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Biochemistry

Stabilization of a primary loop in myosin subfragment 1 with a fluorescent crosslinker

(myosin head/fluorescent modification/dibromobimane/interdomain relationship)

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ABSTRACT **A bifunctional fluorescent alkylating agent, dibromobimane, has been used to stabilize a preexisting primary loop in myosin subfragment 1 (S-1). The crosslink achieved joins Cys-707 (called sulfhydryl group "SH1") of the 20-kDa domain (formerly called "20K" domain) with a thiol of the 50-kDa domain and seems to place the dibromobimane near the ATP-perturbable tryptophan.**

The number and distribution of thiol residues in myosin subfragment 1 (S-1) is slowly emerging. If S-1 is prepared so as to conserve myosin light chain 2 (LC2) (i.e., by using papain in the presence of Mg^{2+}), amino acid analysis (1) shows a total of 12, and these can actually be titrated—8 directly and 4 more after denaturation (2). If the popular chymotrypsin preparation is used, the total number is less certain (possibly because a thiol-containing fragment of LC2 is variously retained). On the other hand, sequence determination (3) has made certain the numbers of thiols in the 20-, 27-, and 50-kDa heavy chain fragments (previously called "20K, 27K, and 50K domains") obtained by trypsinolysis (4–6) of the chymotrypsin preparation: in the 27-kDa domain, 1 thiol (Cys-122); in the 50-kDa domain, 3 thiols (Cys-402, Cys-522, and Cys-540); and in the 20-kDa domain, 4 thiols (Cys-674, Cys-697, Cys-707, and Cys-794). (The tentative numeration of groups in the heavy chain amino acid sequence has been kindly communicated to us by M. Elzinga.) There remains the possibility that some thiols are lost when the short interdomain "connector" regions are destroyed. Because reactions of Cys-697 [formerly called sulfhydryl group 2 ("SH2")] and of Cys-707 [formerly called sulfhydryl group 1 ("SH1")] with various reagents have profound effects on the ATPase activities of myosin (for a review, see ref. 7), these two thiols have been studied most intensively.

Recently, conformational consequences of reacting SH1 and SH2 have been linked to an "intersite communication system" in S-1 (8). Also, a large conformational change that results in "trapping" Mg^{2+}-nucleotide and in reducing actin affinity has been reported to result from crosslinking SH1 and SH2 (9, 10). In the present paper we show that a different interthiol crosslinking reveals another peculiarity of S-1 structure. To produce our crosslinking, we used an interesting fluorescent bifunctional thiol-alkylating reagent, dibromobimane, invented by Kosower *et al.* (11, 12).

MATERIALS AND METHODS

S-1 and Actin Preparation. Rabbit skeletal myosin was prepared as described by Offer *et al.* (13). S-1 was prepared by digestion of myosin filaments with α-chymotrypsin (14) and was purified as described by Mornet *et al.* (6). The split S-1 obtained by a trypsin cleavage (S-1/trypsin, 1:25 molar ratio, at 25°C for 30 min in 0.05 M Tris·HCl, pH 8.0) was also purified as described by Mornet *et al.* (6). Both S-1 concentrations were estimated by using $A_{280}^{1\%} = 7.5$ as described by Wagner and Weeds (15). Cleavage of labeled S-1 in the presence of nucleotide (5 mM MgATP) was as described by Hozumi (16).

Rabbit skeletal muscle actin was prepared as described by Eisenberg and Kielley (17), and its concentration was estimated by using $A_{280}^{1\%} = 11.0$ (18). G-actin was obtained as described by Mornet and Ue (19).

L-1-Tosylamido-2-phenylmethyl chloromethyl ketone-treated trypsin and α-chymotrypsin were from Worthington.

Monobromobimane and dibromobimane were from Calbiochem–Behring. All other reagents were of the highest grade.

Labeling Conditions. For labeling, monobromobimane was used in a 2- to 4-fold molar excess over S-1. The stock solution of dye was made daily in pure methanol; in the presence of S-1, the final concentration of methanol was <5%. The dye was easy to dissolve and immediately became yellow. Dibromobimane was used under similar conditions, but the stock solution was first made in water; however, it was relatively difficult to dissolve it completely, and finally we settled on dissolving it daily in pure methanol. The solution was not fluorescent, but fluoresced after reacting with S-1.

S-1 was in 5 mM 2-{[*N*-tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes) (pH 7.6); 5 mM MgATP was added before either of the bimanes. In our conditions the presence of MgATP prevented dimer formation with dibromobimane, and for comparative purposes we retained the presence of the nucleotide during modification with monobromobimane.

Sodium Dodecyl Sulfate Gel Electrophoresis. Tryptic fragments of modified S-1 were separated by electrophoresis in 0.1% $NaDodSO_4$/polyacrylamide slab gels (20) containing a 5–18% (wt/vol) gradient of acrylamide (21). The running buffer was 50 mM Tris/100 mM boric acid, pH 8.0 (22).

The following S-1 derivatives were used as molecular weight markers: S-1 heavy chain (95 kDa); LC1 (25 kDa); LC3 (17 kDa); the three fragments of tryptically split S-1 (50 kDa, 27 kDa, and 20 kDa); and actin (42 kDa).

Specific Modifications of S-1 Thiols. For modification with iodoacetamide we used procedures of the kind reviewed by Reisler (7). Modified S-1 was separated from excess reagent by precipitation with 60% ammonium sulfate (23).

Thiol Titration with 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB). The thiol content of unmodified and modified S-1 in 0.1 M bicarbonate buffer (pH 8.0) was measured by using Ellman's reagent (24) in the absence and presence of 5 M urea.

Specific Hydroxylamine Cleavage. Specific cleavage of the

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Abbreviations: S-1, subfragment 1; Tes, 2-{[*N*-tris(hydroxymethyl)-methyl]amino}ethanesulfonic acid; 1,5-IAEDANS, *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; LC, myosin light chain.

Asn-Gly peptide bond with hydroxylamine was done as described by Sutoh (25) and by Borstein and Balian (26). Lithium hydroxide was used to adjust the pH to 9.0 in this chemical cleavage. Then, after filtration on a Sephadex G-25 column equilibrated with 9% formic acid and lyophilization, samples corresponding to different cleavage times (10 min, 30 min, 1 hr, 2 hr, 4 hr, 6 hr, and 8 hr) were dissolved in 5% $NaDodSO_4$ and applied to a polyacrylamide gel for electrophoresis.

S-1 ATPase Activities. The K^+/EDTA-, Ca^{2+}- and Mg^{2+}-dependent ATPase activities of S-1 were measured (27). The actin-activated Mg^{2+}-ATPase was assayed in a medium containing 10 mM KCl, 50 mM Tris·HCl (pH 8.0), 1.5 mM $MgCl_2$, 2.5 mM ATP, and 0.050 mg of S-1.

Steady-State Fluorescence Measurements. Steady-state fluorescence was measured on a Hitachi/Perkin–Elmer MPF-4 fluorometer with corrected spectra capability and controlled cell holder temperature (10°C in our case).

RESULTS

Modification of S-1 with Monobromobimane. Upon a 30-min incubation with a typical SH1 modifier such as iodoacetamide, the Ca^{2+}-ATPase was activated by more than 400%, and the K^+/EDTA-ATPase was inhibited by about 30%. However, when the modifier was monobromobimane, *both* activities were inhibited, the former by about 40% and the latter by about 60%. The modifications were conducted in the presence of 5 mM MgATP (so that they could serve as controls in subsequent proteolytic work), but this reagent reduced only slightly the inhibitory effect of the modification on the ATPases.

When S-1 was first modified with iodoacetamide so as to activate Ca^{2+}-ATPase to 80–90% of maximum and then modified with monobromobimane, both the Ca^{2+}- and K^+/EDTA-dependent ATPase activities were essentially abolished.

Examination of the electrophoretograms revealed that, whenever monobromobimane was used as a modifier, its fluorescence appeared mainly in the 95-kDa heavy chain band. Pretreatment with iodoacetamide reduced the fluorescence that monobromobimane imparted to the heavy chain. When, after modification and purification, S-1 was subjected to limited trypsinolysis, the electrophoretogram showed essentially all of the fluorescence of the heavy chain to reside in the 20-kDa domain (Fig. 1*A*). When S-1 was first proteolyzed and then subjected to monobromobimane modification as a function of time (Fig. 1*B*), the fluorescence was at first exclusively on the 20-kDa band; after an extended time, faint fluorescence also began to appear on the 50-kDa band. The result of pretreating split S-1 with iodoacetamide and then modifying it with monobromobimane (not shown) was that less fluorescence developed on the 20-kDa band and relatively more on the 50-kDa band.

Modification of S-1 by Dibromobimane. For up to 30 min, the effect on the ATPases of modification with dibromobimane was essentially identical to that with monobromobimane.

Several experiments were done to investigate the S-1–dibromobimane reaction; two protocols used above with monobromobimane are illustrated here: (*i*) S-1 was incubated with dibromobimane for a considerable time, *viz.* 1 hr; the modification was then quenched, the labeled S-1 was isolated, and progressive trypsinolysis was studied (Fig. 1*C*). (*ii*) S-1 was trypsinolyzed to produce the undissociated 27-, 50-, and 20-kDa fragments, then this "split S-1" was progressively modified with dibromobimane (Fig. 1*D*). Progressive trypsinolysis and progressive modification were studied by electrophoretic analysis. Each electrophoretogram in Fig. 1 was studied by its own fluorescence (*Right*) and after staining it with Coomassie blue (*Left*).

In protocol *i*, the $t = 0$ plates show (Fig. 1*C*) that modification generated a 105-kDa band as well as the expected 95-kDa band. In other experiments (not shown), the 95-kDa

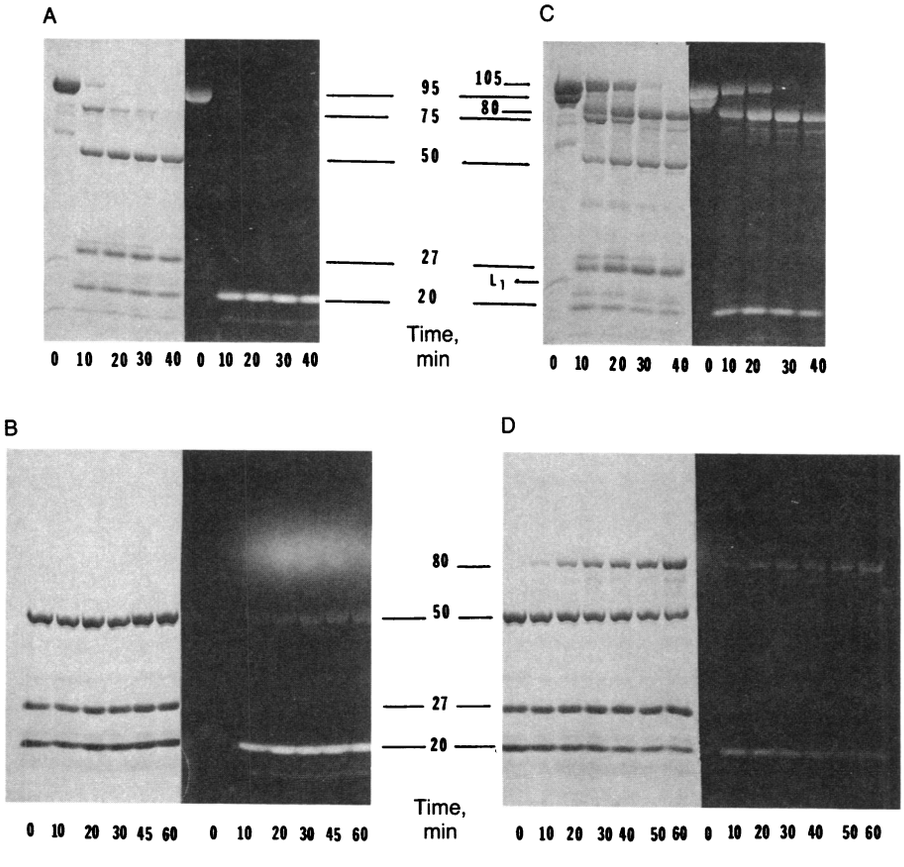

FIG. 1. Electrophoretograms of S-1 treated according to various protocols. Each panel shows an electrophoretogram viewed by its own fluorescence (*Right*) and the same electrophoretogram stained with Coomassie blue (*Left*). In *A* (monobromobimane) and *C* (dibromobimane), S-1 was labeled with a 2- to 4-fold molar excess of dye. The isolated modified protein was washed and then digested by trypsin; at the indicated time, aliquots were taken, boiled in 5% $NaDodSO_4$, and then electrophoresed in a gradient slab gel. In *B* (monobromobimane) and *D* (dibromobimane), tryptically split S-1 (27-kDa/50-kDa/20-kDa fragments) was incubated with a 2–4 molar excess of dye. At the indicated time, protein aliquots were denatured and electrophoresed.

band was observed to decrease and the 105-kDa band to increase during modification. As trypsinolysis proceeded, the usual 20-kDa band appeared, and at least a substantial fraction of it was fluorescent. There also appeared significant 27-kDa and 50-kDa bands, but they were not fluorescent, and neither was the 75-kDa band that disappeared with progressive trypsinolysis. An additional new fluorescent band (besides the 105-kDa band) that was generated in this protocol was an 80-kDa band. The plates of protocol *ii* (Fig. 1*D*) show that the 80-kDa band grew at the expense of both 20-kDa and 50-kDa components. Our interpretation is that the 80-kDa material is the union (crosslinked product) of fluorescent 20-kDa and nonfluorescent 50-kDa materials. Returning to the observations of protocol *i*, we think that the same union occurring intramolecularly in the fluorescent 95-kDa material generates the fluorescent 105-kDa band. Experiments with monobromobimane showed that the 20-kDa band reacts with bimane well ahead of the 50-kDa band; so we think that the fluorescent 95-kDa band at $t = 0$ in protocol *i* consists of material in which dibromobimane has reacted with the 20-kDa region of the heavy chain by one of its functions but has not yet reacted by the other. Since trypsinolysis first attacks heavy chain at the 20-kDa/50-kDa junction, it is easy to see that upon this attack fluorescent 95-kDa material gives rise to nonfluorescent 75-kDa material (as well as fluorescent 20-kDa material), and the 75-kDa material is further attacked to produce nonfluorescent 50-kDa and 27-kDa material. In the foregoing interpretation, we have tacitly assumed nonadditive weights (anomalous electrophoretic migration) for materials in which the dibromobimane is attached by both its functions (105-kDa and 80-kDa components).

A revealing variant of protocol *i* was to use more intensive trypsinolysis (trypsin/S-1, 1:5) in the presence of MgADP. Hozumi (16) has shown that then both the 27-kDa fragment and 50-kDa fragment suffer a further loss of 5 kDa, and Labbé *et al.* (28) have shown that, in the latter case, the loss is from the COOH-terminal region. Between 10 and 15 min of intensive trypsinolysis (initiated by nucleotide addition at 5 min) produced 45-kDa material from 50-kDa material (Fig. 2 *Left*); also fluorescent 65-kDa material was produced from fluorescent 80-kDa material (Fig. 2 *Right*). We interpret these observations as meaning that intensive trypsinolysis degrades 5 kDa from the union of the 50-kDa and 20-kDa fragments and that, when it does so, the resulting structure

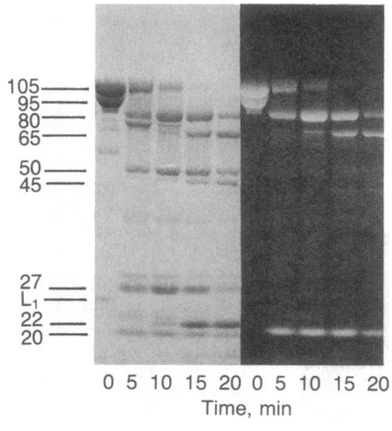

FIG. 2. Time course of intensive tryptic digestion of S-1 modified with dibromobimane. The tryptic cleavage was done initially with a trypsin/S-1 molar ratio of 1:5. After 5 min there arose a major new 80-kDa fluorescent fragment, and MgATP was added to a final concentration of 5 mM (which is known to produce additional cleavage of 5 kDa in the 27-kDa domain as well as in the 50-kDa domain). (*Right*) Self-fluorescence. (*Left*) Same gel as *Right* but stained with Coomassie blue.

no longer migrates anomalously but as a 65-kDa band.

Absorption and Fluorescence Properties. Dibromobimane in absolute methanol, diluted in 5 mM Tes buffer (pH 7.6), is slightly yellow. After reaction with 2-mercaptoethanol, the wavelength of maximum absorption shifted from 402–400 nm to 392–388 nm. The same maximum for modified S-1 was essentially the same, 390 nm; the wavelength of maximum emission was 450 nm; neither maximum appeared to be sensitive to pH.

The extinction coefficient of dibromobimane saturated with 2-mercaptoethanol was $\varepsilon_{390} = 4450 \pm 20\ M^{-1} \cdot cm^{-1}$. Since the absorbance of modified S-1 was the same in buffer as in 5 M urea, we used this ε and the measured A_{390} of dialyzed S-1 to estimate the degree of modification of S-1. After 1 hr of labeling and purification, we found $n \simeq 1$ mol of dibromobimane per mol of S-1.

The emission spectrum of (S-1)-bound dibromobimane excited at 390 nm is a single, well-defined peak with the maximum at 450 nm. On addition of equimolar actin, the peak shifted 5 nm to the blue with no appreciable change in maximal intensity. If then excess (1 mM) MgATP was added, the resulting peak red-shifted 3 nm from the original (i.e., to 453 nm), and its maximum was quenched by 10–15%. This last spectrum is the same as that obtained by adding ATP to labeled S-1; when the ATP concentration increments were graded (0.2–1 μM), the red shift and quenching also were graded.

Also, we studied the turbidity of actomyosin S1 complex formation. The turbidity increased in parallel and to the same plateau on adding either native or dibromobimane-modified S-1. After centrifugation of actomyosin modified S-1 mixtures, we obtained supernatant solutions devoid of modified S-1.

Fig. 3 compares the fluorescence of native and modified S-1 at equal concentrations when the systems were excited at 293 nm (tryptophan absorption). Dibromobimane modification inhibited the MgATP-induced enhancement of the 340-nm tryptophan emission. Dibromobimane itself has a weak emission peaking at 480 nm, but its quenching of the native tryptophan emission and its rather good emission at 450 nm suggest that it may accept energy donated by a tryptophan, possibly Trp-130 (29).

Titration of S-1 Thiol Residues. Titration of thiol residues of S-1 with dithiobis(nitrobenzoate) in 5 M urea showed that in our preparation there were accessible 9.5 + 0.5 mol of SH groups per mol of S-1. After modification with dibromobimane, this number was 7.4 ± 0.5 mol of SH per mol of S-1. Thus, as expected if dibromobimane simultaneously binds two thiols, there were lost 2.1 ± 0.3 mol of SH per mol of bound dye.

DISCUSSION

The simplest and most attractive interpretation of our central results is that a bifunctional alkylating reagent, dibromobimane, can crosslink two thiol residues of S-1—one of these is Cys-707 ("SH1") of the 20-kDa domain and the other is a thiol residue of the 50-kDa domain (Fig. 4).

A priori, however, other interpretations might have been adduced, so it is worth reviewing why these were rejected. Conceivably, crosslinks might have formed between intact heavy chain and LC1, but—apart from the discrepancy between expected and observed molecular masses (120 kDa and 105 kDa)—fluorescence was never imparted to the clearly visible (with Coomassie blue) LC1 bands, and in trypsinolysis the cascade of molecular masses observed was not that expected from LC involvement. Another possibility might have been for the 20-kDa domain to crosslink to the 27-kDa domain, but in that case intensive trypsinolysis should have generated a 42-kDa piece, which it did not. A

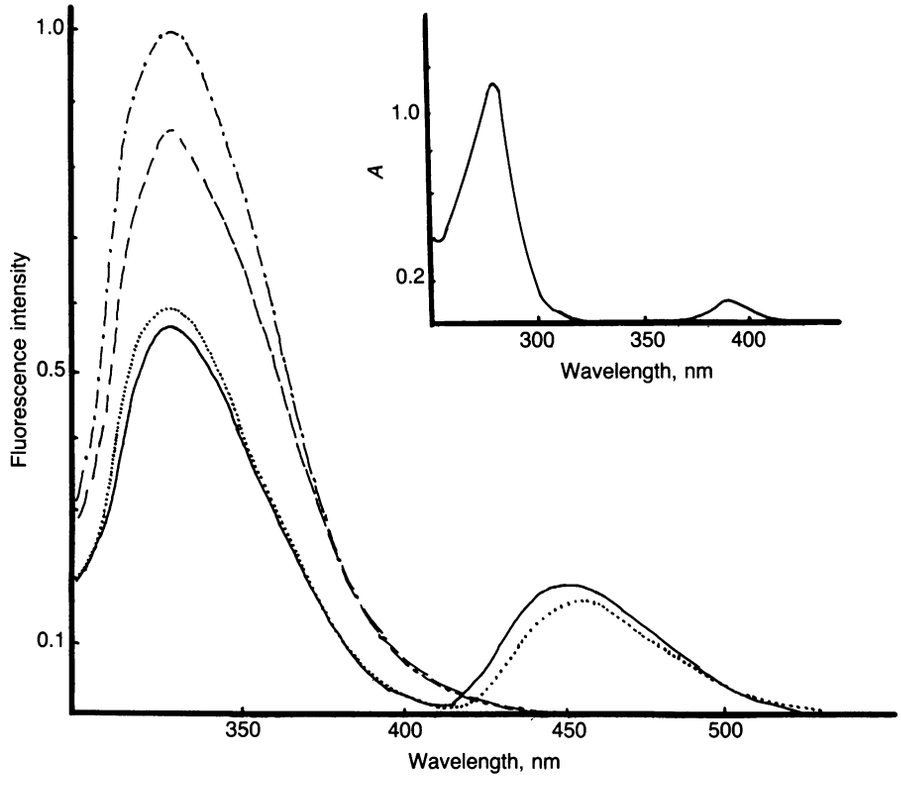

Fig. 3. Comparative tryptophan fluorescence emission spectra of native S-1 and modified S-1 by dibromobimane. Excitation was at 293 nm, and maximum emission was at 330 nm. ---, Native S-1 (2.3 μM); -·-, native S-1 with 160 μM MgATP; —, modified S-1 (2.3 μM); ···, modified S-1 with 160 μM MgATP. (*Inset*) Absorption spectrum of S-1 modified by dibromobimane after purification (labeling time = 1 hr).

third possibility could have been intradomain binding—i.e., the crosslinking of Cys-707 with another 20-kDa thiol residue. This cannot be totally excluded and may have happened to a degree because fluorescence transfer never appears to be completed. However, it could not have been an extensive reaction because then it could have caused some dimerization of whole S-1s, an effect never observed in the Coomassie blue-stained gels.

On the other hand, several observations with split S-1 strongly support our interpretation. Experiments with monobromobimane show that fluorescence is rapidly imparted to the 20-kDa band and then slowly and sparsely to the 50-kDa

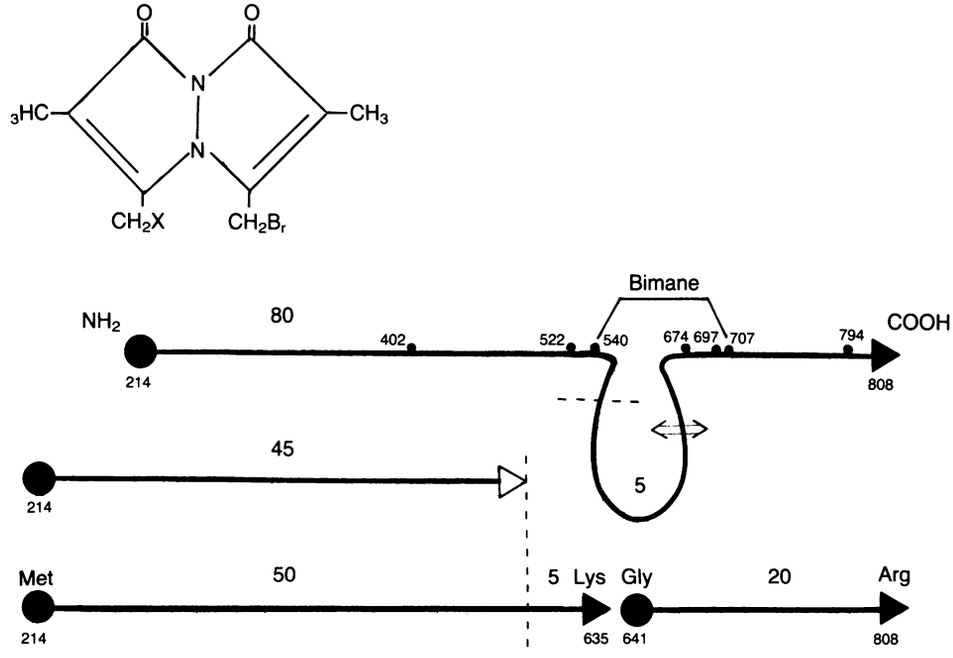

FIG. 4. Linear (amino acid sequence) representations of various heavy chain peptides produced by trypsinolysis and dibromobimane crosslinking. Peptides are named for their apparent molecular masses in PAGE: the 45-kDa fragment is obtained by degrading 5 kDa from the COOH terminus of the 50-kDa fragment (28) by using intensive trypsinolysis and MgADP (16); the 80-kDa fragment is obtained by crosslinking the 50- and 20-kDa fragments with dibromobimane. Large black circles and triangles mark amino- and carboxyl-terminals, respectively. A double-headed arrow marks the 50-kDa/20-kDa junction; dashed lines mark cuts achieved by intensive trypsinolysis and MgADP. Work in this paper shows that dibromobimane crosslinks Cys-707 ("SH1") and one of the three thiols of the 50-kDa domain, but which thiol remains unknown. In this figure Cys-707 is arbitrarily linked to the thiol closest in the linear sequence, thus generating the shortest loop; linkage with either of the other thiols would generate a topologically similar but longer loop. (*Upper Left*) Structure of dibromobimane (X = Br) or monobromobimane (X = H).

band. Similarly, dibromobimane rapidly imparts fluorescence to the 20-kDa band, and then fluorescence begins to appear in the 80-kDa band or in the 65-kDa band if the 50-kDa band has been further proteolyzed. These observations make it natural to conclude that when dibromobimane reacts with intact S-1 heavy chain, it induces a structural alteration that now manifests itself as a 105-kDa component rather than as the usual 95-kDa component.

It has not been directly shown that the bimanes react with Cys-707, but the circumstantial evidence that they do so is rather strong. For example (in a result that is not shown), the fluorescence that they impart to the 20-kDa band stays in the 13-kDa piece (not the 7-kDa piece) when the 20-kDa component is split by hydroxylamine. Furthermore, pretreatment with iodoacetamide (which does react specifically with Cys-707) blocks the reaction of the bimanes with S-1. That the bimanes fail to activate Ca^{2+}-ATPase is not proof that they initially react at thiols other than Cys-707. The reagent *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) certainly reacts initially at Cys-707 and activates Ca^{2+}-ATPase, but some of its isomers do not activate Ca^{2+}-ATPase (unpublished results). As Botts *et al.* (2) have argued, it is likely that the enzymatic effect arises from structural distortions produced by the bulk attached to Cys-707; if so, then the magnitude and direction of the distortion may well depend on the structure of the reagent.

The character of the bromomethyl groups of the bimanes, the loss of two thiols per mol of bound dibromobimane, and the inability of 1,5-IAEDANS to react with either the 105-kDa species of intact heavy chain or the 80-kDa product generated from split heavy chain are all indications that the "other" anchor point of bound dibromobimane is also a thiol—in this case a thiol of the 50-kDa domain—implicated in a crosslink with Cys-707. The 50-kDa domain bears three thiol residues. In Fig. 3 we have arbitrarily assumed that the thiol nearest in the linear sequence (Cys-540) is the second anchor point; however, the topological result—formation of a loop—would be the same whichever thiol of the 50-kDa domain reacted.

That a short-length (0.3–0.6 nm) crosslinker such as dibromobimane joins two thiols does not show conclusively that the thiols are proximal in the native structure because there could be large-scale motion in the peptide chain. However, in this case there are some indications that the deduction of proximity is permissible. Even for the closest thiols, the length of chain between them is rather vast (≈5 kDa), and at least a portion of this length constitutes the actin-binding site. Under these circumstances it seems unlikely that large-scale motion is occurring or that the *imposition* of a chain loop would proceed so easily and would leave the actin affinity unchanged (results of turbidity measurements). For these reasons we think that cross-linking merely stabilizes a primary loop that preexisted in the native structure.

Anomalous gel electrophoretic migration (i.e., a mobility slower than expected from molecular weight) seems to occur whenever polypeptide chains cross, in accordance with the empirical rule. Thus, migration of the intact heavy chain with the stabilized loop or of the union of the 20- and 50-kDa domains is anomalous. When the latter structure loses 5 kDa (intensive trypsinolysis), however, its migration becomes normal, possibly because the enzymatic cut has been near to the crosslink (as it would be if the crosslink involved Cys-540).

The dibromobimane suppression of tryptophan perturbation upon addition of ATP could have two quite distinct origins. (*i*) It might be caused by a direct mechanical effect of the modification, or, indirectly, by an effect on the steady-state concentration of the intermediate known as M**ATP,-P_i. (*ii*) It might be due to extensive energy transfer from the tryptophan residue that is normally perturbed to the bound dibromobimane. In the future this ambiguity might be resolved by modifying with a nonfluorescent analog of dibromobimane.

If further analysis confirms that dibromobimane accepts energy from the ATP-perturbable tryptophan due to spatial proximity, it will be possible to conclude that, at least in some regions, all three domains are contiguous. Hiratsuka (30) has recently also made such a suggestion.

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