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THE INTERNAL CROSSLINKING OF THE S1 HEAVY CHAIN FROM SMOOTH MUSCLE PROBED BY DIBROMOBIMANE

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SUMMARY: The reaction of the crosslinker dibromobimane has recently revealed a functionally important internal loop structure within the skeletal myosin S1 heavy chain where Cys-522 of the 50K domain and Cys-707 (SH1) of the 20K region are spatially juxtaposed. Here we have studied the possible relevance of this topological feature to the architecture and transducing activity of the myosin head in general, by extending the dibromobimane modification to smooth muscle myosin. Treatment of chicken gizzard myosin S1 with dibromobimane resulted in intramolecular crosslinking between the C-terminal 25K and central 50K segments of the S1 heavy chain. The data suggest a conservation at the 50K-25K interface of smooth muscle S1 heavy chain and the importance of the neighboring SH_1 region, whose conformation may play an important role in energy transduction by the myosin head.

During muscle contraction, the head of the myosin molecule (S1) is involved in detachment and reattachment to the actin filament in the presence of Mg^{2+}-ATP. Because the S1 heavy chain contains the actin and nucleotide binding sites and is directly involved in the expression of actomyosin ATPase (1-3), knowledge of its internal structure is essential for understanding the mechanism of energy transduction in motile systems.

Intramolecular crosslinking experiments on the myosin S1 domains reported

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Abbreviations: S1: myosin subfragment-1;EDC: 1-ethyl-3 (3-(dimethylamino) propyl) carbodiimide; K or kDa: kilodalton(s); Mes: 2-(N-morpholinoethane) sulfonic acid; SDS: sodium dodecyl sulfate; Hepes: 4 (2-hydroxyethyl)-1-piperazinethane sulfonic acid; DTNB: 5,5'-Dithiobis (2-nitrobenzoic acid); Tes: N-Tris(hydroxymethyl) methyl-2-aminoethyl sulfonic acid; IAEDANS: N-iodoacetyl-N'-(5-sulfo-1-naphtyl)ethylenediamine;DB:dibromobimane; BPIA: 4-(2-iodoacetamido)benzophenone.

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previously (4-9) showed that dibromobimane, a fluorescent bifunctional reagent, promotes the freezing of a pre-existing loop segment between the 20K and 50K domains of the myosin heavy chain from skeletal muscle (10).

The present work was undertaken to confirm the conservation of this relationship between the actin interaction and the heavy chain topography of the myosin S1 heavy chain from smooth muscle, which is composed of three domain-like fragments of 29K, 50K, and 25K, as in the case of skeletal muscle S1 (11). We report here on the reaction of dibromobimane with myosin S1 and we show that the properties of the crosslinked S1 from smooth muscle are similar of the S1 from skeletal muscle (9).

MATERIALS AND METHODS

Protein preparations :

Myosin was prepared from fresh chicken gizzards and digested into S1 (12) with mercuripapain (Sigma Chemical Co.). In the S1 preparation at least 95% of the 97K heavy chains were intact and 50% of the L-20K light chains were undegraded (13). Skeletal chymotryptic S1 was obtained according to Weeds and Taylor (14). The proteolytic derivative (29K-50K-25K) S1 was isolated as previously described (11). Smooth S-1 was fluorescently labeled with 1,5 IAEDANS (15). Rabbit skeletal muscle F-actin was isolated by the procedure of Eisenberg and Kielly (16). Protein concentrations were determined with an extinction coefficient $E^{1\%}_{280\ nm} = 4.5\ cm^{-1}$ for gizzard myosin and $11.0\ cm^{-1}$ for skeletal actin. The concentrations of S1 and all other cleaved derivatives were determined according to Bradford (17) with skeletal S1 as the standard. Calculations were based on molecular weights of 42,000 for actin and 115,000 for S1.

Labeling of smooth S1 with dibromobimane and monobromobimane:

The stock solutions of the chemical fluorescent agents, dibromobimane (DB) or monobromobimane (Calbiochem.) were prepared daily in pure methanol and used at a 5 molar excess over S1. The reaction was performed for 1h at room temperature in 5 mM Tes buffer, pH 7.6, in the presence of 5 mM Mg^{2+}-ATP(6,9).

S1 ATPase Activities :

To follow the time course of changes in S1 ATPase activities during the dibromobimane reactions, aliquots were withdrawn at various times and assayed for Ca^{2+}-, K^{+} EDTA- and Mg^{2+}-ATPases, as previously described (18).

Cross-linking of F-actin-S1 complexes with carbodiimide :

The association between F-actin and native or dibromobimane-modified S1 from smooth muscle S1 (9) was stabilized using EDC (12).

Sodium dodecyl sulfate gel electrophoresis :

All reactions were monitored by electrophoresis in 0.1 % SDS/polyacrylamide slab gels using a 5-18 % acrylamide gradient (18). The optical densities of Coomassie-blue-stained protein bands were measured with a Shimadzu model S-930 high-resolution gel scanner equipped with a computerized integrator.

Thiol titration with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) :

The thiol content of unmodified and modified S1 was measured using Ellman's reagent in the presence or absence of 5 M guanidinium chloride (4).

Determination of affinity constants :

S1 (6 µM) in 10mM Tris HCl buffer, pH 8.0, containing 10 mM KCl, 2.5 mM $MgCl_2$ and 0.5 mM dithioerythritol was mixed with varying concentrations of

rabbit F-actin (0-150 μM). After cosedimentation at 150,000 x g for 30 min in a Beckman airfuge (19), the supernatants were subjected to SDS polyacrylamide slab gel electrophoresis and the amounts of free native and modified-S-1s were estimated from the intensities of the 97K or 95K and 107K or 105K heavy chains bands, respectively, to determine their actin affinity constants.

RESULTS

Modification of the structure and activity of smooth myosin S1:

When a 5-fold excess of dibromobimane was allowed to react with the native papain smooth myosin S1, the electrophoretogram of the reaction mixture (Fig.1) revealed that the original 97K heavy chain band was converted into a new 107K band after 60 min of modification, with a maximum yield of 40 %. Densitometric measurements of the protein bands further indicated no significant change in the amount of L-20K and L-17K light chains linked with the 97K heavy chain converted to the 107K protein band. Lane B (Fig.1) shows the fluorescence pattern of the 97K and the 107K heavy chains after 60 min of DB treatment; some fluorescence was also incorporated into LC-17K.

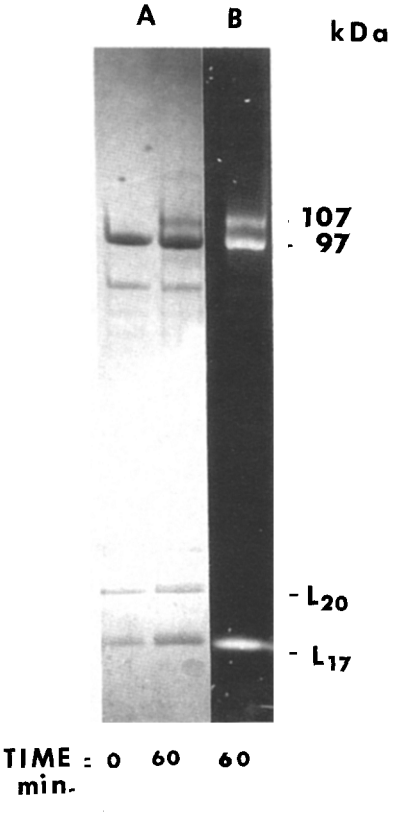

Figure 1: Gel pattern of the modification by dibromobimane of native smooth muscle S1

A 5-molar excess of dibromobimane was used for the modification of subfragment-1 (2 mg/ml) in 5 mM Tes buffer, 5 mM Mg^{2+}-ATP, pH 7.6, at room temperature. At the time indicated (60 min) the samples were analyzed by SDS/polyacrylamide slab gel electrophoresis. The gel viewed under ultraviolet light shows the fluorescence pattern of S-1 treated for 60 min with DB (B).

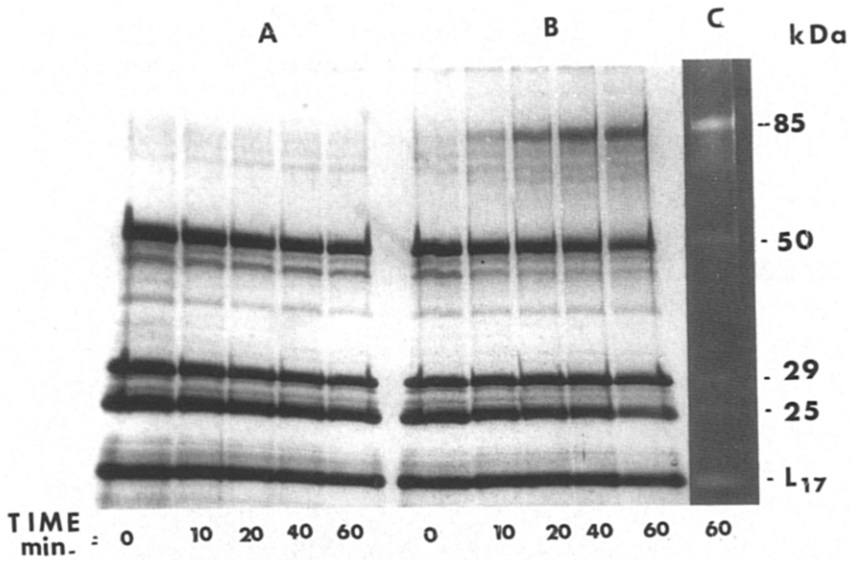

Figure 2 : Comparative time-courses of modification by monobromobimane(A) or dibromobimane(B) of trypsin-split (29K-50K-25K) subfragment-1 :
The conditions described in Figure 1 were used for monobromobimane or dibromobimane modifications of the fragmented smooth muscle subfragment-1. (A) and (B) gels after Coomassie blue staining, (C) same gel seen under ultraviolet light after 60 min of dibromobimane modification.

The time course of the reaction process, involving preformed trypsin-split (29K-50K-25K)S1, was monitored by gel electrophoresis revealed by Coomassie blue staining (Fig.2B) and the inherent fluorescence of incorporated DB (Fig.2C). A new strongly fluorescent protein band was generated at 85K, which developed at the expense of both the 25K and 50K components (as confirmed by densitometric measurements). Most significantly, this entity was not at all produced when monobromobimane was used as the modifier (Fig.2A), or when SH_1 was first modified by IAEDANS in comparative set of experiments. Our interpre-tation is that the 85K protein material results from a covalent union catalyzed by the bifunctional reactivity of DB between the 25K (SH_1 residue) and 50K fragments, whereas the 107K entity arose from the formation of the same crosslink within the intact S1 heavy chain.

In a parallel set of experiments, we investigated the influence of DB substitution on the enzymatic activities of smooth and skeletal muscle S1s. Concomitantly, the intensities of the 107K and 105K bands (9) formed during the reaction were determined by densitometric measurements. As indicated in Table I, DB promoted the inactivation of the Ca^{2+}- and K^{+} -EDTA-ATPases of gizzard S1 and after 60 min we obtained about 45 % inhibition. When the modifier reacted with skeletal muscle S1, the inhibition of ATPase activities was slightly larger (65 %). The yield of DB-crosslinked species was slightly smaller than the percentage of ATPases lost (Table I).

TABLE I

Relationship between the amount of internal crosslinking (107K-105K species) estimated from densitometric measurement of the protein bands and the loss of ATPase activities during dibromobimane modification.

	Time in minutes	Loss of ATPase activities in %	Amount of crosslinked product in %
107K Species	10	8	7
	20	15	12
	30	22	19
	40	30	26
	60	45	40
105K Species	10	11	10
	20	22	17
	30	31	28
	40	43	40
	60	65	60

Titration of the thiol groups in dibromobimane-treated S1 :

Titration of the thiol residues of native S1 with 5,5'-dithiobis (nitrobenzoic acid) in 6 M guanidinium chloride showed that in our preparation there were 11.0 $\pm$ 0.5 moles of SH groups per mole of S1. After DB modification, the value was 8.3 $\pm$ 0.5 SH groups per mole of S1. There was a loss of 2.7 $\pm$ 0.2 moles of SH groups per mole of S1. Assuming that DB simultaneously crosslinks two thiols in S1 (one being SH_1 while the other one is located within the 50K domain) with about 40 % yield, our modification reveals the reactivity of other thiols according to the LC-17K fluorescence pattern observed in Fig.1.

Interaction of actin with dibromobimane-crosslinked S1 :

Having determined that optimum modification conditions gave about 40 % intramolecular bridge formation, we studied the interaction of actin with modified S1 heavy chain by two different methods using ultracentrifugation and chemical crosslinking techniques. First, airfuge centrifugation showed the binding constant (Ka) for native S1 (97K) to be 6.2 ($\pm$ 1.5) x 10^7 M as expected. In comparaison, the actin binding constant of the 107K S1 was 7.2($\pm$1.5) x 10^5 M, i.e. 100 to 200 times weaker than that of native S1.

Second, we studied EDC chemical crosslinking between actin and DB-treated S1 (107K band) which gave rise to no band that could account for the covalent union of actin with the intramolecularly crosslinked 107K S1 heavy chain (result not shown). These data were confirmed by the results of similar

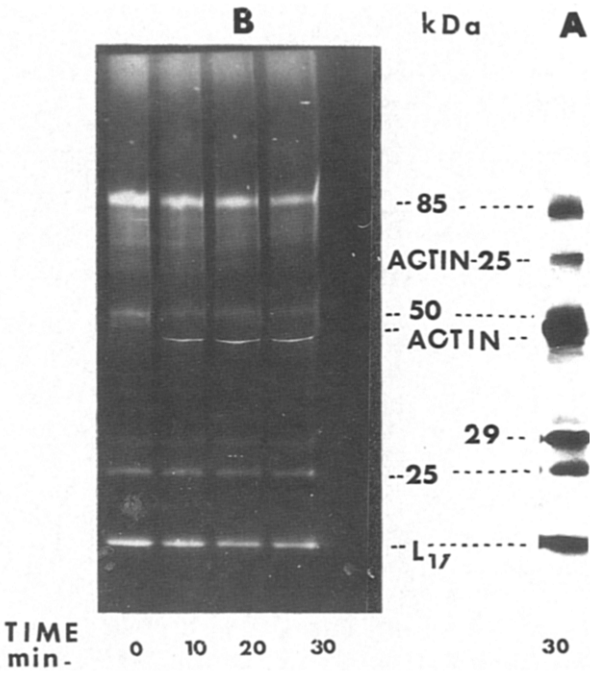

Figure 3 : Crosslinking dibromobimane-modified trypsin split S1 with actin : The crosslinking process was initiated by adding 5 mM carbodiimide in 100 mM Mes buffer, pH 7.0, to the F-actin solution (5mg/ml) and the solution activated for 2 min with EDC was added to the modified S1 in a two-molar excess. (B)-Gel showing the initial dibromobimane-treated S-1 before adding of actin in the first lane. The gel was exposed to ultraviolet light, which shows the fluorescence pattern of the time-course of cross-linking. Lane (A) shows the Coomassie blue staining of the gel corresponding to 30 min of EDC crosslinking of DB-treated S1 with actin (note the presence of actin-25K migratting as the new 65K band; (12)).

experiments carried out using split S1, 40 % of whose the 50K and 25K fragments were covalently linked and converted into the 85K fluorescent entity (Fig.3). The reaction of carbodiimide-activated F-actin with this internally crosslinked split S1 species generated no new products, as seen on fluorescent gel patterns showing the time-course of cross-linking (Fig.3B). In contrast, and as expected (Fig.3A), actin was readily linked to the residual C-terminal 25K domain, forming a 65K entity (12).

DISCUSSION

Previous reports describing proteolytic fragmentation of native smooth and skeletal muscle myosin, heavy meromyosin and S1 (11-12,20) have revealed that the two proteins are composed of three fragments, (N-terminal/central/C-terminal) peptides joined by protease-sensitive loops. Specific homologous sequences are thought to be important for myosin function (21-22). The SH_1 and SH_2 homologous sequence is present in the 25K domain of gizzard smooth myosin S1 heavy chain (15) and contributes to the tight binding of the head to actin

(23) in communicating with the ATPase site and affecting its affinity for the nucleotides (24). This C-terminal heavy chain segment can be in the S1s from vertebrate (6,9) and invertebrate myosins (25) crosslinked to actin by EDC as its N-terminal portion near the 50K-20K junction (13). This suggests that some conserved sub-sites of the 20K heavy chain segment are involved in actin interaction, as indicated by NMR studies (26). This property is lost in DB-treated S1 from smooth muscle myosin. These present data are consistent with our proposal (9) that in both skeletal and smooth muscle S1, the geometry of the actin interaction with this particular area is sensitive to the conformation of the SH_1-containing peptide segment. This is in agreement with the concomitant decrease in the affinity of actin for DB-crosslinked S1. The larger decrease in the case of modified smooth muscle S1 compared to skeletal muscle S1 (9) suggests an even more extensive change in the interaction of actin with the smooth muscle S1 25K domain.

In conclusion, the use of DB as well as other crosslinkers such as BPIA (27), shows that the chemical union of the SH_1-50K region is a general feature for the S1 structure from different muscle sources. With the results presented here, we suggest a possible conformational importance of the neighborhood between SH_1 and SH_x (within 50K domain) for the actin dissociation-association pathway.

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