Location of SH_1 and SH_2 along a Heavy Chain of Myosin Subfragment 1[†]

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ABSTRACT: Two reactive SH groups (SH_1 and SH_2) of myosin subfragment 1 (S-1) were selectively labeled with a fluorescent dye, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM). When the DACM-S-1 was digested with trypsin, 95K heavy chain was cleaved into 50K, 25K, and 20K fragments, and the fluorescent labels incorporated into SH_1 and SH_2 were exclusively found in the 20K fragment, indicating that these SH groups were located in the fragment. When the trypsin-treated DACM-S-1 was subsequently fragmented with hydroxylamine in 6 M guanidine hydrochloride at pH 9.0, the fluorescent 20K fragment was cleaved into two fluorescent segments, the 13K segment containing SH_1 and the 5K segment containing SH_2. Since it is known that SH_1 and SH_2 are only 10 residues apart in the sequence and that SH_1 is nearer the COOH terminus than SH_2, the results show that these reactive SH groups are separated from the COOH terminus of S-1 heavy chain by a 13K-dalton stretch of polypeptide chain.

It is well established that modification of two reactive SH groups within a myosin head significantly alters activity properties of myosin ATPases. Modification of one of these groups, referred to as SH_1, results in a marked elevation of Ca–ATPase activity with a concomitant loss of EDTA[1]–ATPase activity. Subsequent modification of the other SH group, referred to as SH_2, leads to a complete loss of both Ca– and EDTA–ATPase activities, suggesting that these SH groups are located near or at the active site of myosin ATPase (Sekine et al., 1962; Sekine & Kielley, 1962; Sekine & Yamaguchi, 1963; Reisler et al., 1974a). A series of experiments to cross-link SH_1 and SH_2 in the presence of various ligands such as MgADP (Reisler et al., 1974b; Burke & Reisler, 1977; Wells et al., 1979a,b; Wells & Yount, 1979, 1980) have shown that spatial distance between SH_1 and SH_2 changes from >12 to 2 Å, in response to the binding of Mg nucleotide to myosin. It has been also shown that Mg nucleotide is trapped in the active-site cleft when SH_1 and SH_2 are cross-linked in the presence of the ligand (Wells et al., 1979a,b; Wells & Young, 1979, 1980). These studies suggest the structural and functional importance of SH_1 and SH_2 for ATPase activities, even though their direct involvement in MgATP hydrolysis seems unlikely (Wiedner et al., 1978; Botts et al., 1979).

Elzinga & Collins (1977) have isolated a peptide containing both SH_1 and SH_2 from the CNBr digest of myosin and determined its amino acid sequence. The peptide contains 92 amino acids including two cysteinyl residues (SH_1 and SH_2). Although their exact locations along the myosin heavy chains are not known, radioactive labeling of SH_1 and SH_2 and subsequent digestion of the labeled heavy chain with trypsin have shown that these SH groups are located in the 20K fragment which is positioned at the COOH-terminal section of the heavy chain of the myosin head (Balint et al., 1978), indicating that the peptide isolated by Elzinga & Collins (1977) occupies some part of the 20K fragment.

In view of importance of locaing SH_1 and SH_2 along the myosin heavy chain for understanding the structure of the ATPase active site, experiments were carried out to determine their positions, exploiting the facts that an Asn–Gly bond is present between SH_1 and SH_2 which are only 10 residues apart in the sequence (Elzinga & Collins, 1977) and that the bond is very susceptible to hydroxylamine cleavage (Bornstein & Balian, 1977).

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Materials and Methods

Myosin subfragment 1 (S-1) and heavy meromyosin (HMM) were prepared from rabbit skeletal muscle myosin according to Weeds & Taylor (1975). *N*-[7-(Dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) was purchased from Wako Chemical Co.

For demonstration of the specificity of DACM labeling at SH_1 and SH_2, S-1 (2 mg/mL) in 30 mM KCl, 20 mM Tris-HCl, and 1 mM MgADP (pH 7.9) was reacted with varying amounts of DACM (0–3 mol of DACM/mol of S-1) for 10 min at 0 °C. After termination of the reaction by addition of 0.01 volume of 100 mM *N*-acetylcysteine (pH 7.0), EDTA– and Ca–ATPase activities were measured according to Kielley & Bradley (1956) and Kielley et al. (1956) at 37 °C.

Throughout the present work, 1.6 mol of DACM/mol of S-1 was employed to selectively label both SH_1 and SH_2 at the same time. Reaction conditions were the same as those described above. For selective labeling at SH_2, SH_1 was blocked by a Dnp group in advance. S-1 (2 mg/mL) in 30 mM KCl and 20 mM Tris-HCl (pH 7.9) was treated with a 1.4-fold molar excess of fluorodinitrobenzene (FDNB) for 1 h at 0 °C and then exhaustively dialyzed against the same solvent. The resulting Dnp-S-1 was subsequently modified with 0.6 mol of DACM/mol of Dnp-S-1 in 30 mM KCl, 20 mM Tris-HCl, and 1 mM MgADP (pH 7.9) for 10 min at 0 °C. The reaction was terminated by addition of *N*-acetylcysteine as before. After each step of modifications, EDTA– and Ca–ATPase activities were measured to check if reactions proceeded quantitatively.

DACM-modified S-1 (2 mg/mL) was digested with trypsin (0.02 mg/mL) in 30 mM KCl, 20 mM Tris-HCl, and 1 mM MgADP (pH 7.5) for 10 min at 20 °C, and then the reaction was terminated with trypsin inhibitor (0.06 mg/mL). Under the conditions, the 95K heavy chain was fragmented into 75K, 50K, 25K, and 20K fragments.

The 20K fragment was purified electrophoretically. Electrophoresis was carried out according to Laemmli (1970), employing 16% acrylamide–0.43% bis(acrylamide) in the

[1] Abbreviations used: S-1, myosin subfragment 1; HMM, heavy meromysin; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]-maleimide; FDNB, fluorodinitrobenzene; Dnp, dinitrophenyl; $NaDodSO_4$, sodium dodecyl sulfate; AL 1, alkaline light chain 1; AL 2, alkaline light chain 2; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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presence of 6 M urea for a separation gel. After electrophoresis, a fluorescent band corresponding to the 20K fragment was cut out under UV illumination, and the peptide was extracted from the acrylamide gel with 0.1% $NaDodSO_4$ at room temperature for 4 h. The resulting fluorescent solution was dialyzed against 6 M urea, 20 mM sodium acetate, and 1% 2-mercaptoethanol (pH 5.4) to remove $NaDodSO_4$ and then against 6 M guanidine hydrochloride, 20 mM sodium acetate, and 1% 2-mercaptoethanol (pH 5.4).

Fragmentation of polypeptides with hydroxylamine was carried out according to Bornstein & Balian (1977). Protein (2–4 mg/mL) in 6 M guanidine hydrochloride, 20 mM sodium acetate, and 1% 2-mercaptoethanol (pH 5.4) was mixed with an equal volume of 2 M hydroxylamine-6 M guanidine hydrochloride (pH 9.0), and the pH of the resulting solution was quickly adjusted to 9.0 by addition of 0.1 N NaOH. The solution was kept at 45 °C for various times, and the reaction was quenched by addition of 0.1 volume of acetic acid. The resulting solution was dialyzed against water and then against 1% $NaDodSO_4$, 20 mM Tris-HCl, and 0.1% 2-mercaptoethanol (pH 8.3) for gel electrophoresis.

Fragments produced by the hydroxylamine cleavage were analyzed by acrylamide gel electrophoresis (Laemmli, 1970), using 16% acrylamide–0.43% bis(acrylamide) in the presence of 6 M urea for a separation gel. When it was necessary to see materials with high molecular weights, 15% acrylamide–0.2% bis(acrylamide) was used for a separation gel. Apparent molecular weights of various fragments were estimated according to Weber & Osborn (1969) by using the following proteins as standards: troponin T (M_r 30 500), troponin I (M_r 21 000), troponin C (M_r 18 000), myoglobin (M_r 17 200), cytochrome *c* (M_r 11 700), CNBr fragments of myoglobin (M_r 14 900, 8300, 6400, and 2600), and CNBr fragments of cytochrome *c* (M_r 7100, 2800, and 1800).

Results

Selective Labeling of SH Groups with DACM. The specificity of DACM labeling at SH_1 and SH_2 was checked to ensure selective incorporation of the fluorescent dye into these SH groups. When S-1 was reacted with varying amounts of DACM in low ionic strength solvent at pH 7.9 in the presence of MgADP, it was observed that a stoichiometric amount of DACM (2 mol of DACM/mol of S-1) was enough to virtually abolish both Ca– and EDTA–ATPase activities (Figure 1), though a low level of Ca–ATPase activity (~10% of the activity of unmodified S-1) persistently remained even after addition of an excess amount of the fluorescent dye. It was also observed that both Ca– and EDTA–ATPase activities decreased concomitantly at any DACM/S-1 ratio studied. The results show that SH_1 and SH_2 are selectively labeled with DACM as long as less than a stoichiometric amount of the fluorescent dye is used for labeling SH groups. Therefore, throughout the present work, 1.6 mol of DACM/mol of S-1 was used for the labeling reaction. The S-1 whose SH_1 and SH_2 are labeled with DACM is denoted as DACM(SH_1–SH_2)-S-1.

S-1 was first reacted with a 1.4-fold molar excess of FDNB in low ionic strength solvent at pH 7.9 in the absence of MgADP (Burke & Reisler, 1977) in order to label only SH_2. The resulting Dnp-S-1 exhibits a complete loss of EDTA–ATPase activity and 3.7-fold activation of Ca–ATPase activity, attesting to complete and selective modification of SH_1 by the Dnp group. Subsequent modification of the Dnp-S-1 with 0.6 mol of DACM/mol of Dnp-S-1 in the presence of MgADP reduced Ca–ATPase activity by 50–60%, indicating that SH_2 was selectively labeled with the fluorescent dye. The S-1 is denoted as DACM(SH_2)-S-1.

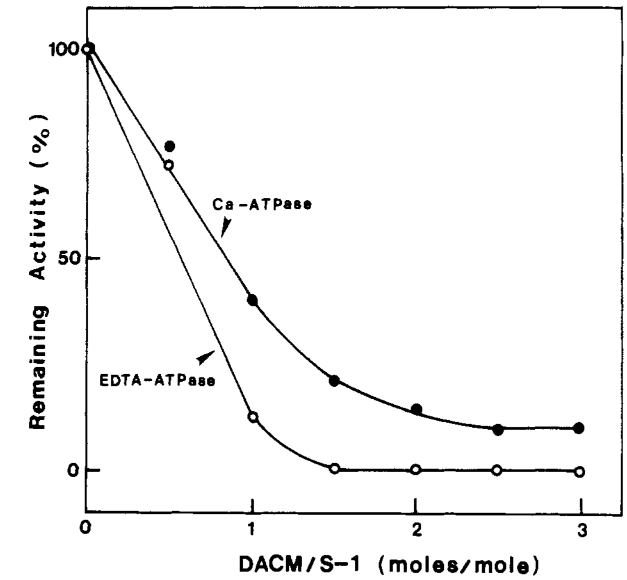

FIGURE 1: Inactivation of Ca– and EDTA–ATPase activities of S-1 with fluorescent dye, DACM. Reaction conditions were as follows: solvent, 30 mM KCl, 20 mM Tris-HCl, and 1 mM MgADP (pH 7.9) at 0 °C; reaction time, 10 min; concentration of S-1, 2 mg/mL; concentration of DACM, 0–3 mol of DACM/mol of S-1. 100% activity is 4.2 μmol of P_i/(mg·min) for EDTA–ATPase activity and 1.8 μmol of P_i/(mg·min) for Ca–ATPase activity. ATPase activities were measured at 37 °C.

Digestion of DACM-Labeled S-1 with Trypsin. When DACM(SH_1–SH_2)-S-1 was digested with trypsin, 95K S-1 heavy chain was cleaved into 20K and 75K fragments at an early stage of digestion, and then the 75K fragment was transformed into 25K and 50K fragments with prolonged digestion, consistent with previous reports (Mornet et al., 1979; Yamamoto & Sekine, 1980). On a $NaDodSO_4$ gel of trypsin-treated DACM(SH_1–SH_2)-S-1, 75K, 50K, 25K, and 20K fragments of S-1 heavy chain together with two alkaline light chains (23K and 17K) were observed (second lane in Figure 2b). When the fluorescence of DACM incorporated in SH_1 and SH_2 was detected on the $NaDodSO_4$ gel, it was observed that only the 20K fragment was fluorescent among these fragments and light chains (second lane in Figure 2a). The result indicates that the 20K fragment contains both SH_1 and SH_2. A similar conclusion has been obtained by Balint et al. (1978), who employed radioactive labeling of SH groups to locate SH_1 and SH_2 along a myosin heavy chain.

Fragmentation of S-1 Heavy Chain and 20K Fragment with Hydroxylamine. For further fragmentation of the 20K fragment containing SH_1 and SH_2, trypsin-treated DACM(SH_1–SH_2)-S-1 was subsequently treated with 1 M hydroxylamine in 6 M guanidine hydrochloride at pH 9.0. Under the conditions, hydroxylamine is known to cleave a polypeptide chain at an Asn–Gly bond (Bornstein & Balian, 1977), and S-1 contains at least one such bond located between SH_1 and SH_2, which are only 10 residues apart in the sequence (Elzinga & Collins, 1977). Therefore, it is expected that hydroxylamine would cleave the 20K fragment at the Asn–Gly bond into at least two segments.

Patterns of fluorescent bands on a $NaDodSO_4$ slab gel shown in Figure 2a indicate that two fluorescent segments with apparent molecular weights of 13 000 and 5 000 are gradually produced with concomitant loss of the fluorescent 20K fragment on incubating the trypsin-treated DACM(SH_1–SH_2)-S-1 with hydroxylamine. The slab gel presented in Figure 2a was stained with Coomassie Blue and resulting band patterns were

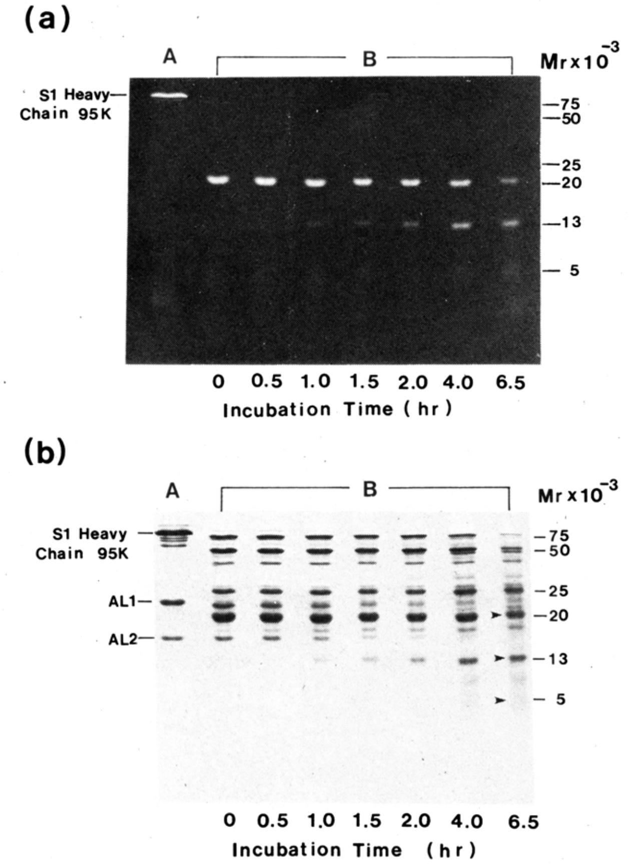

FIGURE 2: Time course of hydroxylamine fragmentation of trypsin-treated DACM(SH_1–SH_2)-S-1. DACM(SH_1–SH_2)-S-1 was digested with trypsin and then treated with 1 M hydroxylamine in 6 M guanidine hydrochloride (pH 9.0) for various times (0–6.5 h) at 45 °C. (a) Fluorescence of DACM incorporated in SH_1 and SH_2 was detected by illuminating a $NaDodSO_4$ slab gel [16% acrylamide–0.43% bis(acrylamide)] with a UV lamp. (b) The same gel shown in (a) was stained with Coomassie Brilliant Blue. Lanes labeled A are intact DACM(SH_1–SH_2)-S-1 as controls. Lanes labeled B are trypsin-treated DACM(SH_1–SH_2)-S-1 after various times of incubation with hydroxylamine. Arrowheads in (b) indicate positions of fluorescent bands (20K, 13K, and 5K) observed in (a).

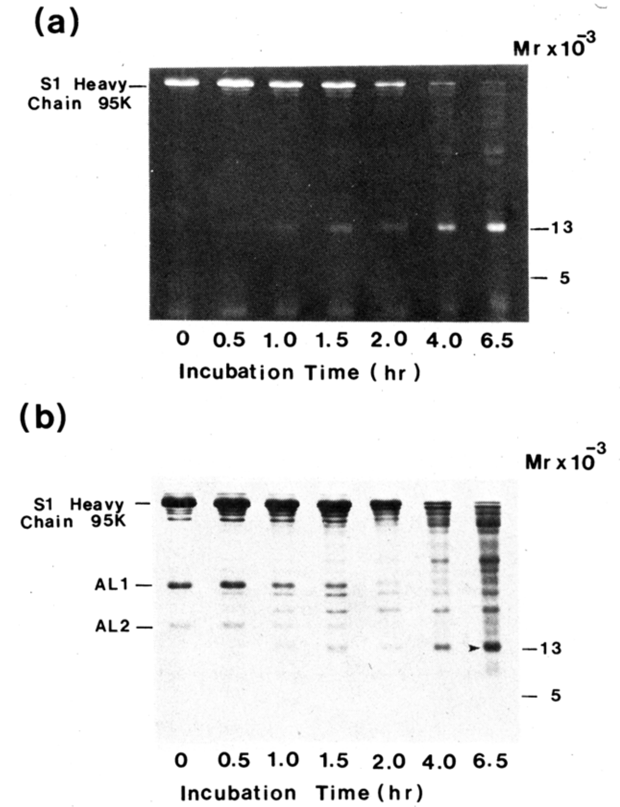

FIGURE 3: Time course of hydroxylamine fragmentation of DACM-(SH_1–SH_2)-S-1. (a) Fluorescence of DACM incorporated in SH_1 and SH_2 was detected by illuminating a $NaDodSO_4$ gel [16% acrylamide–0.43% bis(acrylamide)] with a UV lamp. (b) The same gel shown in (a) was stained with Coomassie Brilliant Blue. Note that the 5K segment is not observed when detected either by fluorescence or by Coomassie staining. Conditions for hydroxylamine treatment were the same as those described in Figure 2. Proteins are incubated with hydroxylamine up to 6.5 h.

shown in Figure 2b. The Coomassie-stained bands corresponding to the fluorescent 20K, 13K, and 5K segments are indicated by arrowheads in the figure. The 13K segment is easily detected even at an early stage of hydroxylamine treatment, while the presence of the 5K segment becomes apparent only at later stage because of its diffuse appearance. As shown later, the 13K segment does not contain SH_2 while the 5K segment does, inferring that the 13K segment is not a precursor of the 5K segment. Thus, it seems likely that hydroxylamine cleaves the 20K fragment into at least two segments (13K and 5K), both of which contain SH_1 and/or SH_2.

It must be noted here that two alkaline light chains were also fragmented with hydroxylamine since they contain an Asn–Gly bond in their sequence (Frank & Weeds, 1974). Fragmentation of two alkaline light chains is also recognized in Figures 3b and 4b.

When intact S-1 heavy chain labeled with DACM was subjected to hydroxylamine treated under the same condition employed for the trypsin-treated DACM(SH_1–SH_2)-S-1, the 13K segment was again produced as a major fluorescent material with concomitant loss of the 95K heavy chain, as shown in Figure 3. On the other hand, no material corresponding to the 5K segment was detected in fragmentation products of intact S-1 heavy chain as shown in Figure 3b. The former finding suggests that the 13K segment originates from the COOH-terminal section of the parent 20K fragment since the 20K fragment and the S-1 heavy chain have the same COOH terminus (Balint et al., 1978). The latter finding suggests that the 5K segment originates from the NH_2-terminal section of the parent 20K fragment and contains its NH_2 terminus.

When DACM(SH_2)-S-1 was digested with trypsin and then fragmented with hydroxylamine, the resulting fluorescence band pattern was different from that of DACM(SH_1–SH_2)-S-1 as shown in Figure 4a. The hydroxylamine fragmentation of trypsin-treated DACM(SH_1–SH_2)-S-1 transformed the fluorescent 20K fragment into the fluorescent 13K and 5K segments (lanes A in Figure 4a) as described before. On the other hand, hydroxylamine fragmentation of trypsin-treated DACM(SH_2)-S-1 produced only the 5K segment as a fluorescent segment (lanes B in Figure 4a). Staining of the $NaDodSO_4$ slab gel shown in Figure 4a by Coomassie Blue revealed that both the 13K and 5K segments were produced by hydroxylamine fragmentation of trypsin-treated DACM-(SH_2)-S-1 as shown in Figure 4b. The fluorescent segments (20K, 13K, and 5K) are indicated by arrowheads in the figure. Thus, the absence of fluorescence in the 13K segment originating from DACM(SH_2)-S-1 means that the segment does not contain SH_2, though it contains SH_1. Moreover, the result shows that the 5K segment contains SH_2.

Hydroxylamine Cleavage of Purified 20K Fragments. The 20K fragment of DACM(SH_1–SH_2)-S-1 and that of DACM-(SH_2)-S-1 were purified electrophoretically and cleaved with hydroxylamine as described above in order to fortify the above

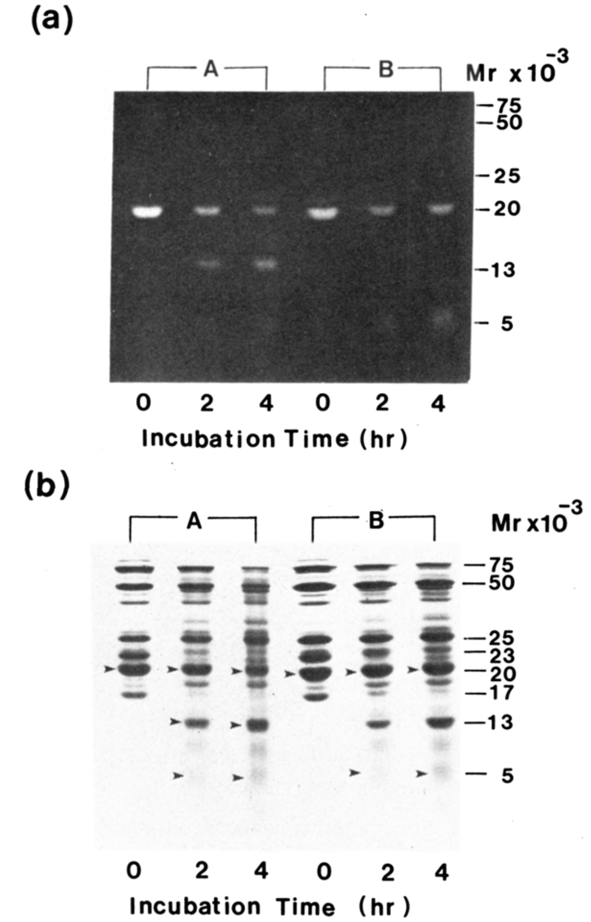

FIGURE 4: Comparison of hydroxylamine fragmentation of trypsin-treated DACM(SH_1–SH_2)-S-1 and that of trypsin-treated DACM-(SH_2)-S-1. (a) Fluorescence of DACM was detected by illuminating a $NaDodSO_4$ gel [16% acrylamide–0.43% bis(acrylamide)] with a UV lamp. (b) The same gel shown in (a) was stained with Coomassie Brilliant Blue. Lanes labeled (A) are for DACM(SH_1–SH_2)-S-1 and those labeled (B) for DACM(SH_2)-S-1. Arrowheads in (b) indicate positions of fluorescent bands observed in (a). Note that the 13K segment is not fluorescent when DACM(SH_2)-S-1 was treated with trypsin and then with hydroxylamine (lanes B). Conditions for hydroxylamine treatment were the same as those described in Figure 2. Proteins are incubated with hydroxylamine for 2 and 4 h.

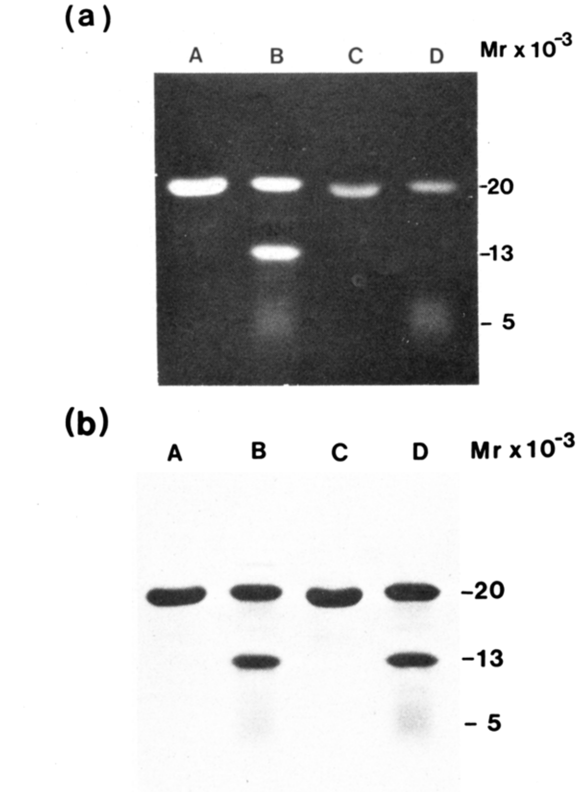

FIGURE 5: Hydroxylamine cleavage of purified 20K fragments of DACM(SH_1–SH_2)-S-1 and DACM(SH_2)-S-1. (a) Fluorescence of DACM was detected by illuminating a $NaDodSO_4$ gel [16% acrylamide–0.43% bis(acrylamide)] with a UV lamp. (b) The same gel shown in (a) was stained with Coomassie Brilliant Blue. (Lane A) Purified 20K fragment of DACM(SH_1–SH_2)-S-1. (Lane B) The 20K fragment shown in lane A treated with hydroxylamine for 4 h. (Lane C) Purified 20K fragment of DACM(SH_2)-S-1. (Lane D) The 20K fragment shown in lane C treated with hydroxylamine for 4 h. Conditions for hydroxylamine treatment were the same as those described in Figure 2.

notion that the 20K fragment is cleaved into the 13K and 5K segments on hydroxylamine treatment. The purified 20K fragments were homogeneous when checked by the acrylamide gel electrophoresis in the presence of $NaDodSO_4$ (lanes A and C in Figure 5). Hydroxylamine treatment of these 20K fragments resulted in the formation of the 13K and 5K segments as shown in lanes B and D in Figure 5b. When the 20K fragment of DACM(SH_1–SH_2)-S-1 was cleaved, both the 13K and 5K segments were fluorescent (lane B in Figure 5a), whereas hydroxylamine cleavage of the 20K fragment of DACM(SH_2)-S-1 produced only the 5K segment as a fluorescent product (lane D in Figure 5a), consistent with the results shown above.

Comparison of Fragmentation Pattern of HMM and S-1. SH_1 and SH_2 of HMM were selectively labeled with DACM in a way similar to that employed for the DACM labeling of S-1 by using 1.6 mol of DACM/mol of myosin head. The DACM-labeled HMM was fragmented with hydroxylamine as before. The resulting fluorescence band pattern on a $NaDodSO_4$ gel (Figure 6) shows that the fluorescent 13K segment is not a product of hydroxylamine fragmentation of HMM heavy chain. Two fluorescent segments with apparent molecular weights of ~80K seem to be major products from the

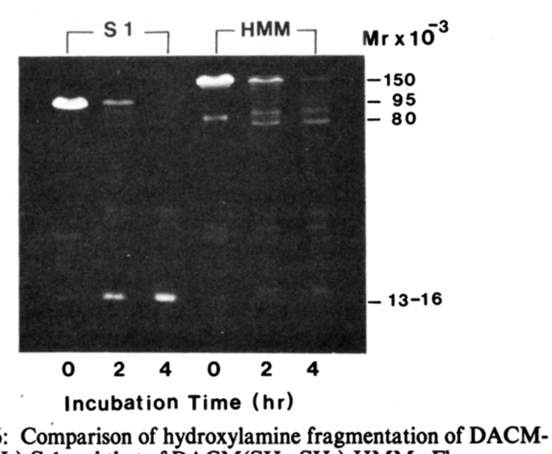

FIGURE 6: Comparison of hydroxylamine fragmentation of DACM-(SH_1–SH_2)-S-1 and that of DACM(SH_1–SH_2)-HMM. Fluorescence of DACM incorporated in SH_1 and SH_2 was detected by illuminating a $NaDodSO_4$ gel [15% acrylamide–0.2% bis(acrylamide)] with a UV lamp. Note that the 13K fluorescent segment is not observed when HMM is treated with hydroxylamine. Conditions for hydroxylamine treatment were the same as those described in Figure 2. Proteins were incubated with hydroxylamine for 2 and 4 h.

150K HMM heavy chain. Since HMM heavy chain has an ~60K-dalton stretch of polypeptide chain beyond the COOH terminus of the 95K S-1 heavy chain (Sutoh et al., 1978), the above result implies that the 13K segment contains the COOH terminus of the S-1 heavy chain and, therefore, is the product of a single-site cleavage of a polypeptide chain at the Asn–Gly

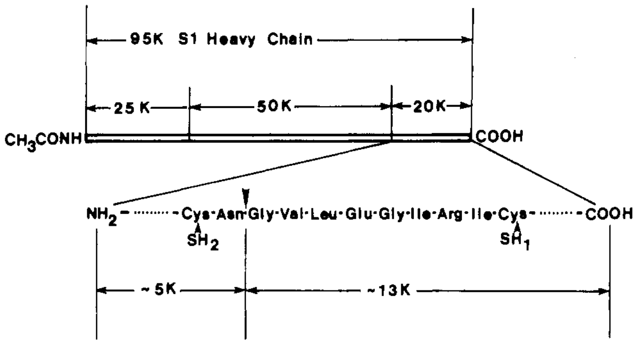

FIGURE 7: Schematic drawing of arrangement of various segments along a S-1 heavy chain. Trypsin digests the 95K S-1 heavy chain into 50K, 25K, and 20K fragments. Hydroxylamine cleaves the 20K fragment into 13K and 5K segments. The cleavage site (an Asn–Gly bond between SH_1 and SH_2) is indicated by an arrowhead in this figure.

bond located between SH_1 and SH_2.

Discussion

Trypsin cleaves the 95K S-1 heavy chain into three fragments with apparent molecular weights of 50K, 25K, and 20K as illustrated in Figure 7 (Balint et al., 1978; Mornet et al., 1979; Yamamoto & Sekine, 1980). Fluorescent labels incorporated in SH_1 and SH_2 were exclusively found in the 20K fragment as shown in Figure 2a, indicating that these SH groups were located in the fragment, consistent with previous reports (Balint et al., 1978; Yamamoto & Sekine, 1980). Further fragmentation of the 20K fragment at the Asn–Gly bond located between SH_1 and SH_2, which are 10 residues apart (Elzinga & Collins, 1977), produced two smaller segments, the 13K segment containing SH_1 and the 5K segment containing SH_2 (Figures 4 and 5). The 13K segment containing the COOH terminus of the parent 20K fragment while the 5K segment contains its NH_2 terminus, indicating that the two segments encompass the whole 20K fragment. Although the sum of molecular weights of the two segments is not equal to that of the parent fragment, the discrepancy seems insignificant considering the limitations in determining molecular weights of polypeptide chains by $NaDodSO_4$ gel electrophoresis. Sites of SH_1 and SH_2 along the S-1 heavy chain have been deduced as shown in Figure 7 by taking the above observations into account.

After completion of the present work, I have learned that the primary structure of the 20K fragment has been reported by Gallagher & Elzinga (1980) in an abstract form. According to their reports, the 20K fragment contains 168 amino acid residues. SH_1 and SH_2 are the 66th and 56th residues, respectively, from the NH_2 terminus. On the basis of their amino acid sequences, molecular weights of the two segments produced by the hydroxylamine cleavage at the Asn–Gly bond located between SH_1 and SH_2 are calculated to be ~13K and ~7K. The 13K segment contains SH_1 and the 7K segment SH_2, consistent with the results presented above, though the molecular weight of the SH_2-containing segment is slightly underestimated in this paper.

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