Cross-Linking of Actin to Myosin Subfragment 1: Course of Reaction and Stoichiometry of Products†

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ABSTRACT: The cross-linking of actin to myosin subfragment 1 (S-1) with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide was reexamined by using two cross-linking procedures [Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306; Sutoh, K. (1983) *Biochemistry 22*, 1579–1585] and two independent methods for quantitating the reaction products. In the first approach, the cross-linked acto–S-1 complexes were cleaved with elastase at the 25K/50K and 50K/22K junctions in S-1. This enabled direct measurements of the cross-linked and un-cross-linked fractions of the 50K and 22K fragments of S-1. We found that in all cases actin was preferentially cross-linked to the 22K fragment and that the overall stoichiometry of the main cross-linked products was that of a 1:1 complex of actin and S-1. In the second approach, actin was cross-linked to tryptically cleaved S-1, and the course of these reactions was monitored by measuring the decay of the free 50K and 20K fragments and the formation of cross-linked products. After selecting the optimal cross-linking procedure and conditions, we determined that the rate of actin cross-linking to the 20K fragment of S-1 was 3-fold faster than the reaction with the 50K peptide. The overall rate of cross-linking actin to S-1 corresponded to the sum of the individual reactions of the 50K and 20K fragments, indicating their mutually exclusive cross-linking to actin. Thus, the reactions with tryptically cleaved S-1 were consistent with the 1:1 stoichiometry of actin and S-1 in the main cross-linked products and verified the preferential cross-linking of actin to the 20K fragment of S-1. These results are discussed in the context of the binding of actin to S-1.

In spite of its obvious importance to the understanding of the mechanism of muscle contraction, the structural information on the acto–myosin interface has been lacking until recently. The electron microscopy and image reconstruction work of Moore et al. (1970), Wakabayashi & Toyoshima (1981), Taylor & Amos (1981), and Amos et al. (1982) produced the first three-dimensional description of the acto–myosin subfragment 1 (S-1)[1] complex. However, the resolution limits of this method preclude at present a detailed assignment of contact areas and domains in the protein complex. This important information may now be within the reach of an alternate approach, the chemical cross-linking of acto–S-1 (Mornet et al., 1981; Yamamoto & Sekine, 1979; Sutoh, 1983). These studies offer a new and intriguing, albeit somewhat controversial, insight into the acto–S-1 interface.

Using a zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), Mornet et al. (1981) have demonstrated that two of the tryptic fragments of S-1, the 50K and 20K peptides, can be cross-linked to actin. Subsequent work by Sutoh (1983) has shown that the portions of the S-1 heavy chain which are involved in such cross-linking to actin span the 18K–20K (on the 20K) and the 27K–35K (on the 50K) regions from the C-terminus of S-1. The cross-linking of the 50K fragment to actin has also been achieved with a different reagent, dimethyl suberimidate (Yamamoto & Sekine, 1979).

When examined on SDS gels, the carbodiimide-cross-linked S-1 migrates as a 180K doublet band, but some higher molecular weight species are present as well (Mornet et al., 1981; Sutoh, 1983). The identity of the main cross-linked products (the 180K doublet) is still in dispute. As judged by their mobility on SDS gels and the results of the ^{3}H–^{14}C double-labeling experiments, Mornet et al. (1981) have suggested that the 180K species contain two molecules of actin simultaneously cross-linked to each S-1. On the other hand, the chemical cleavage experiments of Sutoh (1983) show that the two bands in the 180K doublet represent stoichiometric 1:1 complexes of actin and S-1 formed by cross-linking of actin to either the 50K or the 20K fragment, respectively. It is important to note that the two groups differ not only in their conclusions and methods of analysis but also in the experimental procedures employed in the cross-linking of actin to S-1. Mornet et al. (1981) carry out the reaction in two steps and at pH 6.0. The first step involves the inactivation of actin by carbodiimide and the second its cros-linking to S-1. In contrast, Sutoh (1983) uses a one-step procedure at pH 7.0 in which the carbodiimide is added to the preformed acto–S-1 complex.

The great interest in the results of cross-linking studies stems from their implications to the nature of the acto–myosin interaction. The main questions are the following: do one or two actin molecules bind to each myosin head, are the two actin binding sites on S-1 equivalent, and are they equally

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[1] Abbreviations: S-1, myosin subfragment 1; DTE, dithioerythritol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.

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modulated by the binding of nucleotides to myosin? In this study, we have focused on the first two questions, i.e., the stoichiometry of the cross-linked acto–S-1 complex and the individual cross-linking reactions of actin to the 20K and 50K fragments. We have examined the cross-linking of actin to S-1 by using two independent methods to quantitate the amounts of the 50K and 20K fragments cross-linked to actin. Also, in view of the different results reported in the literature, all reactions were carried out by using both the one-step, pH 7.0 (Sutoh, 1983) procedure and the two-step, pH 6.0 (Mornet et al., 1981) procedure. We find that although the particular reaction conditions affect the efficiency and the rates of cross-linking, in all cases the main products of such reactions are the complexes of actin and the 20K fragment. In addition, we confirm the observation of Sutoh (1983) and conclude that the cross-linked species are primarily 1:1 complexes of actin and S-1.

Materials and Methods

Materials. All the proteases, inhibitors, catalase, the cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), nucleotides, and other reagents were products of Sigma Chemical Co. (St. Louis, MO). N-(Iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS) was obtained from Molecular Probes (Plano, TX). All other reagents used were analytical grade.

Proteins. Actin was extracted from 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 spectrophotometrically at 280 nm using $A^{1\%} = 5.5$ cm^{-1} for myosin, 7.5 cm^{-1} for S-1, and 11 cm^{-1} for actin.

Fluorescent Labeling of S-1 and Actin with IAEDANS. S-1 (3 mg/mL) in 40 mM NaCl–5 mM NaH_2PO_4, pH 7.0, was reacted in the dark with a 20× molar excess of IAEDANS for 1 h at 0 °C (Duke et al., 1976). Unreacted IAEDANS was quenched by the addition of 10 mM DTE and then removed by passing the reaction mixture through two Sephadex G-50 centrifugation columns (Penefsky, 1977) and dialysis against the next buffer.

G-Actin (5 mg/mL) in 0.2 mM $CaCl_2$, 0.2 mM ADP, and 2 mM Tris, pH 7.6, was reacted in the dark with a 20× molar excess of IAEDANS for 90 min at 0 °C. The reaction was stopped, and the excess reagent was removed by the same procedure as described above for S-1.

The concentrations of the modified protein solutions were determined by the Lowry assay.

Cross-Linking of the Acto–S-1 Complex. Labeled or unlabeled S-1 and actin were cross-linked with EDC under two sets of reaction conditions.

Under one-step, pH 7.0 conditions (Sutoh, 1983), actin and S-1 were mixed at final concentrations of usually 0.5 mg/mL each and allowed to form the acto–S-1 complexes. After 30-min incubation time, EDC was added to 1 mg/mL to start the reaction. The cross-linking reaction was carried out in 0.1 M NaCl, 2 mM $MgCl_2$, and 10 mM imidazole, pH 7.0 (the pH 7 buffer).

Under two-step, pH 6.0 conditions (Mornet et al., 1981), actin (2.5 mg/mL) was first activated by EDC (3 mg/mL) for 2 min and then added in the second step of the reaction to 10× volume of 0.25 mg/mL S-1. Both actin and S-1 were equilibrated in 0.1 M MES, pH 6.0, buffer (the pH 6 buffer).

The molar ratio of actin to S-1 was 2:1 unless indicated otherwise.

Digestion of Cross-Linked Acto–S-1 with Elastase. After cross-linking for 2 h according to the one-step, pH 7.0 procedure, or for 20 min under the two-step, pH 6.0 conditions, the reactions were terminated by the addition of 10 mM β-mercaptoethanol. To remove the un-cross-linked S-1, 15 mM MgATP and 2 mM PP_i were added to the reaction mixture which was subsequently centrifuged at 40000 rpm for 1 h. The supernatant contained only S-1. The pellet containing isolated cross-linked acto–S-1 species was homogenized in the pH 7 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.

Cross-Linking of Tryptically Cleaved S-1. S-1 (3 mg/mL) in 0.1 M KCl and 50 mM Tris, pH 8.0, was digested by trypsin (0.03 mg/mL) for 45 min at 22 °C. Soybean trypsin inhibitor was added to 0.09 mg/mL to stop the digestion. Any modification of S-1 was done prior to this digestion. The tryptically cleaved S-1 was cross-linked to actin as described in the preceding section. The time course of cross-linking was followed by removing aliquots at various time points and quenching the reaction with β-mercaptoethanol. These samples were then analyzed on SDS–polyacrylamide gels. If separation of cross-linked and un-cross-linked tryptically cleaved S-1 was desired, the quenched aliquots were made up to 15 mM MgATP and 2 mM PP_i (pH 7.0) and then centrifuged and processed as described above.

Sodium Dodecyl Sulfate–Polyacrylamide 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 Measurements and Dye Staining Considerations. The optical densities of the Coomassie blue stained protein bands were determined with a Helena Quick Scan R&D gel scanner equipped with an integrator. 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 adsorption were corrected by dividing the normalized intensities by the molecular weights of the respective protein bands. Calibration curves indicated that the 20K fragment stains between 1.5- and 2.0-fold more intensly than the 50K peptide. The quantitation of the free 20K and 50K fragments formed in elastase digestions of cross-linked acto–S-1 included such staining correction. However, since parallel quantitations of the 20K–actin and 50K–actin products by dye staining and by monitoring the fluorescence of such products containing labeled actin agreed within 5–10%, the staining correction was not applied to the cross-linked materials. If used, this correction would reduce the rates of formation of the 20K–actin product (in Figures 4B,C and 5A,B) by about 20%. This would bring into closer agreement the rates of fragment decay and product formation reported in Table II. However, the correction would in general increase the difference between the fluorescence and Coomassie blue results to between 20% and 30%. In view of this, and because the calibration procedure involves some additional assumptions (e.g., stoichiometric equivalence and equal proteolytic stability of the 20K and 50K fragments), we have not applied the staining correction to the 20K–actin product in this work.

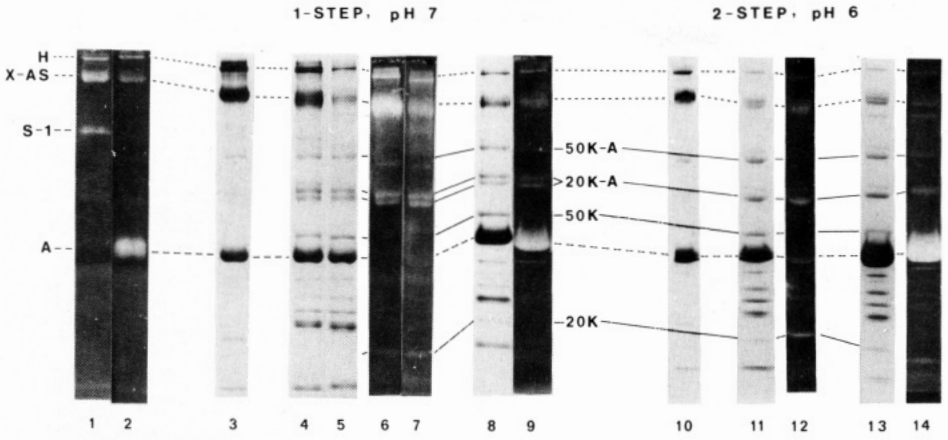

FIGURE 1: Representative electrophoretic patterns of acto–S-1 samples cross-linked by EDC and digested with elastase. Actin and S-1 were cross-linked by EDC according to the one-step, pH 7.0 procedure for 120 min (lanes 3–9) or under two-step, pH 6.0 conditions for 20 min (lanes 1, 2, and 10–14). The reaction mixtures were then centrifuged at 40 000 rpm for 1 h in the presence of 15 mM MgATP and 2 mM PP_i to remove any un-cross-linked S-1. The pellets containing cross-linked acto–S-1 were resuspended in 0.1 M NaCl and 10 mM imidazole, pH 7.0, and digested by elastase (Materials and Methods). Lanes 1 and 2 are the fluorescent gels of acto–S-1 samples after a 20-min cross-linking reaction under two-step, pH 6.0 conditions. Lane 1 contains IAEDANS-labeled S-1, and lane 2 contains IAEDANS-labeled actin. Identical gels for the one-step, pH 7.0 conditions are not shown. Lanes 3–7 contain IAEDANS-labeled S-1. Lane 3 shows the isolated, cross-linked acto–S-1 (one step, pH 7.0) prior to elastase digestion. Lanes 4 and 6 are the 20-min digestions, and lanes 5 and 7 are the 60-min digestions of the sample in lane 3. The faint band above the 20K–actin doublet in lane 4 corresponds to actin cross-linked to light chain 1. Lanes 8 and 9 are the 60-min digestions of cross-linked acto–S-1 (one step, pH 7.0) containing IAEDANS-labeled actin. Lane 10 shows isolated, cross-linked acto–S-1 (two steps, pH 6.0) prior to elastase digestion. Lanes 11 and 12 show the same 60-min digestions of the sample in lane 10 and contain IAEDANS-labeled S-1. Lanes 13 and 14 show the same 60-min digestion of cross-linked acto–S-1 (two steps, pH 6.0) containing labeled actin. H refers to high molecular weight cross-linked species; X-AS refers to the 180K doublet of cross-linked acto–S-1 (Mornet et al., 1981) and to its first cleavage product (cleaved at the 25K/50K junction of S-1); A corresponds to actin, and 50K–actin and 20K–actin correspond to actin cross-linked to the 50K and 20K fragments, respectively.

The apparent molecular weights of bands corresponding to cross-linked species were determined by comparing their mobilities on the SDS gels with those of protein standards of known molecular weights. To measure the distribution of fluorescence among different protein bands, the fluorescent gels were photographed under UV illumination, and the negatives were scanned with a Helena Quick scanner.

RESULTS

Stoichiometry of Cross-Linked Acto–S-1 Complexes. (a) Digestions with Elastase. The first approach toward analysis of the cross-linked acto–S-1 complexes involved their proteolytic digestion with elastase and subsequent examination of the resulting products on SDS–polyacrylamide gels. These experiments are based on the previous observation that elastase can cleave the 50K/22K junction in acto–S-1 complexes (Applegate & Reisler, 1983). It follows then that S-1 cross-linked to two actins and digested with elastase should yield only the 50K–actin and 22K-actin products, and no free 50K and 22K fragments (the 22K fragment produced by elastase corresponds to the 20K tryptic peptide in S-1). On the other hand, S-1 cross-linked to a single actin molecule and then digested with elastase should produce either the 50K–actin or the 22K–actin species and the free 22K or 50K fragments, respectively.

Representative results of elastase digestions carried out on acto–S-1 complexes cross-linked according to the one-step, pH 7.0 (Sutoh, 1983) and two-step, pH 6.0 (Mornet et al., 1981) procedures are shown in Figure 1. In both cases, the isolated cross-linked acto–S-1 complexes are predominantly in the form of 180K species although higher order products (e.g., 260K; Mornet et al., 1981) are apparent on the top of all lanes in Figure 1. Upon cleavage of the cross-linked acto–S-1 species, we note the formation of the 50K–actin and the 22K–actin products and the liberation of the free 50K peptides and a smaller amount of free 22K peptides (Figure 1). As judged by the time course of elastase digestion reactions (not shown) and the stability of the generated products, the optimal proteolysis time in these experiments is between 40 and 60 min. By this time, almost complete cleavage of the cross-linked acto–S-1 is achieved (Figure 1). The identification of the electrophoretic bands in Figure 1 has been achieved with the help of actin or S-1 labeled with IAEDANS. IAEDANS-labeled S-1 (on the 20K fragment) identifies the 22K–actin product and the free 22K peptide (lanes 6, 7, and 12 in Figure 1). Similarly, labeled actin serves to identify the 50K–actin and 22K–actin products.

Table I: Fractions of 22K and 50K Fragments Cross-Linked to Actin in EDC-Cross-Linked Acto–S-1[a]

reaction conditions	f_{22K}	f_{50K}
one step, pH 7.0		
S-1 + actin	0.88	0.39
S-1[b] + actin	0.87 (0.78)	0.26
S-1 + actin[b]	0.80	0.31
two steps, pH 6.0		
S-1 + actin	0.68	0.46
S-1[b] + actin	0.68 (0.67)	0.40
S-1 + actin[b]	0.67	0.45

[a] The calculated fractions of cross-linked fragments represent the distribution of the 22K and 50K peptides between the 22K–actin and 22K and between 50K–actin and 50K bands on SDS gels. These distributions were quantitated following an almost complete (40 or 60 min) digestion of cross-linked acto–S-1 samples. The data in parentheses were obtained by measuring the fluorescence intensity of the labeled 22K fragment in the 22K and 22K–actin bands. The data for unlabeled proteins represent an average over several independent reactions. Experimental variation in these experiments was ±10%. [b] IAEDANS-labeled proteins.

Visual comparison of the digestion products obtained by elastase cleavage of acto–S-1 cross-linked by either the one-step, pH 7.0 or the two-step, pH 6.0 procedures reveals only small differences between these systems. These include different ratios of the free 50K and 22K peptides (Figure 1), and the presence of a well-defined electrophoretic doublet for the 22K–actin complex in the one-step, pH 7.0 reaction (lanes 6,

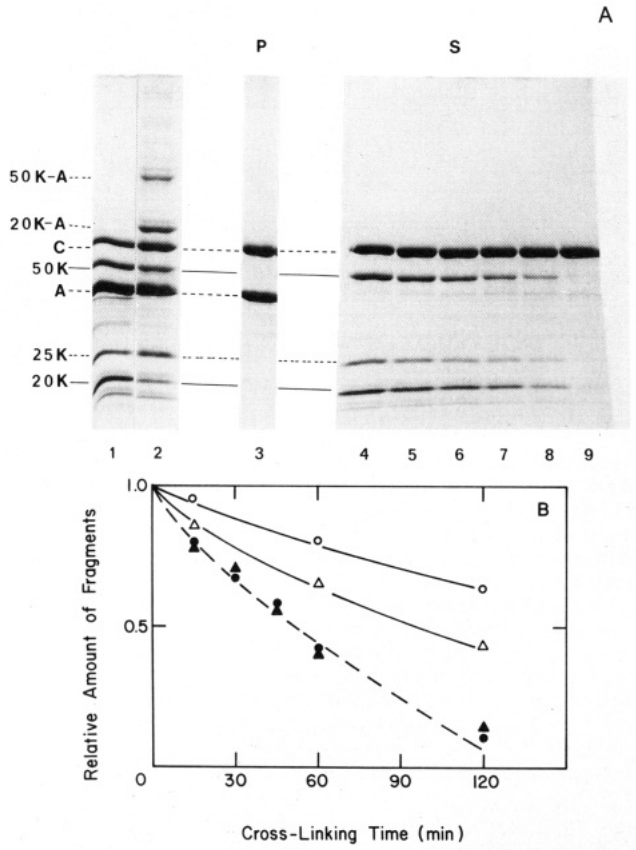

FIGURE 2: (A) Representative electrophoretic gels showing the time course of cross-linking actin to tryptically cleaved S-1. Actin and tryptically cleaved S-1 were cross-linked under one-step, pH 7.0 conditions. Aliquots were removed after 0, 15, 30, 45, 60, and 120 min, and the reaction was terminated with β-mercaptoethanol. Un-cross-linked tryptically cleaved S-1 was separated from cross-linked acto–S-1 by centrifugation of the reaction aliquots in the presence of 15 mM MgATP and 2 mM PP_i. Lanes 1 and 2 show the total reaction mixture after 0 and 120 min of cross-linking, respectively. A more detailed time profile of such a reaction is shown in Figure 3. Lane 3 shows the pellet of the sample in lane 1, and it demonstrates the complete separation of un-cross-linked S-1 fragments from actin by the employed separation procedure. Lanes 4–9 are the supernatants containing un-cross-linked S-1 obtained after centrifugation of the 0-, 15-, 30-, 45-, 60-, and 120-min aliquots of the cross-linking reaction. C refers to catalase and A to actin. Other symbols are the same as in Figure 1. (B) Comparison of the decay of the 50K and 20K fragments with the decay of un-cross-linked tryptically cleaved S-1 during the cross-linking reaction. The data for the 50K (○) and 20K (△) fragments were obtained by monitoring their decay in the total reaction mixture (as shown in lanes 1 and 2 of panel A) and in Figure 3). The data for the un-cross-linked tryptically cleaved S-1 were obtained by following the simultaneous and equimolar decay of the 50K (●), 20K (▲), and 25K fragments (not shown) in the supernatants shown in lanes 4–9 of (A). The dashed curve represents the sum of the individual decyas of the 50K and 20K fragments in the total reaction mixture (i.e., ○ + △). Thus, it is also the theoretical curve for the decay of S-1 fragments in the supernatants assuming independent and simultaneous cross-linking of actin to the 20K and 50K peptides.

7, and 9). The identity of this doublet is verified by its appearance on gels monitoring fluorescently labeled actin and S-1.

Quantitative analysis of the partitioning of the 22K and 50K peptides between their free fragment form and the 22K–actin and 50K–actin complexes is presented in Table I. These data summarize the distributions measured on gels shown in Figure 1 and on several other gels monitoring the elastase digestion of cross-linked unlabeled acto–S-1 complexes. Table I shows that while most of the 22K fragment is cross-linked to actin (about 85%), much of the 50K peptide (60–70%) is left un-

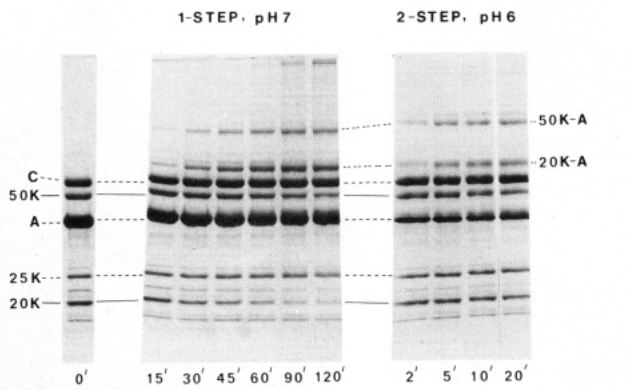

FIGURE 3: SDS–polyacrylamide gels showing the time course of cross-linking actin to tryptically cleaved S-1 under the one-step, pH 7.0 and two-step, pH 6.0 conditions. Reaction times in minutes are indicated under each lane. C refers to catalase, A refers to actin, and 50K–actin and 20K–actin correspond to actin cross-linked to the 50K and 20K fragments, respectively.

cross-linked. This difference in the cross-linking of the 22K and 50K peptides is less pronounced under the two-step, pH 6.0 reaction conditions, although the 22K fragment remains the preferred site of crosslinking to actin. In all reactions, the combined fractions of cross-linked 22K and 50K peptides ($f_{22K} + f_{50K}$) are between 1.0 and 1.25. This clearly indicates that 1:1 actin:S-1 stoichiometry is prevalant in the cross-linked acto–S-1 complexes. It is very likely that the digestion of the higher order species (e.g., the 260K cross-linked products) is responsible for the somewhat higher than expected sum total (1.0) of combined fractions of cross-linked peptides. The higher order cross-linked species may well contain complexes of 2:1 stoichiometry of actin to S-1.

(*b*) *Cross-Linking of Tryptically Cleaved S-1.* In order to check and confirm the results reported in the previous section, we have examined the time course of cross-linking the tryptically cleaved S-1 to actin. Such experiments provide two sets of data. The rates of cross-linking the 20K and 50K peptides to actin are derived by monitoring the decay in the intensity of the free fragments in the total reaction mixture as a function of cross-linking time (Figure 2). This method is demonstrated and used more extensively in the next section (Figure 3). The overall rate of S-1 cross-linking is determined on the isolated and un-cross-linked S-1 by measuring the simultaneous (and equal) decay in the intensity of all three fragments (25K, 50K, and 20K). In the initial stages of the reaction (one step, pH 7.0, up to 60 min), the rate of S-1 cross-linking is adequately described by the sum of the individual cross-linking reactions of the 20K and 50K fragments (Figure 2). This result again indicates that the initial cross-linking of acto–S-1 yields complexes of predominantly 1:1 protein stoichiometry. At prolonged reaction times (longer than 60 min), this correlation frequently breaks down, most likely because of secondary cross-linking reactions.

Rates of Cross-Linking the 20K and 50K Fragments in S-1 to Actin. Elastase digestions of acto–S-1 complexes revealed preferential cross-linking of actin to the 22K fragment in S-1, in particular in the one-step, pH 7.0 reaction (Table I). In order to verify this observation and to quantitatively compare the cross-linking of the 20K and 50K fragments to actin, we have followed the reactions of tryptically cleaved S-1 with actin. In a typical experiment shown in Figure 3, we monitor the decay in the intensity of the free 50K and 20K fragments due to their cross-linking to actin, as well as the formation of the 20K–actin and 50K–actin cross-linked products. These complexes migrate on SDS gels with the mobility of 63K and

Table II: Effects of pH and Actin Preactivation on the Course of Acto-S-1 Cross-Linking: Ratios of Reaction Rates and Reaction Products[a]

reaction conditions	$k_{20}:k_{50}$	$k_{20\text{-A}}:k_{50\text{-A}}$	20K-actin: 50K-actin
one step, pH 7.0 (Sutoh, 1983)	2.0–2.5	2.7–3.2 (2.9)	
two steps, pH 7.0			3.0–3.5
one step, pH 6.0	1.0–1.5		1.5–2.0
two steps, pH 6.0 (Mornet et al., 1981)			2.0–2.5

[a] The rate constants k_{20} and k_{50} were determined from the semilogarithmic plots of the time-dependent decay of the free 20K and 50K fragments during the cross-linking of actin to tryptically cleaved S-1 (e.g., Figure 4A). The rates of product formation, $k_{20\text{-A}}$ and $k_{50\text{-A}}$, which are a more direct measurement of the cross-linking of actin to the 20K and 50K fragments, were obtained from the initial slopes of product formation curves shown in Figure 4B. These data were not corrected for the more intense staining of the 20K than the 50K fragment (see text). The ratio in parentheses was obtained by measuring the fluorescence intensity of labeled actin in 20K-actin and 50K-actin bands. The range of values for the ratios of the respective rates or amounts of products corresponds to an experimental spread in several independent experiments (four reactions for the 1-step, pH 7.0 and the two-step, pH 6.0 conditions and two reactions for the one-step, pH 6.0 and the two-step, pH 7.0 conditions). Rates of reactions could not be reliably determined for the biphasic two-step reactions at pH 6.0 and 7.0. The 20K-actin:50K-actin ratios were calculated from the amounts of the 20K-actin and 50K-actin cross-linked products accumulated by the end of the respective reactions. These ratios were determined whenever direct measurements of $k_{20\text{-A}}$ and $k_{50\text{-A}}$ were impractical. They are not corrected for the different staining of the 20K and 50K fragments. Such a correction would decrease these ratios by about 20%.

98K species, and their identification relies on comparison with the migration of similar products obtained in reactions employing IAEDANS-labeled actin or S-1. It should be noted, however, that the modification of the SH_1 groups on cleaved S-1 decreases the rate of actin cross-linking to the 20K fragment.

In most of our kinetic work on the cross-linking of tryptically cleaved S-1 to actin, we have followed the one-step, pH 7.0 procedure. In such reactions, the decay in the intensities of the 50K and 20K fragments can be fitted by single exponentials (Figure 4) and yields the first-order rate constants for the cross-linking of these fragments (k_{20} and k_{50}). The semilogarithmic plots appear linear over the first 45–60 min of the reaction. Control cross-linkings of cleaved S-1 in the absence of actin do not show any significant loss of the free fragments or formation of any higher order products over the same period of time. It also appears that actin does not induce any measurable intramolecular cross-linking in S-1, as judged by the constant amount of free 25K fragment present on SDS gels over the first 60 min of the reaction.

In all experiments similar to those shown in Figures 3 and 4A, actin is cross-linked to the 20K fragment at a rate about 2-fold faster than to the 50K peptide (Table II). Parallel measurements of the rates of 20K-actin ($k_{20\text{-A}}$) and 50K-actin ($k_{50\text{-A}}$) formation through direct scanning of their respective bands show the same general pattern. The production of cross-linked species is linear with time over the first 40–60 min of the reaction, and the $k_{20\text{-A}}$ rates are about 3-fold faster than the values for $k_{50\text{-A}}$ (Figure 4B). For reasons discussed under Materials and Methods, these values were not corrected for the stronger staining of the 20K than the 50K fragment. If applied, such a correction would reduce the value of $k_{20\text{-A}}$ by 20%, and the respective ratios of $k_{20\text{-A}}:k_{50\text{-A}}$ in Table II would range between 2.2 and 2.6. Thus, irrespective of the staining correction, the two ways of monitoring the kinetics of the cross-linking reaction lead to the same conclusion about

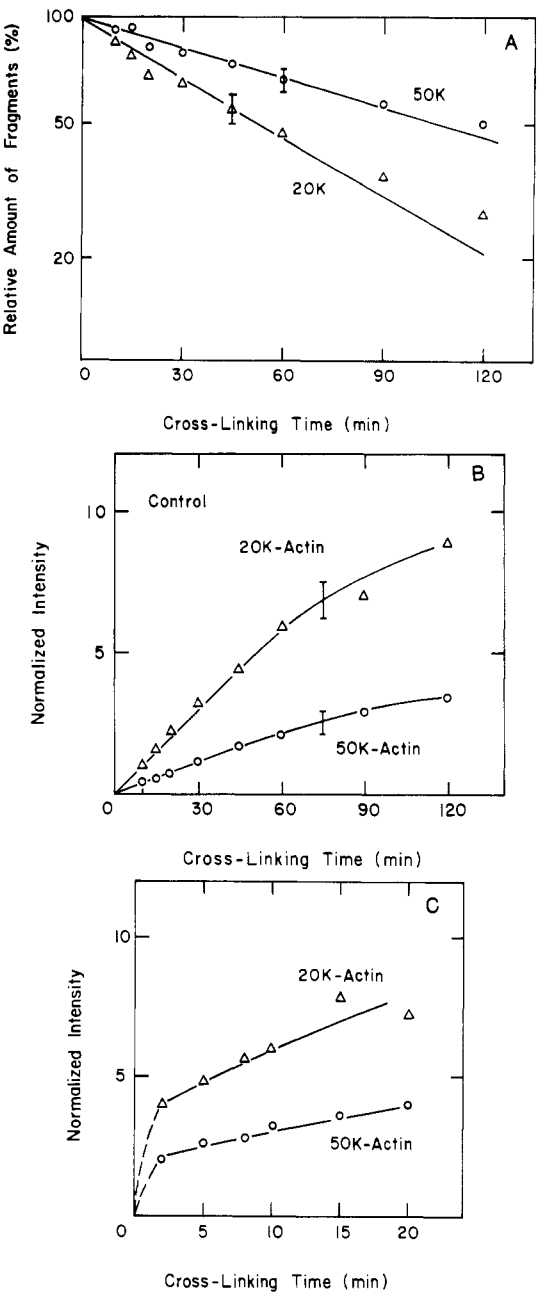

FIGURE 4: (A) Time course of decay of the 50K (○) and 20K (△) fragments in the one-step, pH 7.0 cross-linking reactions. The decay data were obtained from SDS gels similar to those shown in Figure 3. The bars indicate experimental error. $k_{50K} = 0.40 \pm 0.04\ h^{-1}$; $k_{20K} = 0.85 \pm 0.10\ h^{-1}$. (B) Time course of formation of the cross-linked 50K-actin (○) and 20K-actin (△) products in the one-step, pH 7.0 reaction. The cross-linked products were quantitated as described under Materials and Methods, without correcting for the more intense staining of the 20K fragment, and are shown here in arbitrary units. (C) Time course of formation of the cross-linked 50K-actin (○) and 20K-actin (△) products in the two-step, pH 6.0 reaction.

preferential cross-linking of actin to the 20K fragment in S-1.

The kinetic analysis of acto-S-1 cross-linking cannot be done equally well for reactions carried out according to the two-step, pH 6.0 procedure. As shown in Figure 4C, the initial rapid formation of the 20K-actin and 50K-actin products (in the first 2 or 4 min) is followed by a second phase in which they accumulate very slowly. The sharply biphasic product formation appears to result from using EDC-preactivated actin in these reactions. Decay curves for the free 20K and 50K fragments are similarly biphasic (not shown) and do not show any significant disappearance of these peptides after the initial few minutes into the cross-linking reaction. Although neither

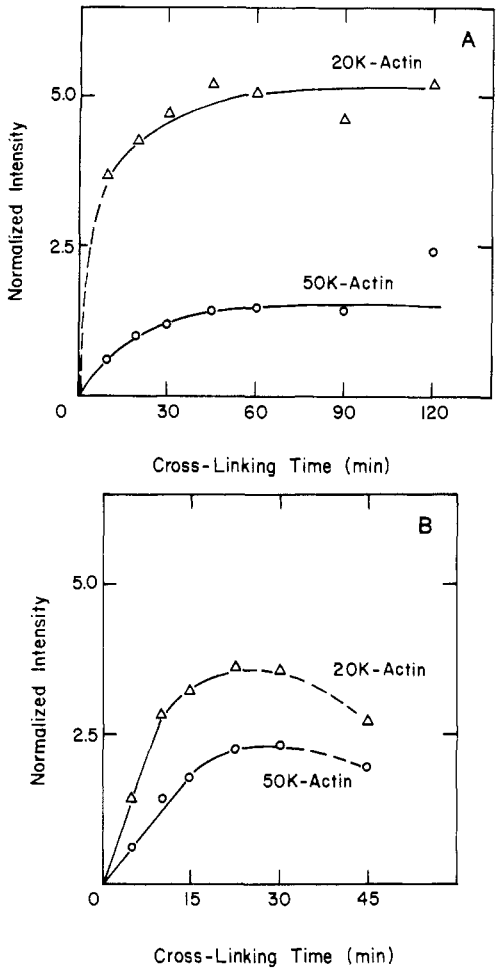

FIGURE 5: (A) Time course of formation of the cross-linked 50K–actin (○) and 20K–actin (△) products in the two-step, pH 7.0 reaction. This cross-linking reaction involved the addition of EDC-preactivated actin to S-1 equilibrated in 0.1 M NaCl, 2 mM $MgCl_2$, and 10 mM imidazole, pH 7.0, buffer. (B) Time course of formation of the cross-linked 50K–actin (○) and 20K–actin (△) products in the one-step, pH 6.0 reaction. This cross-linking reaction was initiated by the addition of EDC to acto–S-1 complex preformed in 0.1 M MES, pH 6.0, buffer.

k_{20} and k_{50} nor $k_{20\text{-A}}$ and $k_{50\text{-A}}$ can be confidently determined for the two-step, pH 6.0 cross-linking reactions, nevertheless, the 20K fragment seems to again be the primary cross-linking target. This is deduced from the fact that the amount of the 20K–actin product is between 2- and 2.5-fold greater than that of the 50K–actin material (Table II).

Comparison of Methodologies. The differences observed in the cross-linking of cleaved S-1 to actin in the one-step, pH 7.0 and two-step, pH 6.0 procedures merit some attention, in particular to assist in selecting the optimal reaction conditions for future studies on the acto–S-1 interface. As shown in the previous section, the cross-linking of S-1 with preactivated actin at pH 6.0, although fast, is hardly suitable for kinetic studies. The somewhat lower ratio in this reaction of the 20K–actin to 50K–actin product appears to be due to the pH conditions, rather than the preactivated state of actin. Thus, for example, when preactivated (two steps) and nonactivated actins (one step) are cross-linked to S-1 at the same pH, the ratios of the generated products are about the same for both reactions (Table II). However, since the two-step cross-linking at pH 7.0 yields clearly biphasic profiles for product formation (Figure 5A) and the decay of the free 50K and 20K fragments, it appears less useful than the one-step, pH 7.0 cross-linking procedure.

Although the cross-linking with EDC is faster at pH 6.0 than at pH 7.0, the one-step reaction at the lower pH has some drawbacks. First, the decrease in the amount of the 20K–actin and 50K–actin products at the later time points (Figure 5B) suggests their incorporation into higher order species. In addition, consistent with the results of the two-step, pH 6.0 reactions, the difference between the cross-linking of actin to the 20K and 50K peptides is reduced compared to similar reactions at pH 7.0 (Table II).

In contrast to the above reservations about cross-linkings at pH 6.0, or the two-step reaction at pH 7.0, the one-step cross-linking at pH 7.0 is well suited for kinetic analysis. The k_{20}, k_{50}, $k_{20\text{-A}}$, and $k_{50\text{-A}}$ rates derived from such reactions are surprisingly reproducible. Their ratios, k_{20}:k_{50} and $k_{20\text{-A}}$:$k_{50\text{-A}}$, remain close to 3:1 even when the individual rates are varied by the imposed changes in the experimental conditions. Thus, by increasing the EDC concentration (to 2 mg/mL) to increase the cross-linking rates, and by reducing the standard 2:1 ratio of actin to S-1 to 1:1 and 0.5:1 in order to decrease the reaction rates, a 5-fold range of cross-linking rates has been probed. Over this range, neither k_{20}:k_{50} nor $k_{20\text{-A}}$:$k_{50\text{-A}}$ shows any significant changes. This is particularly helpful for probing the acto–S-1 interface in the presence of nucleotides. Such cross-linking experiments are now in progress in our laboratory.

Discussion

One of the main objectives of this study was to reexamine the stoichiometry of the chemically cross-linked complexes of acto–S-1. The original finding of Mornet et al. (1981) on the simultaneous and high-yield cross-linking of actin to both the 20K and 50K fragments on S-1 suggests the potential for functional binding of two molecules of actin to each myosin head (Mornet et al., 1981). Such an interpretation of the cross-linking experiments paves the way for various schemes of cross-bridge action based on transitions between myosin heads attached to two and one actins (Goody & Holmes, 1983).

The results of this study confirm the basic observation of Sutoh (1983) on the 1:1 stoichiometry of acto–S-1 complexes cross-linked with carbodiimide. We reach the same conclusion irrespective of the reaction conditions employed to fix the protein complex, i.e., using both the two-step, pH 6 (Mornet et al., 1981) and the one-step, pH 7.0 (Sutoh, 1983) cross-linking procedures. Moreover, we reach this conclusion by employing two independent methods to quantitate the cross-linked acto–S-1 fragments. In the first approach, digestions of the isolated and cross-linked acto–S-1 with elastase led to a direct measurement of the cross-linked and un-cross-linked fractions of the 20K and 50K fragments. We show that for any preparation or cross-linking reaction, the overall sum of the cross-linked 20K and 50K fragments is as expected for a 1:1 complex of actin and S-1.

Two features of the above-mentioned experiments merit some attention. First, the isolated, cross-linked acto–S-1 complexes invariably contain small amounts of higher order cross-linked species (including the 260K; Mornet et al., 1981). Their presence in our samples is a direct consequence of driving the cross-linking reactions to completion. Only then can the cross-linked acto–S-1 be viewed as representative of the entire S-1 population. Although the higher order cross-linked species may well represent 2:1 complexes of actin and S-1, and are most likely responsible for the increased fractions of cross-linked 20K and 50K fragments, they have not been analyzed in any detail. Such analysis is forgone since the significance of the secondary reactions yielding the 260K species is unknown at present. The second feature of this set of cross-

linking experiments is that the respective ratios of 20K–actin and 50K–actin products are unchanged with time of digestion, in spite of the gradual accumulation of all proteolytic products. This ensures an unbiased analysis of the entire acto–S-1 sample.

Because of the small and yet likely contribution of the higher order species to the above analysis, we have verified the stoichiometry of the cross-linked acto–S-1 complexes in a set of independent experiments. The observation that the rate of cross-linking of tryptically cleaved S-1 to actin can be fitted very well by the sum of the cross-linking rates of the two fragments (Figure 2). is consistent with their independent and mutually exclusive cross-linking to actin. In addition, these experiments show that intact S-1 and tryptically cleaved S-1 yield similar information on the nature and stoichiometry of the cross-linked products.

The second conclusion of this study is that the two cross-linking reactions of actin to the 20K and 50K fragments are nonequivalent. Under all conditions, the 20K fragment is the preferred site of cross-linking, although the actual differences between the reactions of the 20K and 50K fragments depend to some extent on the employed experimental procedures and the application of dye-staining correction. The preferential cross-linking of actin to the 20K unit in intact S-1 is clearly seen in the elastase-digested samples of acto–S-1. The analysis and comparison of such samples with actin cross-linked to trypsin-treated S-1 verify that the cleavage of S-1 has only a small, if any, effect on the inherent differences in the cross-linking of the 20K and 50K fragments. Thus, comparative quantitation of the two cross-linking reactions has been done for the complexes of actin with tryptically cleaved S-1, for which the entire reaction course can be followed in kinetic terms. Nevertheless, the relevant rate constants are derived from the initial phase of the cross-linking reactions, thus limiting any contributions due to secondary and nonspecific events. Also, we put more trust in the measurements of the formation of the cross-linked 20K–actin and 50K–actin products rather than in the decay rates of the free fragments. The latter rates may be affected in some cases by reactions producing the higher order species seen on SDS gels. The optimal conditions for monitoring the cross-linking of actin to S-1 are those of the one-step, pH 7.0 reactions. Their initial smooth and monophasic progress leads to reliable determinations of reaction rates, as demonstrated by the closely similar k_{20}:k_{50} and $k_{20\text{-A}}$:$k_{50\text{-A}}$ ratios. Other experimental conditions, including the two-step, pH 6.0 reactions, are less suitable for following the cross-linking of actin to S-1. Nevertheless, it can be concluded that in general the rate of cross-linking actin to the 20K fragment is between 2- and 3-fold faster than that to the 50K fragment.

Perhaps the most important and intriguing question with respect to the cross-linking experiments is whether they indeed monitor binding affinities of actin to the 20K and 50K fragments of S-1. The fact that under all experimental conditions, and over a severalfold range of reaction rates, the cross-linking of actin is primarily directed at the 20K fragment strongly suggests that this fragment contains the high-affinity binding site for actin. At least two independent lines of evidence support such a contention. First, the cross-linking of the reactive SH_1 and SH_2 groups on the 20K peptide, and the resulting changes in this portion of S-1, decreases the affinity of actin for S-1 by several orders of magnitude (Burke et al., 1976; Chalovich et al., 1983). Also, the binding of nucleotides to S-1, which decreases its affinity for actin, is known to affect the SH_1–SH_2 peptide on the 20K fragment. Such effects are not restricted to changes in the reactivity of these cysteines (Yamaguchi & Sekine, 1966; Reisler et al., 1974; Wells et al., 1980) but include their motions with respect to each other (Burke & Reisler, 1977; Dalbey et al., 1983). In other words, nucleotides may weaken the acto–S-1 interaction by causing conformational changes in the 20K fragment.

Second, more direct support for the results of our cross-linking experiments comes from a recent study of Muhlrad & Morales (1984). These authors have separated and renatured the 20K and 50K fragments of S-1 and find that the former has a high binding constant for actin (at least 10^{-6} M), whereas the latter shows only a weak affinity for actin. Thus, it appears that the preferential cross-linking of actin to the 20K fragment is a direct consequence of their strong binding to each other.

The molecular interpretation of the independent cross-linking of actin to the 20K and 50K fragments on S-1 is by no means straightforward. The simplest view of such results is that actin can occupy only one of its two binding sites on S-1, either on the 20K peptides or on the 50K peptides. Since these peptides may represent separate structural domains (Karn et al., 1983; Applegate & Reisler, 1983; Mornet et al., 1984; Muhlrad & Morales, 1984), the extent of steric overlap between such putative sites is hard to predict. However, neither the binding of actin to separated S-1 fragments (Muhlrad & Morales, 1984) nor the cross-linking studies can precule a more traditional model of acto–S-1 that involves a single binding site for actin generated by strong and weak interactions of this protein with the 20K and 50K fragments, respectively. It is possible that some protein–protein contacts in this interdomain binding site are differentially affected by nucleotides, which would allow for conformational and structural transitions in acto–S-1. This view of the acto–S-1 complex can be reconciled with the results of carbodiimide cross-linking by postulating *alternate* interactions of the presumably flexible N-terminal portion of actin with the S-1 fragments. Such speculation is consistent with the assignment by Sutoh (1983) of the same N-terminal residues on actin as the site of cross-linking to both the 20K and the 50K fragments. It implies that carbodiimide cross-linking of acto–S-1 detects only one or two out of several contact areas between these proteins. Further work is necessary to resolve between the different possible models of acto–S-1 binding.

In conclusion, our results suggest that the predominant stoichiometry of the cross-linked acto–S-1 complexes is 1:1, with the 20K fragment being the preferred site for binding and cross-linking to actin. We also show that under appropriate reaction conditions, the cross-linking methods can provide a quantitative tool for probing the acto–S-1 interface.

After submission of this work for publication and using approaches different from ours, Greene (1984) and Heaphy & Tregear (1984) reported on a similar finding that one S-1 is cross-linked to one F-actin monomer.

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Drug–Protein Interactions: Binding of Chlorpromazine to Calmodulin, Calmodulin Fragments, and Related Calcium Binding Proteins†

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ABSTRACT: The quantitative binding of a phenothiazine drug to calmodulin, calmodulin fragments, and structurally related calcium binding proteins was measured under conditions of thermodynamic equilibrium by using a gel filtration method. Plant and animal calmodulins, troponin C, S100α, and S100β bind chlorpromazine in a calcium-dependent manner with different stoichiometries and affinities for the drug. The interaction between calmodulin and chlorpromazine appears to be a complex, calcium-dependent phenomenon. Bovine brain calmodulin bound approximately 5 mol of drug per mol of protein with apparent half-maximal binding at 17 μM drug. Large fragments of calmodulin had limited ability to bind chlorpromazine. The largest fragment, containing residues 1–90, retained only 5% of the drug binding activity of the intact protein. A reinvestigation of the chlorpromazine inhibition of calmodulin stimulation of cyclic nucleotide phosphodiesterase further indicated a complex, multiple equilibrium among the reaction components and demonstrated that the order of addition of components to the reaction altered the drug concentration required for half-maximal inhibition of the activity over a 10-fold range. These results (1) confirm previous observations using immobilized phenothiazines [Marshak, D. R., Watterson, D. M., & Van Eldik, L. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6793–6797] that indicated a subclass of calcium-modulated proteins bound phenothiazines in a calcium-dependent manner, (2) demonstrate that the interaction between phenothiazines and calmodulin is more complex than previously assumed, and (3) suggest that extended regions of the calmodulin molecule capable of forming the appropriate conformation are required for specific, high-affinity, calcium-dependent drug binding activity.

The calcium-modulated proteins are a class of proteins that bind calcium ions reversibly at physiological ionic strength and pH (Van Eldik et al., 1982). Members of this class include calmodulin, troponin C, parvalbumin, S100α, S100β, intestinal calcium binding protein, and myosin light chains. We previously demonstrated (Marshak et al., 1981) that a subclass of calcium-modulated proteins interacts with immobilized phenothiazines in a calcium-dependent manner. These results suggested that calmodulin, troponin C, S100α, and S100β contain similar structural domains for drug binding. Phenothiazines also inhibit the calcium-dependent activation of enzymes by calmodulin [for a recent review, see Klee & Vanaman (1982)] and therefore are useful probes of the rela-

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