neybee development. Important questions regarding VHDL synthesis, potential uptake, and storage by tissue and its role in honeybee lipid metabolism can now be addressed.

ACKNOWLEDGMENTS

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# Intramolecular Cross-Linking of Myosin Subfragment 1 with Bimane†

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ABSTRACT: We previously showed that the fluorescent inter-thiol cross-linker dibromobimane (DBB) [Kosower, N. S., Kosower, E. M., Newton, G. L., & Ranney, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3382–3386] cross-links two [50 and 20 kilodaltons (kDa)] of the three major fragments of myosin subfragment 1 (S-1); on intact S-1, DBB quenches tryptophans and inhibits all ATPases [Mornet, D., Ue, K., & Morales, M. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1658–1662]. Here we characterize the modification chemically: DBB cross-links Cys-522 (50 kDa) with Cys-707 (20 kDa), thereby sealing a large preexisting heavy-chain loop containing important functionalities. Cross-linking rate is insensitive to nucleotides, but apparently sterically, either monobromobimane or DBB reduces $Ca^{2+}$-ATPase to low, nonzero levels.

In a previous paper (Mornet et al., 1985), we reported that the inter-thiol cross-linker dibromobimane (DBB)[1] (Kosower et al., 1979) cross-links Cys-707 ("SH1") of the myosin head with a second thiol whose sequence position[2] was not identified except for ascertaining that it resided on the proteolytic fragment of S-1 known as "50 kDa" (Cys-707 resides on the proteolytic fragment, "20 kDa"). We showed that this intramolecular cross-linking seals off a myosin heavy-chain "loop" whose length was shown to exceed 44 residues (5 kDa).

This new modification of S-1 is interesting for several reasons: when in place, DBB (a) strongly accepts resonance

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[1] Abbreviations: S-1, chymotryptic subfragment 1 of myosin; DBB, dibromobimane; MBB, monobromobimane; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; $NaDodSO_4$, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; $M_r$, molecular weight; $LC_1$, alkali light chain 1; $LC_3$, alkali light chain 2; SH, thiol residue; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; dansyl, 5-(dimethylamino)-1-naphthalenesulfonate.

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energy from a nearby tryptophan(s) and (b) strongly inhibits all ATPase. Since our paper, the research of many workers has focused on events occurring on or near the loop. For example, both Morales (1986) and Chaussepied et al. (1986a) have called attention to the possibility that energy transduction occurs in the loop. Kasprazak (1986) has performed experiments reassuring that the DBB quenching of tryptophan(s) emission is true resonance transfer and that the close proximity of Cys-707 and the 50-kDa thiol preexists. Lu (Lu et al., 1986) and Sutoh (personal communication) have reported that nucleotide binding draws certain regions near, or in, the loop closer together. Hiratsuka (1986) has achieved certain glutaraldehyde (inter-lysine) cross-links between 50 and 20 kDa that appear to be in the loop, and Mornet et al. (1986) have found that the DBB modification weakens actin binding and abolishes the EDC-induced cross-link between the N-terminal region of actin and 20 kDa of the myosin head.

In view of the growing interest in the loop region of the myosin head, it seemed desirable to characterize further the modification with DBB. So in this paper we attempt to (a) identify the 50-kDa thiol that DBB cross-links to SH1, (b) inquire whether nucleotide binding affects the rate of DBB cross-linking, and (c) examine quantitatively the $Ca^{2+}$-ATPase inhibition.

## Materials and Methods

*Materials.* $\alpha$-Chymotrypsin was from Worthington. DBB was from Calbiochem-Behring. Sephadex G-25 (pD-10) column was prepacked from Pharmacia. Cyanogen bromide was from Sigma. $NaDodSO_4$, acrylamide, and all other gel electrophoresis chemicals were from Bio-Rad. *N*-Ethylmorpholine, dansyl chloride, dimethyl formamide, and the F-1700 micro polyamide plate (5 × 5 cm) were from Pierce.

*Proteins.* Myosin was prepared from the back muscles of rabbits according to Tonomura et al. (1966). S-1 was prepared by digestion of myosin filaments with $\alpha$-chymotrypsin (Weeds & Taylor, 1975). The concentration of intact S-1 was estimated by using $A_{280nm} = 7.5\ cm^{-1}$ (Wagner & Weeds, 1977). The concentration of modified S-1 was determined by the method of Bradford (1976).

*Labeling of S-1.* DBB and MBB labeling conditions were as described in a previous paper (Mornet et al., 1985). DBB or MBB was used in a 4-fold molar excess over S-1. S-1 was labeled at 25 °C in 20 mM *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES) (pH 7.6); 5 mM MgATP was added before either of the bimanes. The presence of MgATP prevented dimer formation of S-1 with DBB in higher concentration and did not affect the rate of labeling with either DBB or MBB. For comparative purposes, we retained the presence of the nucleotide during modification with DBB and MBB unless indicated. 1,5-IAEDANS labeling condition was as described in Takashi et al. (1976).

*Titration of Thiols of S-1.* This titration was done with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Thiol content of modified and unmodified S-1 was titrated with this reagent, in the presence of 8 M urea (urea was added just before titration to avoid oxidation) assuming $\epsilon_{412nm} = 1.36 \times 10^4\ cm^{-1}$ (Ellman, 1958).

*Cyanogen Bromide Cleavage.* Modified S-1 was subjected to CNBr cleavage (Elzinga & Collins, 1977), in 70% formic acid at room temperature for 16–18 h, using at least a 100-fold molar excess of CNBr over the methionine content.

*Specific Hydroxylamine Cleavage.* Specific cleavage of the Asn–Gly peptide bond (between Cys-697 and Cys-707) with hydroxylamine was done according to Borstein and Balian (1977) and Sutoh (1981). Lyophilized protein (in our case, MBB modified split S-1) was dissolved in 6 M guanidinium chloride, 1% 2-mercaptoethanol, and 20 mM potassium bicarbonate, pH 8.0, and then mixed with an equal volume of 2 M hydroxylamine in 6 M guanidinium chloride, pH 9.0 (4.5 M lithium hydroxide was used for adjusting the pH) at 45 °C. The reaction was carried out for 3–4 h and was then quenched by adding 0.1 volume of 90% formic acid. The resulting solution was applied to a Sephadex G-25 column equilibrated and eluted with 9% formic acid. After lyophilization, the sample was dissolved in 8 M urea and further diluted with 1% $NaDodSO_4$, 1% 2-mercaptoethanol, and 20 mM Tris-HCl (pH 8.8) for gel electrophoresis.

*Polyacrylamide Gel Electrophoresis (PAGE).* Modified S-1 fragments produced by CNBr cleavage and hydroxylamine cleavage were analyzed by PAGE using a 0.1% $NaDodSO_4$ and 10–18% gradient acrylamide slab gel (Studier, 1973). The running buffer was 0.1% $NaDodSO_4$, 50 mM Tris, and 100 mM boric acid (Mornet et al., 1981).

*Polyacrylamide Gel Scanning.* The fluorescence intensity of modified S-1 heavy chain and the optical densities of Coomassie blue stained S-1 heavy chain in the polyacrylamide gel were measured with a Shimadzu Model CS-930 high-resolution gel scanner equipped with a computerized integrator.

*Purification of Dibromobimane-Cross-Linked Peptides.* After CNBr cleavage, DBB-labeled S-1 was brought to dryness by lyophilization. These cross-linked peptides were then dissolved in a small amount of 8 M urea, and buffer (containing 5% $NaDodSO_4$, 5% 2-mercaptoethanol, 25% glycerol, 0.01% bromophenol blue, and 50 mM Tris-HCl, pH 8.8 v/v) was added. Three-milligram aliquots of peptide were electrophoresed on a preparative, 3-mm-thick 10–18% gradient acrylamide–$NaDodSO_4$ gel in a Bio-Rad protein slab cell. The running buffer contained 0.1% $NaDodSO_4$, 50 mM Tris, and 100 mM boric acid. Following electrophoresis, the fluorescent band representing DBB-cross-linked peptides was carefully cut out and transferred to a 1.8-cm diameter tube with a 0.6-cm diameter outlet. The outlet was plugged by a 5% acrylamide gel, and the tube was placed in a disc gel electrophoresis cell. A small dialysis sack filled with 1 mL of running buffer was fastened to the outlet of the tube (Muhlrad et al., 1986). The running buffer is the same as the slab gel system. The electrophoresis was run at 50 V until all the fluorescent material was transferred to the dialyzing sack. The fluorescent sample was then removed, dialyzed against $H_2O$, and lyophilized to dryness.

*End-Group Analysis.* Analysis of N-terminal end groups was according to Gray (1972) with modification. Dried DBB-cross-linked peptides were placed in a small test tube and dissolved in 50 $\mu$L of 1% $NaDodSO_4$ during heating in a boiling water bath for 2–3 min. After the sample cooled, it was mixed thoroughly with 50 $\mu$L of *N*-ethylmorpholine. Seventy-five microliters of dansyl chloride (25 mg/mL in anhydrous dimethyl formamide, freshly made) was then added and mixed thoroughly. The reaction proceeded for 1 h or more at room temperature. The dansylated peptides were precipitated by adding 500 $\mu$L of acetone. After mixing thoroughly, it took several minutes or a much longer time for a suitable precipitate to form. When the pellet had been compressed by centrifugation, it was washed once with 500 $\mu$L of 80% acetone, centrifuged again, and dried in vacuo over NaOH pellets. The sample was then hydrolyzed in 6 N HCl inside a sealed

[2] The tentative numeration of groups in the heavy-chain amino acid sequence has been kindly communicated to us by M. Elzinga.

Table I: Time Dependence of the Disappearance of Free Thiol and Binding of Label When S-1 Is Modified with MBB and DBB[a]

| protein | labeling time (min) | mol of free SH/mol of S-1 | mol of blocked SH/mol of S-1 | mol of dye/mol of S-1 |
|---|---|---|---|---|
| MBB–S-1 | 10 | 9.26 | 0.76 | 0.98 |
| MBB–S-1 | 60 | 9.08 | 0.94 | 1.18 |
| DBB–S-1 | 10 | 8.93 | 1.09 | 1.07 |
| DBB–S-1 | 60 | 8.32 | 1.70 | 1.14 |

[a] Unmodified S-1 had 10.02 mol of free thiol/mol of S-1. All entries are averages of duplicate samples.

tube for 6–18 h at 105 °C, and the HCl was removed at the end of the hydrolysis. Identification of dansyl amino acids was by two-dimensional chromatography on polyamide layers. The dansylated sample was dissolved in 10–20 μL of ethanol; 1–2 μL of sample was spotted on an F-1700 micro polyamide plate (5 × 5 cm) and chromatographed in the following solvent systems: first solvent, water/90% formic acid (200:3 v/v) run in the first dimension. After the solvent front moved to the end, the sheet was dried, turned through 90°, and run in the second solvent [second solvent, benzene/acetic acid (9:1 v/v)]. After developing and drying, the sample was rerun in a third solvent in the same dimension [third solvent, ethyl acetate/methanol/acetic acid (20:1:1 v/v)]. After the third solvent, because Glu and Tyr are not always well separated in the third solvent, a fourth solvent was used in the same dimension as the third solvent [fourth solvent, heptane/1-butanol/acetic acid (3:3:1 v/v)].

*S-1 ATPase Activities.* The $Ca^{2+}$-dependent ATPase activities of modified S-1 were determined to 25 °C in 250 mM KCl, 5 mM $CaCl_2$, 2.5 mM ATP, and 50 mM Tris-HCl, pH 7.5 (Mornet et al., 1979).

## Results

To know the stoichiometry of modification of S-1, MBB and DBB were dissolved in absolute methanol and then diluted in 20 mM TES buffer (pH 7.6). After their reaction with 2-mercaptoethanol, the wavelength of maximum absorption was 390 nm for MBB and 393 nm for DBB. When these compounds modify S-1, the wavelength of maximum absorption essentially does not change. The absorbance of MBB is stable after reaction with 2-mercaptoethanol, but for DBB, the absorbance increases with time and takes about 3–4 h to reach its maximum.

The extinction coefficient is $E_{390} = 5230\ M^{-1}\ cm^{-1}$ for MBB saturated with 2-mercaptoethanol and is $E_{393} = 5350\ M^{-1}\ cm^{-1}$ for DBB similarly saturated. We used these $E$ values to estimate the degree of modification of S-1. After 10–60 min of incubation, ca. 1 mol of MBB or of DBB reacts with 1 mol of S-1 (Table I).

Titration of S-1 thiols with DTNB in 8 M urea (Table I) shows that after 60 min of incubation with MBB, 0.94 mol of thiol/mol of S-1 is blocked. When the same incubation time is used with DBB, ca. 1.70 mol of thiol/mol of S-1 is blocked; this indicates that DBB cross-links with a second thiol. If incubation with DBB is only 10 min, then only 1.09 mol of thiol/mol of S-1 is blocked. This shows that the cross-linking process takes a longer time to develop.

The 20-kDa fragment of S-1 contains four thiols: Cys-674, Cys-697, Cys-707, and Cys-794. As is shown in Figure 1, lanes d–f, when MBB-labeled split S-1 is CNBr cleaved, the fluorescence that it imparts to the 20-kDa band stays in the internal 10-kDa piece (Elzinga & Collins, 1977), which contains only Cys-697 and Cys-707. On the other hand, if the same labeled split S-1 is cleaved by hydroxylamine, the fluorescence stays only in the 13-kDa piece, containing only Cys-707 and Cys-794. Clearly, Cys-707 in 20 kDa of S-1 is the thiol that MBB attacks. A parallel experiment was done by CNBr cleavage and hydroxylamine cleavage of 1,5-IAEDANS-labeled split S-1 (Figure 1, lanes a–c). It is known that 1,5-IAEDANS specifically labels SH1 (Cys-707) of S-1 (Takashi et al., 1976). In Figure 1, lanes a–c, it is shown that after CNBr cleavage and hydroxylamine cleavage, the fluorescence stays in the internal 10-kDa piece and in the 13-kDa piece, respectively. This is the same as in the MBB-labeled split S-1 case.

The 50-kDa fragment of S-1 contains three thiols: Cys-402, Cys-522, and Cys-540. From the Elzinga sequence, the possible DBB unions with Cys-707 are shown in Figure 2A by dashed lines. First we consider the predicted fragmentation of DBB-labeled S-1 by CNBr (CNBr cleavage does not break the DBB cross-linking). Each thiol of 50 kDa resides in a different fragment, so the fluorescent (containing DBB) structure expected would have a different molecular weight, depending on which union is made (Figure 2B). When DBB-labeled S-1 is CNBr cleaved, two fluorescent bands are seen in $NaDodSO_4$–PAGE (Figure 3, lanes b–d). The faster band is just a "10-kDa" piece of 20 kDa containing Cys-697 and Cys-707 that has not been joined (the DBB cross-linking reaction is never complete). So the cross-linked piece is the

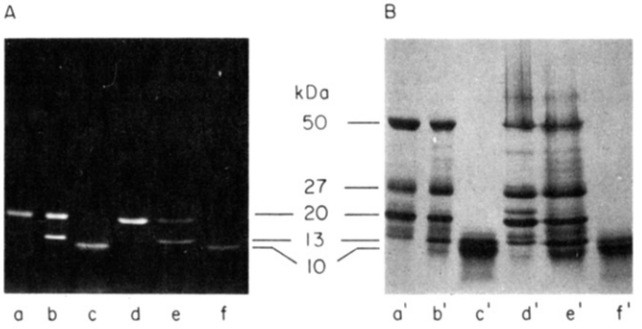


FIGURE 1: PAGE patterns of S-1 labeled with either 1,5-IAEDANS (a, a′, b, b′, c, c′) or MBB (d, d′, e, e′, f, f′). The materials in (A) are photographed by their own fluorescence; in (B), they are Coomassie stained. (a, a′) S-1 labeled with 1,5-IAEDANS and then tryptically split; (b, b′) sample in (a, a′) cleaved with $NH_2OH$; (c, c′) sample in (a, a′) cleaved with CNBr; (d, d′) S-1 labeled with MBB and then tryptically split; (e, e′) sample in (d, d′) cleaved with $NH_2OH$; (f, f′) sample in (d, d′) cleaved with CNBr.

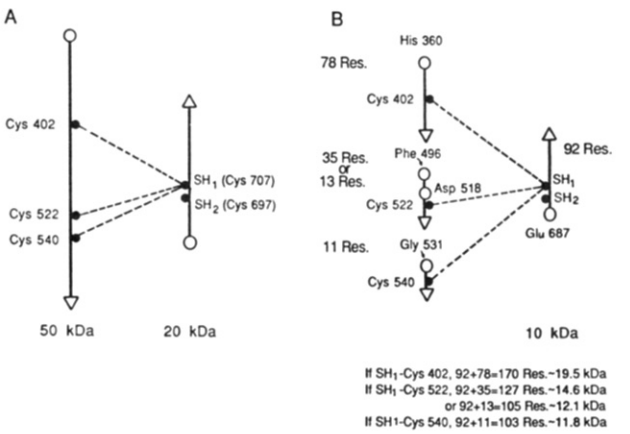


FIGURE 2: Anticipated distribution of inter-thiol cross-links when a thiol of 50 kDa is joined by DBB to Cys-707 of 20 kDa. (A) The three possible unions between 50 kDa and 20 kDa. (B) Characteristics of the structures resulting after each of the joined structures of (A) is CNBr cleaved. Below (B) are shown the anticipated molecular weights of the three joined structures of (B).

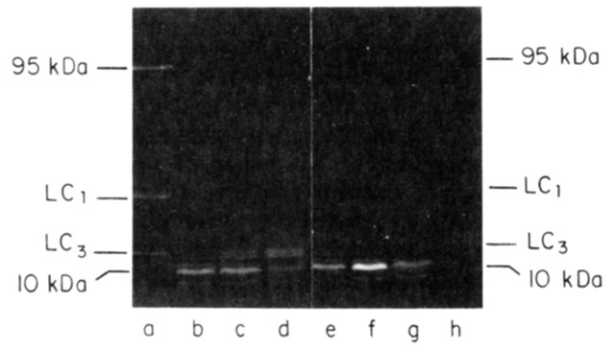


FIGURE 3: Fluorescence-observed $NaDodSO_4$ gel electrophoretograms of the CNBr fragments of myosin S-1 modified with various fluorophores. (b, c, d) S-1 modified for 10, 20, and 60 min, respectively, with DBB. (e, f) S-1 modified for 10 and 60 min, respectively, with MBB. (g) S-1 modified for 60 min with 1,5-IAEDANS. In lanes a and h is intact chymotryptically prepared S-1 labeled for 60 min with 1,5-IAEDANS in the presence of 8 M urea; their light chains 1 and 3 serve as molecular weight markers.

slower band. The slower band practically comigrates with the 16.5-kDa $LC_3$ (Figure 3, lane a). However, we know that cross-linking slows up electrophoretic migration; therefore, the cross-linked structure certainly weighs less than 16.5 kDa. Cys-402 would have created a structure weighing 19.5 kDa, so we feel that Cys-402 is probably not the cross-linked thiol. On the basis of this experiment, the most probable thiol is Cys-522, but the structure weights that would be created by Cys-522 and Cys-540 are too close to distinguish on this basis. Instead, we have made the identification by comparing the N-terminal end group actually found with the predicted end group for each possible cross-link. The slower fluorescent band is very clearly distinguished, as is the corresponding Coomassie blue band. So this slower band can be cut out from the gel and its contents eluted electrophoretically for end-group analysis. Whether the link is made with Cys-522 or Cys-540, the structure contains "10 kDa", whose N-terminal end group is Glu. However, the other N-terminal end group is Phe[3] if the link is with Cys-522 and Gly if the link is with Cys-540. The result was Glu and Phe. So the conclusion is that DBB joins Cys-707 of 20 kDa and Cys-522 of 50 kDa.

To study the kinetics and stoichiometry of DBB labeling, S-1 was progressively modified with DBB and was studied by PAGE. Each electrophoretogram (Figure 4A) was studied by its own fluorescence, and after staining it with Coomassie blue. As usual (Mornet et al., 1985), the modification generated a novel 105-kDa band and the expected 95-kDa band; the 95-kDa band decreased and the 105-kDa band increased during modification.

In order to study the kinetics of modifying S-1, we scanned the fluorescence of the 95-kDa band plus 105-kDa band of dibromobimane-modified S-1 as functions of incubation time and the fluorescence of the 95-kDa band of MBB-modified S-1 as functions of incubation time (Figure 4B). Both curves show that in a short time (within 10 min) modification almost reaches a maximum. The reaction of DBB can be shown in a diagram (Figure 4C). First DBB reacts rapidly with just one thiol (Cys-707 of 20 kDa) and then slowly with the second thiol (Cys-522 of 50 kDa); it is the second reaction which cross-links. In order to study the relation between cross-linked S-1 and modified S-1, we scanned the fluorescence of the 105-kDa band and the 95-kDa band as functions of incubation time, and to study the relation between cross-linked S-1 and total S-1 (modified plus unmodified S-1), we scanned the Coomassie blue stained 105-kDa band and the 95-kDa band as functions of incubation time. The results at 60-min incubation time (Figure 4D) show that 71% of the total S-1 is cross-linked and that 80% of the modified S-1 is cross-linked.

To study the effect of nucleotide on the kinetics of labeling, S-1 was progressively modified with dibromobimane or MBB in the presence of the following solutions: (1) MgATP; (ii) Mg; (iii) MgADP; (iv) Mg–pyrophosphate (all dissolved first in a small amount of methanol and then diluted in 20 mM TES, pH 7.6, buffer). First we studied whether the degrees of cross-linking are different in the four conditions of modification. We scanned the fluorescence of the 95-kDa band and 105-kDa band of the modified S-1 as functions of incubation time as described in the previous paragraph. The results (Figure 5) show that the degrees of cross-linking are similar. Second we studied whether the $Ca^{2+}$-ATPase activities are different in the presence or absence of nucleotide. Figure 6 shows the courses of $Ca^{2+}$-ATPase inhibition upon DBB or MBB modification in the presence or absence of nucleotide are essentially the same. Note, however, that the inhibition is never totally complete.

## DISCUSSION

The conclusions that seem to be established by the foregoing work are as follows: MBB reacts with a thiol that resides in the CNBr fragment known as p10; this fragment is known (Elzinga & Collins, 1977) to contain only the Cys-697 and Cys-707 thiols. MBB reacts with a thiol which after hydroxylamine cleavage resides in the 13-kDa fragment of 20 kDa; 13 kDa is known (Sutoh, 1981) to contain Cys-707 and one other thiol (not Cys-697). Only thiol Cys-707 meets both requirements, so we must conclude that MBB reacts with Cys-707 ("SH1"). The reaction of MBB with myosin S-1 is fast (as is usual for SH1 reactions). DBB is a molecule identical with MBB except that a bromine atom (DBB) replaces a hydrogen atom (MBB) at the second reactive position. When DBB reacts with S-1, it rapidly (at a rate comparable to MBB) imparts fluorescence to the protein, but the rate at which 50 kDa becomes cross-linked to 20 kDa is slow. We conclude from these observations that DBB reacts with S-1 in two phases—a rapid reaction with Cys-707 (of 20 kDa) followed by a slow reaction with a residue that resides on 50 kDa. In our usual observational time (ca. 60 min), very nearly 1 mol of DBB reacts with 1 mol of S-1. When the fluorescence distribution of the gel is also considered, there is no reason to think that DBB significantly engages in any reaction other than what we have just described. DBB is a compound designed (Kosower et al., 1979) to react specifically with two thiols, but the possibility that it reacts with Cys-707 and with

[3] Elzinga has indicated a Met-495, a Met/Glu heterogeneity at position 517, and a Met-530; so, if position 517 is occupied by Glu, then Cys-522 resides on a 35-residue peptide contributed by 50 kDa, the migrating structure is of 14.6 kDa, and Phe is the N end group contributed by 50 kDa, and if position 517 is occupied by Met, then Cys-522 residues on a 13-residue peptide contributed by 50 kDa, the migration structure is of 12.1 kDa, and Asp is the N end group contributed by 50 kDa. The bimane-containing band that is observed in PAGE suggests a 14.6-kDa component rather than a 12.1-kDa component. In the plate for end-group analysis, a spot for Phe is definite and bright, while a spot for Asp is only marginally detectable and diffuse; it is also possible to discern a second, very faint cross-linked structure band. So we are inclined to think that Glu is the predominant residue at position 517, though Met is no doubt present in a small amount. The conclusion that Cys-522 is the attachment for dibromobimane on 50 kDa is unaffected by the uncertainty of the heterogeneity, since the end groups predicted if Cys-402 or Cys-540 was the attachment would be His or Gly, whereas if Cys-522 is the attachment, they would be Phe or Asp (Glu would be an end group in any case because it is contributed by P10). The results were Phe (strong) and Asp (faint). So the conclusion is that DBB joins Cys-707 of 20 kDa and Cys-522 of 50 kDa.

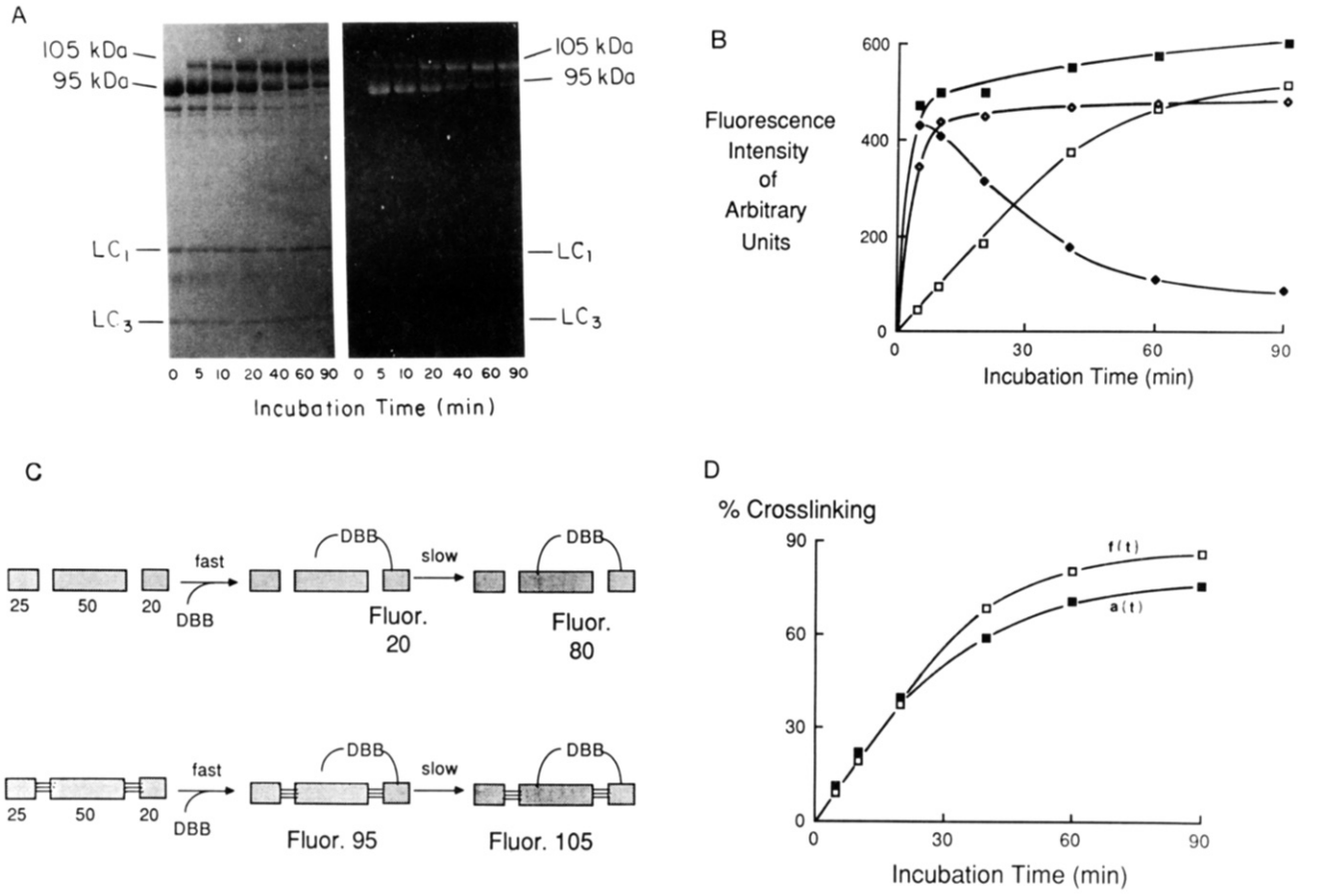


FIGURE 4: Time course of bimane modification of S-1, observed by making $NaDodSO_4$ gel electrophoretograms of aliquots drawn at various incubation times. (a) Experiment with DBB (analogous experiments with MBB were also done, but electrophoretograms are not shown); (left panel) Coomassie stained; (right panel) fluorescence observed. With the aid of a scanner, the Coomassie absorbance, or the fluorescence intensity, was integrated across each heavy-chain band of interest, producing an integral of value $S(t)$ where $t$ is the incubation time indicated below each lane. When DBB is the modifier, there are two fluorescent heavy-chain bands, an $S_{95}(t)$ and an $S_{105}(t)$; when MBB is the modifier, $S_{105}(t) = 0$. $S$ is proportional to the amount of a component—of a heavy-chain species in the case of absorbance and of modifier in the case of fluorescence; in fluorescence, however, the constant of proportionality may be different from different components. (B) Fluorescent (◇) $S_{95}(t) + S_{105}(t)$ for MBB modification [recall $S_{105}(t) = 0$ for MBB]. Also shown is the fluorescence (♦) $S_{95}(t)$, (□) $S_{105}(t)$, and (■) $S_{95}(t) + S_{105}(t)$ for DBB. Of course, $S_{95}(t) + S_{105}(t)$ is the same (roughly a constant) for MBB and DBB, but with DBB, it is clear that fluorescence is first rapidly imparted to the 95-kDa component, which then loses it to the 105-kDa component. The fluorescence $S_{95}(t)$ is so similar for DBB and MBB that it is reasonable to think that in both cases the same single thiol (in Figure 1 is evidence that this is Cys-707) is reacted. The transfer of fluorescence from 95 to 105 kDa, i.e., cross-linking, appears to be a much slower process. Thus, the model (C) seems to account for our observations. The upper portion in (C) shows DBB that reacts with split S-1; the lower portion shows DBB that reacts with S-1. In (D) are plotted $100S_{105}/(S_{105} + S_{95}) = a(t)$ for absorbance (■) and $100S_{105}/(S_{105} + S_{95}) = f(t)$ for fluorescence (□). $a(t)$ is the percent of heavy chain that is cross-linked, and $f(t)$ is the percent of bimane that is doubly ligated. The ratio $a(t)/f(t)$ would be "moles of bound dye per mole of heavy chain" if the constant of proportionality mentioned above were the same for singly and doubly bound dye; the limiting value with time of this ratio would be the stoichiometry of labeling, but this number is best obtained otherwise (see Table I). In fact, the highest value of the ratio provided by the gels of (D) is near unity.

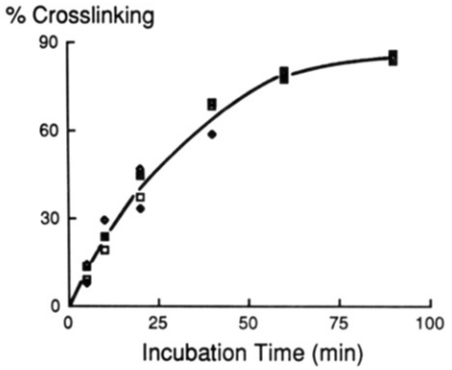


FIGURE 5: Examination of nucleotide effect upon DBB labeling kinetics. This is a plot of $f(t)$ (Figure 4D). During labeling, 5 mM each of MgATP (□), $MgCl_2$ (♦), MgADP (■), and $MgPP_i$ (◇) was present.

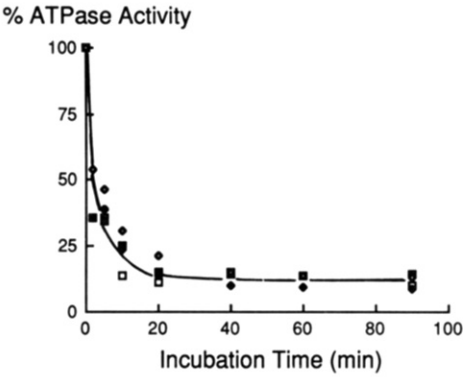


FIGURE 6: Effect upon $Ca^{2+}$-ATPase activity of labeling with nucleotide. (□) DBB + 5 mM MgATP; (♦) DBB + 5 mM $MgCl_2$; (■) MBB + 5 mM MgATP; (◇) MBB + 5 mM $MgCl_2$.

a non-thiol residue of 50 kDa cannot be excluded a priori. However, our thiol titration shows that a result of reacting DBB with S-1 is that approximately two thiols of S-1 become inaccessible to the titrant. This leads us to conclude that the residue of 50 kDa that is cross-linked is also a thiol. Fragment

50 kDa contains only three thiols, Cys-402, Cys-522, and Cys-540. Following CNBr fragmentation of the cross-linked system into various pieces, the piece containing the cross-link moves too fast in PAGE to be the piece that would be generated by cross-linking Cys-402 and moves with the speed that might be anticipated from a cross-link with Cys-522. Furthermore, the N end group in this fluorescent piece (other than the end group of P10) is Phe-496, precisely the N end group expected if the cross-link is with Cys-522. So we conclude that DBB cross-links Cys-522 of 50 kDa with Cys-707 of 20 kDa. The distance between the two reactive groups of DBB is about 0.7 nm, so, after cross-linking, Cys-522 and Cys-707 are in close proximity. However, a priori this proximity need not be a feature of the unmodified structure. However, the experiments of our colleague Kasprzak (1986), indicating that the distances between MBB (which does not cross-link) and DBB (which does cross-link) to certain tryptophans are indistinguishable, suggest that the proximity preexists; i.e., the proximity is a feature of the *native* structure. Considered as an "SH1 reagent", MBB is interesting and unusual because it *inhibits* $Ca^{2+}$-ATPase; moreover, it inhibits to a *nonzero* asymptotic level. DBB behaves essentially the same way. From the similarity in this behavior, we conclude that the inhibition is not due to cross-linking but perhaps to the foreign bulk being introduced into this region (Botts et al., 1979). Possibly the bimanes sterically hinder, but do not totally abolish, movements that are part of the ATPase process. Finally, the rate of labeling with MBB and DBB is independent of the presence of Mg–nucleotide. Since the rate of cross-linking determines the rate of labeling, we conclude that the residence of Mg–nucleotide does not appreciably alter the spatial relations (either separation distance or relative orientation) between Cys-522 and Cys-707. This result and the result of Lu et al. (1986), indicating that Mg–nucleotide *does* affect cross-linking with 50 kDa by a Cys-707-based photoaffinity cross-linker [Sutoh's personal communication has shown that Lu's analogue (benzophenone iodoacetamide) reacts somewhere on the stretch 500–522], together define regions that are static and mobile during the process.

If we accept the foregoing conclusions, we can say that in the native S-1 structure there is a "loop" of at least 185 residues, made in part by 50 kDa and in part by 20 kDa. Whether the interfragment proximity extends to <522 and >707 is not known, but the discovery of another 50-kDa to 20-kDa cross-link involving Cys-697 and possibly Cys-540 (Chaussepied et al., 1986b) suggests that the proximity may continue in the opposite direction (>522 and <707). This latter case is interesting because it has been reported (Chaussepied et al., 1986) that a "30-kDa" fragment extending from >561 to the C-terminus of S-1 can be renatured and exhibits actin binding as well as dissociation by polyphosphate. This report has led to speculations that both polyphosphate and actin interact on the loop (Morales, 1986).

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