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Identification of Two Segments, Separated by ~45 Kilodaltons, of the Myosin Subfragment 1 Heavy Chain That Can Be Cross-Linked to the SH-1 Thiol†

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ABSTRACT: The thiol-specific photoactivatable reagent 4-(2-iodoacetamido)benzophenone (BPIA) can be selectively incorporated into the SH-1 of myosin subfragment 1 (S1), and upon photolysis an intramolecular cross-link is formed between SH-1 and the N-terminal 25-kDa region of S1. If a Mg^{2+}–nucleotide is present during photolysis, cross-links can be formed either with the 25-kDa or with the central 50-kDa region [Lu, R. C., Moo, L., & Wong, A. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6392–6396]. Heavy chains with these two types of intramolecular cross-links and un-cross-linked heavy chain have different mobility on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels and therefore can be purified electrophoretically. Each type of heavy chain was cleaved with *Staphylococcus aureus* protease, chymotrypsin, or lysyl endopeptidase. The cleavage points were determined on the basis of the molecular weights of peptides containing the N-terminus, which was identified with the use of an antibody. Locations of the cross-links were deduced by comparing the peptide maps of cross-linked and un-cross-linked heavy chains. The results indicate that the segment located about 12–16 kDa from the N-terminus of the heavy chain can be cross-linked to SH-1 via BPIA independently of the presence of a nucleotide, whereas the segment located 57–60 kDa from the N-terminus can be cross-linked to SH-1 only in the presence of a Mg^{2+}–nucleotide. With use of the avidin–biotin system, it has been shown that SH-1 is located 13 nm from the head/rod junction [Sutoh, K., Yamamoto, K., & Wakabayashi, T. (1984) *J. Mol. Biol. 178*, 323–339]. Since BPIA spans less than 1 nm, our results show that two regions, separated by ~400 amino acid residues and located in the 25- and 50-kDa domains of S1, respectively, are also part of the head structure about 12–14 nm from the head/rod junction.

The head portion of myosin, subfragment 1 (S1),[1] is a key component of the machinery of muscle cells that converts the chemical energy of ATP to mechanical energy. The heavy chains of S1 can be nicked with proteolytic enzymes into three distinct fragments with apparent molecular masses of 50, 25, and 20 kDa on SDS–PAGE (Balint et al., 1975). The nicked S1 retains the K^+- and Ca^{2+}-ATPase activities and the ability to bind actin. The most reactive thiol, SH-1, is located in the C-terminal 20-kDa region (Lu et al., 1978; Balint et al., 1978; Gallagher & Elzinga, 1980); the modification of SH-1 affects the ATPase activities of S1 (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966), but a direct involvement of SH-1 in nucleotide binding has been excluded (Burke & Reisler, 1977;

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[1] Abbreviations: S1, subfragment 1; BPIA, 4-(2-iodoacetamido)-benzophenone; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; kDa, kilodalton(s); $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LEP, lysyl endopeptidase; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Wells & Yount, 1979; Wells et al., 1980; Tao & Lamkin, 1981; Perkins et al., 1984; Botts et al., 1984). It has been shown that some nucleotide analogues bind to the N-terminal 25-kDa region (Szilagyi et al., 1979; Okamoto & Yount, 1985) whereas others bind to the central 50-kDa region (Mahmood & Yount, 1984; Hiratsuka, 1985). Several groups have reported that actin preferentially cross-links to the 20-kDa region and to a lesser extent to the 50-kDa region (Yamamoto & Sekine, 1979; Monet et al., 1981; Sutoh, 1983; Chen et al., 1985). The view that the three proteolytic fragments correspond to three independently folded domains appears to be attractive but awaits definitive evidence. Using biotinylated thiol reagent and a nucleotide analogue, Sutoh and his colleagues have demonstrated by electron microscopy that the SH-1 and nucleotide binding sites are located 13 and 14 nm from the S1/rod joint, respectively, and probably are on opposite sides of S1 (Sutoh et al., 1984, 1986).

It has been shown recently that the photoactivatable reagent 4-(2-iodoacetamido)benzophenone (BPIA) can be selectively incorporated into SH-1 in the absence of nucleotide. Upon photolysis, an intramolecular cross-link is formed between SH-1 and the 25-kDa region. Further, if a Mg^{2+}–nucleotide is present during photolysis, the cross-link can be formed either to the 25-kDa region or to the 50-kDa region (Lu et al., 1986). In this paper we report on the locations of two regions containing the cross-linking sites in the primary structure and draw some conclusions about the folding of the S1 heavy chain in the tertiary structure. The use of an antibody specific for the N-terminus of myosin heavy chain and comparison of peptide maps of the heavy chains with and without cross-links led to the identification of two segments that can be cross-linked to SH-1, one located in the 12–16-kDa region and the other in the 57–60-kDa region from the N-terminus of myosin heavy chain.

Materials and Methods

Proteins and Reagents. Myosin was prepared from rabbit back muscle, and S1 was made by chymotrypsin digestion according to Weeds and Pope (1977). BPIA was purchased from Molecular Probes (Junction City, OR). *N*-[7-(Dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) was from Wako Chemical Co. (Tokyo, Japan). Vectastain and a biotinylated anti-rabbit IgG antibody were purchased from Vector Laboratories (Burlingame, CA). Protease (*Staphylococcus aureus*, strain V8), chymotrypsin, and lysyl endopeptidase were purchased from Miles Scientific (Naperville, IL), Sigma (St. Louis, MO), and Wako Chemical Co. (Tokyo, Japan), respectively.

A polyclonal antibody against the N-terminus of the myosin heavy chain was raised as follows. A peptide with the sequence Ac-Ser-Ser-Asp-Ala-Asp-Met-Ala-Val-Lys was synthesized by the solid-phase method (Stewart, 1969). The sequence of this peptide corresponds to the first eight residues of the N-terminal region of the myosin heavy chain (Starr & Offer, 1973; Tong & Elzinga, 1984) except that a lysine residue was added at the C-terminus. Keyhole limpet hemocyanin (KLH) was coupled to the lysine residue of the synthetic peptide by glutaraldehyde (Bulinski et al., 1983). The epitope density in the KLH–peptide complex was 10. Antibody was raised in New Zealand White rabbits as previously described (Bulinski et al., 1983). The titer of the antibody was estimated to be 10^5 by the enzyme-linked immunosorbant assay (ELISA). Details of the peptide synthesis and the characterization of the antibody will be published elsewhere (Sutoh et al., 1987).

Modification and Photolysis of S1 with BPIA. SH-1 was modified at a 1:1.5 molar ratio of S1 to BPIA in a buffer containing 40 mM KCl and 20 mM sodium phosphate, pH 7.0. The reaction was allowed to proceed for 30 min at room temperature in the dark and then stopped by adding 2-mercaptoethanol (4 mol/mol of BPIA). The modified protein was dialyzed overnight against 40 mM KCl and 10 mM Hepes, pH 7.0, at 4 °C in the dark. Photolysis was carried out for 10 min at 4 °C in a Rayonet RPR-100 photochemical reactor as described earlier (Lu et al., 1986).

Purification of Cross-Linked Heavy Chains. The BPIA-modified S1 was divided into three parts: one part was used unphotolyzed (U), the second part was photolyzed without the addition of any nucleotide (P^-), and the third one was photolyzed in the presence of 1 mM Mg^{2+}-ATP (P^+). All three samples were denatured in 1% $NaDodSO_4$ and 62.5 mM Tris-HCl, pH 6.8, and then labeled with DACM at an equal molar ratio of S1. The reaction was carried out for 2 h at room temperature and then quenched by the addition of 2-mercaptoethanol to 2% (v/v). The DACM-labeled samples were electrophoresed on slab gels [2 mm thick, 10% acrylamide and 0.25% bis(acrylamide)] in the presence of $NaDodSO_4$ according to the procedure of Laemmli (1970). The fluorescent-labeled polypeptide chains were visualized by illumination with a UV lamp. The bands corresponding to two types of cross-linked (X1 and X2) and un-cross-linked heavy chains were cut out from gels and eluted with an electroelution apparatus. Each of the eluted samples was subjected to $NaDodSO_4$–PAGE again to show their purity.

Peptide Mappings. Five micrograms of each of the purified heavy chains (1 mg/mL in 1% $NaDodSO_4$, 1% 2-mercaptoethanol, 25 mM Tris, and 250 mM glycine, pH 8.8) was loaded in the wells of a slab gel [12.5% acrylamide and 0.3% bis(acrylamide)], and then 20 ng of the enzyme, V8 protease, chymotrypsin, or lysyl endopeptidase, was added to each well. The electrophoresis was performed in a normal manner except that the current was turned off for 20 min after the peptides and proteases entered the stacking gel, allowing the enzymatic digestion to proceed (Cleveland et al., 1977).

Fragments of the heavy chains on the gel were transferred onto a Durapore membrane (type GV, Millipore) electrophoretically (Towbin et al., 1979). Fragments containing the N-terminus of the heavy chain were selectively visualized on the membrane as follows. The membrane was washed twice with a solution containing 0.15 M NaCl, 20 mM sodium phosphate, pH 7.0, and 0.05% Tween 20 (TPBS) for 15 min and then incubated with the antiserum against the N-terminus of the heavy chain for 1 h; the antiserum had been diluted 200-fold wiith TPBS. The membrane was washed 3 times with TPBS for 5 min and then incubated with a secondary antibody, a biotinylated anti-rabbit IgG, followed by treatment with an avidin–peroxidase complex (Vectastain) and color development with 4-chloronaphthol as a chromogen (Sutoh & Mabuchi, 1986).

Miscellaneous Procedures. Apparent molecular weights of peptides were determined on the basis of the mobilities of the following markers: myosin (200 000), β-galactosidase (116 000), phosphorylase *b* (97 400), bovine serum albumin (66 000), the 50-kDa fragment of the S1 heavy chain, egg albumin (45 000), actin (42 000), aldolase (40 000), carbonic anhydrase (29 000), the 25-kDa fragment of the S1 heavy chain, and molecular weight standards (17 000, 14 400, 8200, 6200, and 2500) from BDH Chemicals Inc., West Germany.

Results

Purification of Heavy Chains of S1 with Intramolecular Cross-Links. Previous studies have shown that the mobility of the heavy chain with an intramolecular cross-link between

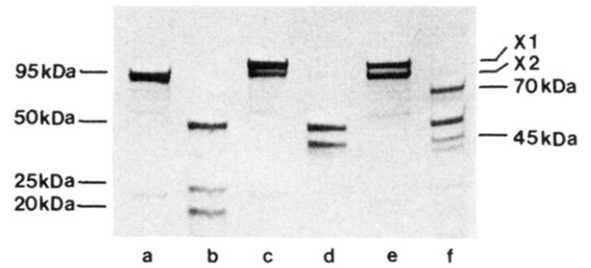

FIGURE 1: $NaDodSO_4$–PAGE of unphotolyzed S1 (a and b), S1 photolyzed in the absence of a nucleotide (c and d), and S1 photolyzed in the presence of 1 mM Mg^{2+}-ATP (e and f). Lanes a, c, and e are intact S1, and lanes b, d, and f are S1 nicked with trypsin for 30 min at an enzyme to S1 ratio of 1:50 by weight. Gel electrophoresis was carried out with a home-made minigel apparatus. The gel contained 7.5% acrylamide and 0.2% bis(acrylamide); the gel was run in 0.1% $NaDodSO_4$ and 0.1 M Tris–0.1 M bicine buffer (Lu et al., 1986).

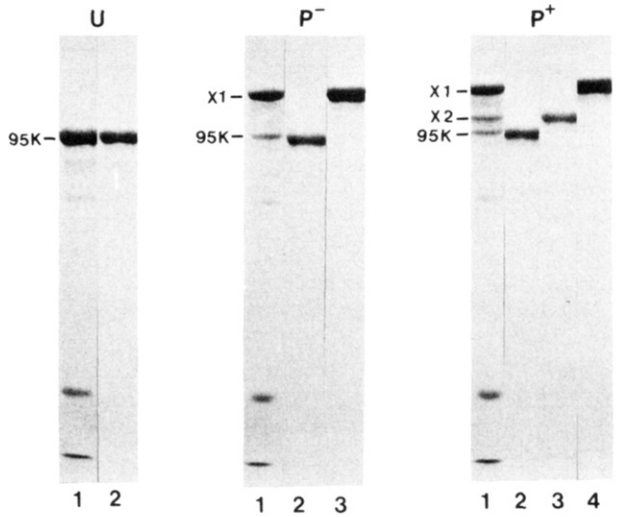

FIGURE 2: Gel electrophoresis of BPIA-modified and photo-cross-linked S1 and purified heavy chains according to the procedure of Laemmli (1970). Samples are grouped into three panels, and U, P^-, and P^+ stand for unphotolyzed, photolyzed in the absence of nucleotide, and photolyzed in the presence of 1 mM Mg^{2+}-ATP, respectively. For all three panels, (lane 1) S1 samples and (lane 2) heavy chains with an apparent molecular mass of 95 kDa purified from S1 shown in lane 1. (Lane 3 in panel P^-) X1-type heavy chains purified from S1 photolyzed in the absence of nucleotide (P^--X1); (lane 3 in panel P^+) X2-type heavy chains purified from S1 photolyzed in the presence of nucleotide; (lane 4 in panel P^+) X1-type heavy chains purified from S1 photolyzed in the presence of nucleotide (P^+-X1).

SH-1 and the 25-kDa region (designated X1) is lower than that of the un-cross-linked heavy chain. The heavy chain with a cross-link between SH-1 and the 50-kDa region (designated X2), however, comigrates with the un-cross-linked heavy chain [Figure 1 and references cited in Lu et al. (1986)]. With the gel system used in the present study, both types of cross-linked heavy chains are well resolved from the un-cross-linked heavy chain (Figure 2, P^+1). Heavy chains with and without intramolecular cross-links were eluted, and their purity was assured by their homogeneity on SDS–PAGE (Figure 2, U2, P^-3, P^+3, and P^+4). Since it is unclear whether or not the cross-link formed between SH-1 and the 25-kDa region in the presence of Mg^{2+}-ATP is the same as the one formed in the absence of a nucleotide, X1 was purifed by both procedures (Figure 2, P^-3 and P^+4).

Strategy for Identifying the Regions That Are Cross-Linked to SH-1. With a probe attached either at the N- or C-terminus, peptide mapping techniques have been used successfully to determine the intermolecular cross-linking sites between actin and actin binding proteins (Sutoh, 1982a,b, 1984; Sutoh & Mabuchi, 1984, 1986; Sutoh & Hatana, 1986). Taking advantage of the facts that SH-1 is located at the C-terminal region of the S1 heavy chain, that cross-links are formed at the N-terminal side of SH-1, and that an antibody that recognizes the N-terminus of the S1 heavy chain is available, we employed a similar peptide mapping method to identify the segments of S1 heavy chains that are involved in cross-linking with the SH-1 thiol.

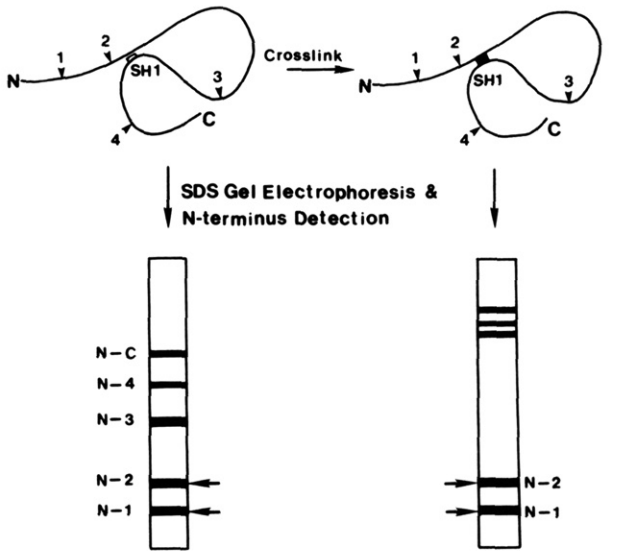

FIGURE 3: Schematic representation of peptide mapping technique. The two upper drawings represent intact S1 heavy chains before (left) and after (right) photo-cross-linking. arrowheads on the heavy chains indicate cleavage points by a proteolytic enzyme. The two lower drawings show the peptide patterns on $NaDodSO_4$ gels after the heavy chains have been partially cleaved, and only those peptides with the N-terminus of the heavy chain were detected by use of an antibody. N-1, N-2, N-3, N-4, and N-C represent peptides starting from the N-terminus and ending at point 1, 2, 3, and 4 and the C-terminus, respectively. Peptides appearing in the digests of both un-cross-linked and cross-linked samples are indicated by arrows.

The rationale of the method is described by the following hypothetical example in which there are four sites susceptible to a proteolytic enzyme (Figure 3, arrowheads 1–4). Partial enzymatic cleavage of the un-cross-linked heavy chain will generate five peptides, N-1, N-2, N-3, N-4, and N-C, that start from the N-terminus. They can be visualized with the use of an antibody that recognizes the N-terminus, and locations of the cleavage sites can be determined on the basis of the apparent molecular weights of the fragments. If SH-1 is located between the third and fourth cleavage point and if it is cross-linked to a segment between the second and third cleavage point, on partial cleavage of the cross-linked heavy chain, two fragments (Figure 3, N-1 and N-2) could appear in the same positions as in the case of the un-cross-linked heavy chain. All other fragments containing the segment between the second and third cleavage point (Figure 3, N-3, N-4, and N-C) will have a lower mobility because they have an additional mass resulting from cross-linking to the segment containing SH-1; some of them may happen to have a mobility similar to that of the larger fragments produced in the un-cross-linked sample. Thus the segment involved in cross-linking is that between the cleavage point producing the largest unperturbed peptide and the next point of cleavage that would produce the smallest fragment among the missing ones. In addition, it is possible to narrow down the size of the segments that contain cross-linking sites by using maps resulting from different kinds of enzymatic cleavage.

Mapping of Cross-Linked and Un-Cross-Linked Heavy Chains. Six types of heavy chains (U-95kDa, P^--95kDa,

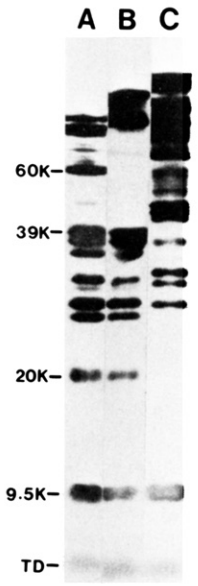

FIGURE 4: Peptide maps of un-cross-linked and cross-linked heavy chains derived from the digestion with V8 protease on NaDodSO$_4$–PAGE. (Lane A) Unphotolyzed heavy chains; (lane B) heavy chain with X2-type intramolecular cross-link; (lane C) heavy chain with X1-type intramolecular cross-link. Note that only peptides wiih the N-terminus of the heavy chain are visualized here. The band with the slowest mobility in lane A corresponds to the intact heavy chain, 95 kDa. TD, tracking dye; K, kDa.

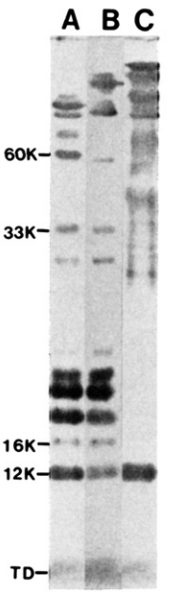

FIGURE 5: NaDodSO$_4$–PAGE of peptides of un-cross-linked and cross-linked heavy chains produced by chymotryptic cleavages. Other conditions are the same as in Figure 4. (Lane A) Un-cross-linked heavy chains; (lane B) heavy chain with X2-type intramolecular cross-link; (lane C) heavy chain with X1-type intramolecular cross-link.

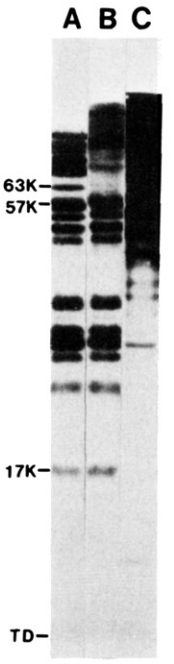

FIGURE 6: NaDodSO$_4$–PAGE of peptides of un-cross-linked and cross-linked heavy chains cleaved with lysyl endopeptidase. Other conditions are the same as in Figure 4. (Lane A) Un-cross-linked heavy chains; (lane B) heavy chain with X2-type intramolecular cross-link; (lane C) heavy chain with X2-type intramolecular cross-link.

P^+-95kDa, P^--X1, P^+-X1, and P^+-X2; refer to Figure 2) were subjected to partial cleavage with V8 protease, chymotrypsin, or lysyl endopeptidase. The peptide mixtures were resolved on SDS–PAGE and then transferred onto a membrane for immunological detection of the peptides containing the N-terminus. The 95-kDa peptides from S1 photolyzed either in the absence or in the presence of Mg^{2+}-ATP (P^--95kDa and P^+-95kDa) generated the same map as that from unphotolyzed S1 (U-95kDa) for each of the three enzymes used in this study (results not shown), indicating they are the heavy chains without an intramolecular cross-link. To save space, only the maps derived from U-95kDa are shown in the following figures.

The peptide maps generated from P^--X1 and P^+-X1 by three enzymes were also indistinguishable (results not shown), indicating that the same segment of the 25-kDa region participated in cross-linking the SH-1 whether or not Mg^{2+}-ATP was present during photolysis. In the following figures, only the results from P^+-X1 will be shown.

The peptide map of un-cross-linked heavy chains produced by V8 protease digestion shows distinct antibody-reacting fragments ranging from 9.5 to 95 kDa (Figure 4, lane A). Identical fragments up to 39 kDa were generated from X2, and among the missing fragments, the one with M_r 60K was the smallest (Figure 4, lanes A and B), indicating that the segment between 39 and 60 kDa from the N-terminus is involved in cross-linking in the presence of Mg^{2+}-ATP. In the case of X1 only the 9.5-kDa fragment was in alignment with that of the un-cross-linked heavy chain (Figure 4, lanes A and C), indicating that the segment between 9.5 and 20 kDa from the N-terminus was cross-linked to SH-1 whether or not a nucleotide is present.

Similarly, a comparison of the chymotryptic maps of un-cross-linked and cross-linked heavy chains shows that the fragments derived from X2 aligned with those of un-cross-linked heavy chains up to 33-kDa peptide, and the 60-kDa peptide was the smallest missing fragment (Figure 5, lanes A and B), indicating the segment between 33 and 60 kDa from the N-terminus is cross-linked to SH-1 in X2. On the map of X1, the 12-kDa fragment was in alignment with a peptide of the un-cross-linked heavy chain, and the 16-kDa fragment was the smallest missing fragment (Figure 5, lanes A and C), indicating that the region between 12 and 16 kDa from the N-terminus is involved in cross-linking between the SH-1 thiol and the 25-kDa region.

The third set of maps result from the digestion with lysyl endopeptidase. In this case, among the fragments of X2 that were in alignment with those of the un-cross-linked heavy chain, the 57-kDa peptide was the largest, and the one with 63 kDa was absent (Figure 6, lanes A and B), showing that

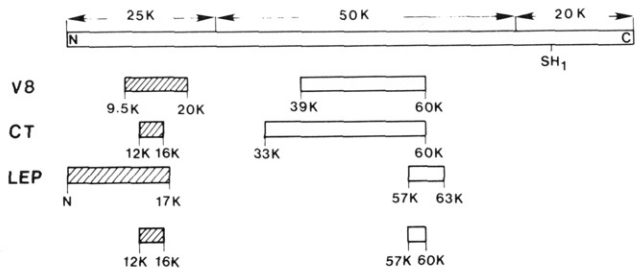

FIGURE 7: Summary of peptide mapping results derived from three enzymes. The S1 heavy chain is shown as an open bar on the top. The 25-, 50-, and 20-kDa fragments produced by tryptic nicking are marked with arrows. N, N-terminus; C, C-terminus; K, kDa. V8, CT, and LEP represent V8 protease, chymotrypsin, and lysyl endopeptidase, respectively. (Hatched boxes) Segments participating in cross-linking with SH-1 heavy chains whether or not the nucleotide is present (X1). (Open boxes) Segments that are involved in cross-linking with SH-1 heavy chains (X2), which takes place only in the presence of a Mg^{2+}–nucleotide. The regions that are shared by segments derived from all three kinds of enzymatic cleavage are shown at the bottom of the figure. Numbers at both sides of the boxes are estimated molecular masses from the N-terminus of the heavy chain.

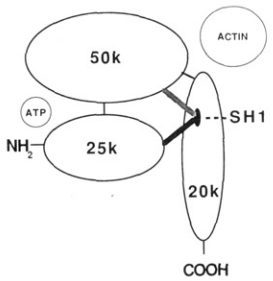

FIGURE 8: Schematic diagram illustrating the topological relationship among three proteolytic fragments of myosin S-1 heavy chain and the putative actin and nucleotide binding regions. A cross-link (solid bar) can be formed between SH-1 and the segment at 12–16 kDa from the NH_2 terminus regardless whether or not the nucleotide is present. The cross-link (dotted bar) between SH-1 and the segment at 57–60 kDa from the NH_2 terminus can be formed only in the presence of a Mg^{2+}–nucleotide. For the sake of simplifying the drawing, the regions corresponding to the fragments are shown as discrete entities; we do not intend to deny or imply the existence of domains in S1.

the region at 57–63 kDa from the N-terminus is cross-linked to SH-1. The smallest peptide observed on the map of uncross-linked heavy chains was the 17-kDa fragment, which was absent from peptides derived from X1 (figure 6, lanes A and C), indicating that in X1 the segment cross-linked to SH-1 is located within 17 kDa from the N-terminus.

The results of three sets of maps are summarized in Figure 7. It is clear that three proteolytic enzymes generate peptide patterns that lead to similar conclusions. For the heavy chain containing a cross-link between the SH-1 and the 25-kDa domain, maps resulting from V8, chymotrypsin, and LEP digestion show that regions located at 9.5–20, 12–16, and 17 kDa from the N-terminus, respectively, participate in cross-linking (Figure 7, hatched boxes). Since all three regions contain a common segment, 12–16 kDa from the N-terminus, we can further conclude that the segment between 12 and 16 kDa from the N-terminus is cross-linked to SH-1 in X1. In the case of heavy chains with a cross-link between SH-1 and the 50-kDa domain, the results derived from V8, chymotryptic, and LEP digestion indicate that the regions located 39–60, 33–60, and 57–63 kDa from the N-terminus, respectively, are involved in cross-linking (Figure 7, open boxes). The segment that is overlapped by all three types of cleavage is 57–60 kDa, and thus we can conclude that the segment between 57 and 60 kDa from the N-terminus is cross-linked to SH-1 in X2.

Discussion

Peptide mapping shows that one segment spanning 12–16 kDa from the N-terminus of myosin heavy chain is close to SH-1 and the cross-linking of this region via BPIA to SH-1 is nucleotide independent, whereas the second segment spanning 57–60 kDa from the N-terminus can be cross-linked to SH-1 only in the presence of a Mg^{2+}–nucleotide. These two segments are located in the 25- and 50-kDa regions, respectively, and are separated by about 400 amino acid residues in the primary structure (Figure 8). The finding that SH-1 can be cross-linked to either of these two segments in the presence of a Mg^{2+}–nucleotide is consistent with other reports (Seidel et al., 1970; Seidel & Gergely, 1971; Wells & Yount, 1979; Wells et al., 1980) that the structure around the SH-1 is more flexible when a nucleotide is bound. Since the same segment in the 25-kDa region, which is 12–16 kDa from the N-terminus, is involved in cross-linking whether or not a nucleotide is present, it appears that no major change in the distance between this segment and the reactive thiol occurs as a result of the nucleotide binding. However, the fact that the second segment becomes cross-linkable to SH-1 only in the presence of a nucleotide is most likely due to a conformational change in the 50-kDa region. It has been shown that the presence of a nucleotide can protect the 50-kDa region from proteolytic susceptibility caused by treatment with heat (Muhlrad & Hozumi, 1982; Mocz et al., 1984) or methanol (Burke & Sivaramakrishnan, 1986).

The SH-1 thiol, which is Cys-707 in the primary structure (Gallagher & Elzinga, 1980; Strehler et al., 1986), is located about 13 nm from the head/rod junction (Sutoh et al., 1984). Since BPIA spans less than 1 nm, the 12–16 and 57–60-kDa segments from the N-terminus in the primary structure must be part of the head structure that is about 13 nm from the head/rod junction (Figure 8). Three-dimensional reconstruction of electron microscopic images of the S1–actin rigor complex (Moore et al., 1970; Toyoshima & Wakabayashi, 1979, 1985a,b; Wakabayashi & Toyoshima, 1981; Taylor & Amos, 1981; Amos et al., 1982) showed that the actin binding site is closer than the SH-1 thiol to the tip of myosin head. Peptide mapping of the cross-linked actin–S1 complex showed that two regions of S1, 75–77 and 60–68 kDa from the N-terminus, located in the 20- and 50-kDa regions, respectively, are involved in cross-linking with actin (Sutoh, 1982a,b, 1983). If these actin-cross-linking sites are the same binding sites as those deduced from three-dimensional reconstruction of electron micrographs of decorated thin filaments and taking into consideration that the C-terminus of the S1 heavy chain starts at the head/rod junction, the 20-kDa region must extend toward the tip of the head by passing the SH-1, the actin-cross-linking site on the 20-kDa region (75–77 kDa from the N-terminus), and the other actin cross-linking site on the 50-kDa region (60–68 kDa from the N-terminus). At some point the 50-kDa region would have to fold back toward the head/rod junction, so that the 57–60-kDa segment will be close to SH-1 (Figure 8). It should be pointed out that although an isolated cyanogen bromide peptide containing SH-1 binds to actin (Katoh et al., 1985), SH-1 is clearly outside the actin binding site on the myosin head (Yamamoto et al., 1984).

The primary structure of the 25-kDa fragment reveals that the stretch from Tyr-109 to Tyr-142 contains 18 hydrophobic residues and only two charged amino acids, suggesting that this part of the polypeptide chain may form a pocket for the binding of the nucleotide (Tong & Elzinga, 1983). Recently, Okamoto and Yount (1985) reported that Trp-130 is the major

site of labeling by the nucleotide analogue *N*-(4-azido-2-nitrophenyl)-2-aminoethyl triphosphate. The 12–16-kDa segment, which we find is close to SH-1, appears to overlap the putative nucleotide binding area. However, whether or not the nucleotide binding site is indeed close to SH-1 in the tertiary structure must await more precise determination of the residues that are cross-linked to SH-1. More work is also needed to know whether the segment in the 50-kDa region (57–60 kDa) coincides with the binding site of some other nucleotide analogues (Mahmood & Yount, 1984; Hiratsuka, 1985).

Recently it has been shown that dibromobimane can cross-link SH-1 and another thiol in the 50-kDa region of the S1, but it cannot form any cross-link between SH-1 and the 25-kDa region. This is probably due to the lack of a thiol at a proper position in the 25-kDa region for dibromobimane to cross-link with, whereas benzophenone can react with any C–H bond once it is photoactivated.

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