of Mornet et al. (1981) and Yamamoto & Sekine (1979), who successfully cross-linked the acto–S-1 complex. Since then it has become clear that under limited reaction conditions only one molecule of actin is cross-linked to S-1 by carbodiimide. The chemical link between these two proteins may indeed occur via either the 20K or the 50K peptides of S-1 (Sutoh, 1983; Greene, 1984; Heaphy & Tregear, 1984; Chen et al., 1985).

Two additional important aspects of the acto–S-1 cross-linking reaction came to light in these studies. First, the same N-terminal portion of actin was shown to cross-link to two different sites on S-1 (Sutoh, 1983). Second, kinetic analysis of acto–S-1 cross-linking by carbodiimide has revealed that actin is preferentially cross-linked to the 20K fragment of S-1 and that only 25% or 30% of myosin heads are linked to actin via the 50K fragment (Chen et al., 1985).

It is tempting to assume that the differences in the cross-linking of actin to the 20K and 50K fragments are somehow related to the binding constants of actin to two distinct sites on S-1. Circumstantial support for this assumption is discussed by Muhlrad & Morales (1984), Katoh & Morita (1984), and Chen et al. (1985). An alternative explanation of the preferential cross-linking of actin to the 20K fragment of S-1 would view the EDC reaction as focusing on a single contact area between actin and S-1, and perhaps not the most important one. In other words, as discussed previously (Chen et al., 1985), the N-terminal portion of actin may indeed preferentially interact with the 20K fragment, but the actual binding of actin to S-1 is determined along a multipoint and perhaps discontinuous contact surface. To put it in trivial terms, carbodiimide cross-linking is a probe of acto–S-1 interaction, but does it probe the area of "action"?

The results of this work shed light on some of the above considerations. First, it is quite obvious that the rates of acto–S-1 cross-linking are related to the binding affinities of these proteins. Using tryptically cleaved S-1, we determine that while MgADP slows the formation of cross-linked 20K–actin and 50K–actin products by about 20% and 10%, respectively, MgAMPPNP brings about a 3- or 4-fold inhibition of these reactions. pPDM-modified S-1, which at least in terms of its interaction with actin may be considered an analogue of S-1·MgATP (Burke et al., 1976; Chalovich et al., 1983; Shriver, 1984), can hardly be cross-linked to actin. Thus, qualitatively, the rates of acto–S-1 cross-linking in the presence of nucleotides are ordered according to the effect of these nucleotides on acto–S-1 binding (Greene, 1981; Chalovich et al., 1983). We have not made any attempt to extend this correlation between the rates of acto–S-1 cross-linking and the affinity of actin for S-1 beyond its present qualitative state. One of the reasons for avoiding such attempts is the sensitivity of the cross-linking reaction to experimental conditions. Thus, for example, ionic strength has a bigger impact on the rate of cross-linking of actin to S-1 than MgADP, although the nucleotide is known to have a greater effect on the affinity of actin for S-1.

The second issue, the effect of nucleotides on the cross-linking of actin to its two putative sites on S-1, was examined in this work with the help of cross-linking reactions carried out in the presence of MgADP and MgAMPPNP and by using pPDM-modified S-1 instead of S-1·MgATP. Strikingly, we found that in all cases the formation of the 20K–actin and 50–actin products was inhibited to a similar although not identical extent. Since the individual rates of cross-linking were inhibited manyfold, particularly when using pPDM-modified S-1, we do not attach special significane to a 20% decrease in the ratio of the $k_{20\text{-}A}$ and $k_{50\text{-}A}$ rates (from $k_{20\text{-}A}:k_{50\text{-}A}$ = 3.0 to 2.4) noted in some reactions (Table II). Clearly, actin is preferentially cross-linked to the 20K fragment of S-1 in all reactions tested in this work, in the presence and absence of nucleotides, in modified and unmodified S-1. Thus, if there are two distinct actin sites on S-1, then either these are equally affected by the binding of nucleotides to acto–S-1 or the EDC cross-linking sites on S-1 are removed from the "prime binding areas" and record only the net binding of the two proteins. Cross-linking distribution of actin between the

20K and 50K fragments would express, in this latter case, the affinity of only one portion of actin for these fragments.

In the absence of any significant changes in the ratio of the two cross-linking rates of actin to the 20K and 50K fragments of S-1 ($k_{20\text{-A}}$:$k_{50\text{-A}}$), we do not have any good evidence to support the existence of two distinct actin binding sites on S-1, i.e., sites that could be differentially affected by nucleotides. However, clearly it is not possible from our results to exclude the possibility of two distinct binding sites. It is very likely that a single cross-linking method cannot conclusively test such a possibility. The findings of this work call for considerable caution in interpreting the results of actomyosin cross-linking experiments and point to the need for additional cross-linking methods and other approaches in order to characterize the acto–S-1 interface.

Registry No. MgADP, 7384-99-8; MgAMPPNP, 82050-25-7.

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