Abolition of ATPase Activities of Skeletal Myosin Subfragment 1 by a New Selective Proteolytic Cleavage within the 50-Kilodalton Heavy Chain Segment†

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ABSTRACT: We have isolated and chemically characterized several 5-thio-2-nitrobenzoate–subfragment 1 derivatives (TNB–S-1) generated by the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB, up to 10-fold molar excess) with native S-1, *N*-acetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine–S-1 (AEDANS–S-1), and *N*,*N*′-*p*-phenylenedimaleimide–S-1 (pPDM–S-1) at 4 °C, pH 8.0. The reaction of the reagent with AEDANS–S-1, which has a blocked $-SH_1$ group, induced the formation of an intramolecular cystine disulfide between two vicinal –SH groups in S-1; in contrast, the treatment of pPDM–S-1 with DTNB resulted in the formation of TNB mixed disulfides only. The incorporation of the TNB groups (up to 3 mol/mol of S-1) into the native or premodified S-1 led to a local conformational change in the 50K heavy chain region that was fully reversed upon disulfide reduction. Exploiting this peculiarity of the DTNB-modified S-1's, we have realized a highly selective proteolysis of the S-1 heavy chain by thrombin and chymotrypsin, which do not act at all on the normal S-1. The 95K heavy chain was cut by thrombin into two fragments with apparent masses of 68K and 30K, whereas the "connector segments" and the light chains were unaffected. The two new fragments were issued from a primary peptide-bound cleavage between Lys-560 and Ser-561 within the amino acid sequence of the 50K region (M. Elzinga, personal communication). After total deblocking of its thiol groups, the isolated (68K–30K)-S-1 derivative exhibited completely inactivated K^+-, Ca^{2+}-, and actin-dependent ATPases; in contrast, the nondigested DTNB-modified S-1 recovered most of its enzymatic activity upon thiol reduction. The properties of this new S-1 form, which we detail in the accompanying paper [Chaussepied, P., Mornet, D., Barman, T. E., Travers, F., & Kassab, R. (1986) *Biochemistry* (following paper in this issue)], suggest that the proteolyzed portion of the 50K segment is linked to the hydrolysis of ATP and the binding of actin in the myosin head.

The subfragment 1 moiety of myosin exhibits the two biochemical functions that are essential for muscle contraction, namely, the hydrolysis of ATP and the interaction with F-actin. These two properties are also expressed by the isolated S-1[1] heavy chain, which behaves as the "catalytic" subunit of the myosin head molecule (Wagner & Giniger, 1981; Sivaramakrishman & Burke, 1982). The coupling between the nucleotide and actin recognition sites is thought to be the crucial feature of the mechanism by which chemomechanical transduction is accomplished by the myosin S-1 (Morales & Botts, 1979; Botts et al., 1984). The two important functionalities of the S-1 heavy chain are actually distributed over its three proteolytically generated fragments denoted "27K", "50K", and "20K", respectively (Balint et al., 1978); these fragments are held together by strong noncovalent forces and are joined by two short connector segments that are severed by various proteases (Mornet et al., 1981a, 1984; Chaussepied et al., 1983; Applegate & Reisler, 1984).

The largest central 50K fragment is of particular interest as it seems to be concerned with the binding of both actin and nucleotides to S-1. Thus, chemical cross-linking investigations have led to the covalent union between actin and the 50K heavy chain segment (Yamamoto & Sekine, 1981; Mornet et al., 1981b; Labbé et al., 1982; Sutoh, 1983). The binding of nucleotides and some polyanions to S-1 induces a local unfolding of the 50K region that is revealed by an accelerated tryptic degradation restricted to its COOH-terminal portion (Kassab, 1981; Mocz et al., 1982; Hozumi, 1983; Labbé et al., 1984; Mornet et al., 1984); the nucleotide influence is likely to be related to the direct interaction between the substrate and the 50K region as demonstrated by the ability of this region to be selectively labeled by the recently employed photoaffinity ATP analogues (Mahmood & Yount, 1984; Hiratsuka, 1985). The relationship between the 50K peptide and the myosin active site was further suggested by the presence in this region of a carboxyl group whose modification causes loss of ATPase activity (Körner et al., 1983). Moreover, the 50K peptide structure was found to undergo some kind of temperature-dependent melting, which allows extensive tryptic degradation of the entire segment and which proceeds concomitantly with loss of the ATPase activity of S-1; both processes are prevented upon the addition of nucleotides or actin to S-1 (Setton & Muhlrad, 1984; Mocz et al., 1984). Structural perturbations occasioned by ambient factors, such as pH changes, and affecting the tryptic susceptibility of only the 50K fragment have also been observed with the S-1 from rat cardiac V3 isomyosin (Lompré et al., 1984). Recently, we have reported on the intrinsic conformational instability of the 50K region of skeletal S-1 (Mornet et al., 1985a); this

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[1] Abbreviations: S-1, chymotryptic subfragment 1 of myosin; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); pPDM, *N*,*N*′-*p*-phenylenedimaleimide; TNB, 5-thio-2-nitrobenzoic acid; DTE, dithioerythritol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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feature can have functional implications in regard to the idea that the elastic and contractile properties of the cross-bridges arise primarily in the S-1 portion of myosin (Cross et al., 1984). Finally, the 50K region bears intramolecular contacts with the adjacent 20K segment, which contains the second class of actin sites of the S-1 heavy chain (Mornet et al., 1981a,b; Sutoh, 1983). The two peptides have been cross-linked by a variety of chemical cross-linking agents (Yamamoto & Sekine, 1979; Mornet et al., 1981a; Labbé et al., 1982; Hiratsuka, 1984); in particular, recent inter-thiol cross-linking experiments with dibromobimane have suggested the proximity of the reactive SH_1 thiol group (cysteine-707) (M. Elzinga, personal communication) present in the 20K region to another cysteine side chain located in the 50K region (Mornet et al., 1985b).

In an effort to explore further the substructure and functional role of the particular 50K heavy chain region, we have undertaken the present investigation, which is founded on the thiol modification dependent proteolytic digestion of S-1. The results show that the reversible blocking of a limited number of SH groups in S-1 with DTNB under mild conditions induces a new enzyme form that is selectively attacked by thrombin and chymotrypsin, which are proteases that do not cut at all normal S-1. With thrombin, the heavy chain is converted into a complex of two peptides with masses of approximately 68K and 30K by a limited peptide bond cleavage occurring only within the 50K segment at Lys^{560}-Ser^{561} as determined by amino acid sequence analyses. ATPase measurements performed on the isolated (68K–30K)-S-1 derivative after total regeneration of the protein thiol groups indicated that this proteolytic scission of the 50K region results in a concomitant abolition of the enzymatic activities of S-1. In the accompanying paper (Chaussepied et al., 1986b), we describe the properties of the inactive thrombin-cut S-1, and the results further suggest that the ATPase loss is linked to the suppression of the kinetic step of ATP hydrolysis.

Materials and Methods

Chemicals. Thrombin from bovine plasma was from Serva; α-chymotrypsin and trypsin [treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone] were purchased from Worthington Biochemicals. Carboxypeptidases A and B, soybean trypsin inhibitor, 5,5′-dithiobis(2-nitrobenzoic acid), and *N,N′-p*-phenylenedimaleimide were obtained from Boehringer-Mannheim. All other chemicals were of the highest analytical grade.

Preparations of Native and Modified Proteins. Rabbit skeletal myosin was prepared according to Offer et al. (1973). S-1 was obtained by digestion of myosin filaments with chymotrypsin as described by Wagner & Weeds (1977) and purified over Sephacryl S-200 eluted with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 mM NaN_3. The concentration of S-1 was determined from its absorbance at 280 nm with an absorption coefficient $A^{1\%}$ of 7.5 cm^{-1}. The concentration of the proteolytic S-1 derivatives was determined according to Bradford (1976) with S-1 as a standard.

The SH_1 group of S-1 was labeled with IAEDANS as described previously (Mornet et al., 1981a). The concentration of the fluorescent label was determined spectrophotometrically with a molar absorption coefficient of 6.2×10^3 M^{-1} cm^{-1} at 340 nm (Hudson & Weber, 1973). Trypsin-cut (27K–50K–20K)-S-1 was obtained essentially as reported by Mornet et al. (1980). pPDM–S-1 was prepared in the presence of Mg^{2+}-ADP as described by Wells and Yount (1979). The protein samples showed less than 5% of all ATPase activities and incorporated 0.80 mol of [^{14}C]ADP/mol of S-1.

The reaction of DTNB with native or premodified S-1 was carried out following the procedure of Wells and Yount (1980). S-1 (35 μM) in 40 mM Hepes, pH 8.0, was incubated at 4 °C with 2–50 molar excess of DTNB, in the absence and in the presence of Mg^{2+}-ADP (2.5 mM). The disulfide exchange was allowed to proceed for 2–15 h. The production of TNB groups was monitored by the change in absorbance at 412 nm (E_{412nm} = 13600 M^{-1} cm^{-1}; Ellman, 1959). The DTNB-treated S-1 was isolated by $(NH_4)_2SO_4$ precipitation (to 60% saturation), at pH 7.0, followed by gel filtration over Pharmacia PD-10 columns in 50 mM Hepes (pH 8.0) and 0.1 mM NaN_3 at 4 °C. Some TNB–S-1 preparations were purified directly by gel filtration with similar results. The number of TNB groups bound to S-1 was determined from their absorbance at 412 nm after treatment of a sample of the purified TNB–S-1 (17 μM) with 5 mM DTE in 50 mM Hepes, pH 8.0, for 30 min at 20 °C.

Proteolytic Reactions. For all digestions the reaction mixture contained a final S-1 concentration of 2–4 mg/mL. The tryptic digestion of S-1 was performed for 20 min at 25 °C in 50 mM Tris-HCl (pH 8.0) with a trypsin to S-1 ratio (w/w) of 1:50 (Mornet et al., 1984). The reaction was terminated by the addition of a 2-fold excess of trypsin inhibitor (w/w).

The digestion of S-1 with chymotrypsin was carried out for 15 min at 25 °C in 50 mM Hepes (pH 7.0), with a protease to S-1 weight ratio of 1:100. The reaction was quenched by the addition of phenylmethanesulfonyl fluoride to 0.2 mM.

The digestion of S-1 or (27K–50K–20K)-S-1 with thrombin was conducted at a weight ratio of enzyme to protein of 1:25 in 50 mM Hepes buffer, pH 8.0, at 25 °C in the interval time 0–180 min. The reaction was stopped by the addition of DTE to 5 mM, and the protein was isolated by gel filtration over Sephadex G-25 eluted with the Hepes buffer.

The cleavage pattern of S-1 generated by the various proteases was analyzed by electrophoresis in 0.1% $NaDodSO_4$ (5–18%) gradient polyacrylamide gels as described by Mornet et al. (1981a). The optical densities of Coomassie blue stained protein bands and the respective mass distributions were measured with a Shimadzu Model CS-930 high-resolution gel scanner equipped with a computerized integrator. Molecular sizes of fragments were estimated by comparing their electrophoretic mobilities to those of commercial marker proteins.

Amino Acid Sequence Analysis. The heavy chain fragments of thrombin-cut S-1 were isolated by gel filtration over Sephacryl S-300 under the dissociating conditions reported by Mornet et al. (1980). Automated amino acid sequence analyses of the 30K peptide were performed in a liquid-phase sequencer (Socosi) as previously described (Chaussepied et al., 1983); phenylthiohydantoins were identified by high-pressure liquid chromatography (Bhown et al., 1978); the peptides (100 nmol) were sequenced up to the tenth residue. The digestion of the 68K peptide with carboxypeptidase B (4 h at 40 °C) was followed by a carboxypeptidase A degradation (4 h at 40 °C) according to Morgan and Henschen (1969). Amino acid analyses were carried out on a Beckman analyzer (Model 119B).

S-1 ATPase Activity. The K^+-, Ca^{2+}-, Mg^{2+}- and actin-activated Mg^{2+}-dependent ATPase activities were determined as specified earlier (Mornet et al., 1979). Before the activity assay, aliquots of TNB–S-1 or native S-1 (5 μM) were reduced with 5 mM DTE in Hepes buffer, pH 8.0, at 20 °C for 30 min.

Results

Reaction of DTNB with Native and Premodified S-1. We have isolated several TNB–S-1 derivatives formed by reacting

Table I: Characterization of the TNB–S-1 Derivatives Formed upon Reaction of Various Concentrations of DTNB with Native and Premodified Chymotryptic S-1[a]

	molar excess of DTNB over S-1	TNB produced during the reaction	TNB released by DTE	% of tryptic 68K fragment
S-1	2	3.1	0.95	20
	3.5	4.1	2.0	70
	5	*5.0*	*3.0*	*95*
	10	5.9	3.8	96
AEDANS–S-1	*5*	*4.2*	*2.1*	*95*
	10	5.1	3.0	95
pPDM–S-1	2	2.2	1.8	45
	5	*2.8*	*2.7*	*75*
	10	3.5	3.6	85

[a] The reaction of DTNB with native or premodified S-1 was carried out as follows: S-1 (35 μM) in 40 mM Hepes, pH 8.0, was incubated at 4 °C with a 2–10 molar excess of DTNB, in the presence of Mg^{2+}-ADP (2.5 mM). The disulfide exchange was allowed to proceed 12 h at 4 °C, and production of TNB groups was monitored at 412 nm as described under Materials and Methods. The number of moles of TNB groups are average values from two to four experiments. Standard deviation is ±0.2 mol of TNB. The italicized data are schematically represented in Figure 2.

the native enzyme at 4 °C, pH 8.0, with DTNB employed at 2–50 molar excess. We have directly determined the amount of TNB groups produced during each reaction and the number of protein-bound TNB released by DTE reduction of the purified derivatives; the results are presented in Table I. In addition to the intramolecular SH_1–SH_2 cystine disulfide that readily forms upon a mixing of S-1 with a 2 mol excess of DTNB (Wells & Yount, 1980), the reaction led to the progressive blocking of maximally four other thiols, which were converted into TNB mixed disulfides. This value was reached on use of a 10-fold excess of DTNB; three of these thiols were particularly reactive as they could be substituted by reacting S-1 with only 5-fold excess of reagent. As shown in Figure 1, no further incorporation of TNB groups was noted even when the S-1 was treated overnight with up to 50-fold excess of DTNB. The addition of 5 mM Mg^{2+}-ATP or Mg^{2+}-ADP to the reaction mixtures did not change the results; also, the reaction of DTNB with the trypsin-cut (27K–50K–20K)-S-1 was essentially similar to that found for native S-1 (Figure 1). All the DTNB-modified S-1 preparations we obtained could be fully reduced by incubation in 5 mM DTE, pH 8.0, at 20 °C for 30 min as judged by the release of the bound TNB groups and the recovery of at least 70% of the Mg^{2+}-, Ca^{2+}-, and K^+-dependent ATPase activities. These data indicate that modification of native chymotryptic S-1 with DTNB, under the conditions specified, results in the reversible blocking of six thiols out of a total of nine cysteine residues present in the enzyme (Elzinga, personal communication; Frank & Weeds, 1974).

In another set of experiments, we reacted DTNB with S-1 premodified at its SH_1 group by IAEDANS and with S-1 intramolecularly cross-linked by pPDM at the pair of SH_1–SH_2 groups; the results, presented in Table I, were compared to those obtained for native S-1. In the case of AEDANS–S-1, there was loss of about one SH group as clearly indicated by a corresponding decrease of the TNB produced during the DTNB reaction, a result in agreement with the presence of 0.9 mol of AEDANS directly measured in the protein sample. However, and most importantly, the isolated TNB–AEDANS–S-1 contained only two TNB groups when it was formed by reaction with a 5-fold excess of DTNB and only three TNB groups when the modification was effected with a 10-fold excess of reagent. For both derivatives, the difference

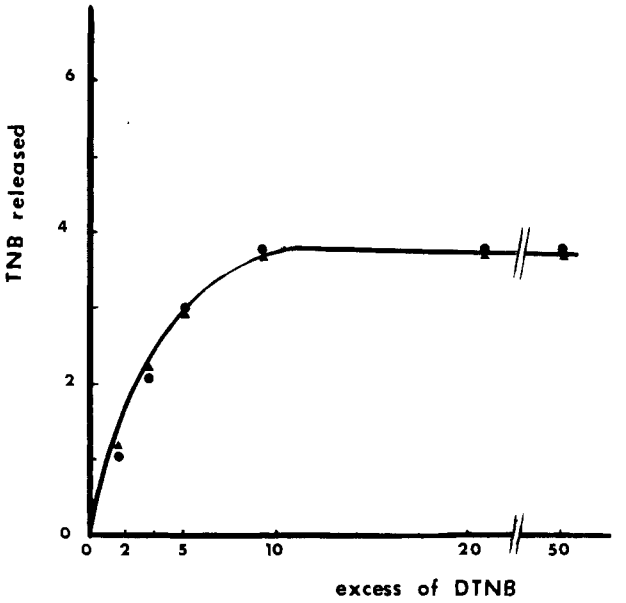

FIGURE 1: Determination of the number of TNB groups incorporated into native S-1 and trypsin-cut (27K–50K–20K)-S-1 as a function of the DTNB concentration. The enzymes (40 μM) in 40 mM Hepes, pH 8.0, 4 °C, were incubated overnight with a 2–50-fold excess of DTNB. The modified protein samples were isolated by gel filtration and treated with DTE as indicated under Materials and Methods; the released TNB groups were estimated at 412 nm. (●) Native S-1; (▲) split S-1.

between the amount of TNB produced by the chemical reaction and the number of enzyme-bound TNB was equal to nearly 2 mol of TNB/S-1. This particular result strongly suggests that, as observed with native S-1, DTNB promotes also the formation within AEDANS–S-1 of one intramolecular cystine disulfide between two vicinal thiols. To assess further the possible nature of these thiols, we reacted various concentrations of DTNB with pPDM–S-1. The data presented in Table I show that for the three different derivatives tested there was a close correlation between the number of TNB groups liberated during the reaction and those displaced by the DTE treatment. This indicates that the reaction involves only the formation of mixed TNB–S-1 disulfides with no occurrence of a cystine disulfide bridge. It suggests that the SH_2 group is participating in the establishment of the cystine disulfide bond observed with DTNB-modified AEDANS–S-1 as will be described in detail elsewhere (Chaussepied et al., 1986a). On the other hand, only three thiols could be fully substituted instead of the expected four thiols with the fourth –SH group to be titrated exhibiting a relatively lower reactivity toward DTNB than observed with the native S-1 or AEDANS–S-1. These differences are probably related to the specific conformation of pPDM–S-1. For clarity, a scheme is presented in Figure 2 summarizing the proposed reactions of DTNB (used at 5-fold excess) with native and premodified S-1.

Tryptic Sensitivity of the Heavy Chain of DNTB-Modified S-1. In a second phase of this work we investigated the influence of the S-1 modification with DTNB on its conformational properties; this was done by first analyzing the cleavage pattern of the enzyme heavy chain with trypsin. Four isolated TNB–S-1 derivatives formed by reacting S-1 with 1-, 2-, 5-, and 20-fold excesses of DTNB, respectively, were studied. The results of $NaDodSO_4$ gel electrophoresis of the four digestion mixtures after 10- and 20-min trypsinolysis are presented in Figure 3. The heavy chain of S-1 treated with up to a 2-fold excess of DTNB generated the NH_2-terminal

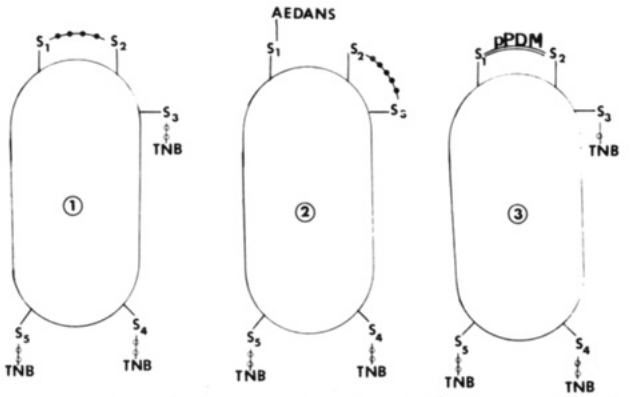

FIGURE 2: Scheme of the proposed reactions of DTNB, used at 5 molar excess, with native S-1 and premodified S-1: (1) native S-1; (2) S-1 blocked at its SH_1 with 1,5-IAEDANS; (3) S-1 blocked at its SH_1 and SH_2 groups by intramolecular cross-linking with pPDM. (●–●) A cystine disulfide bond; (O–O) a protein SH–TNB mixed disulfide bond; (–O–) a SH–TNB mixed disulfide bridge, the formation of which does not go to completion within pPDM–S-1. This scheme fits all the italicized data presented in Table I.

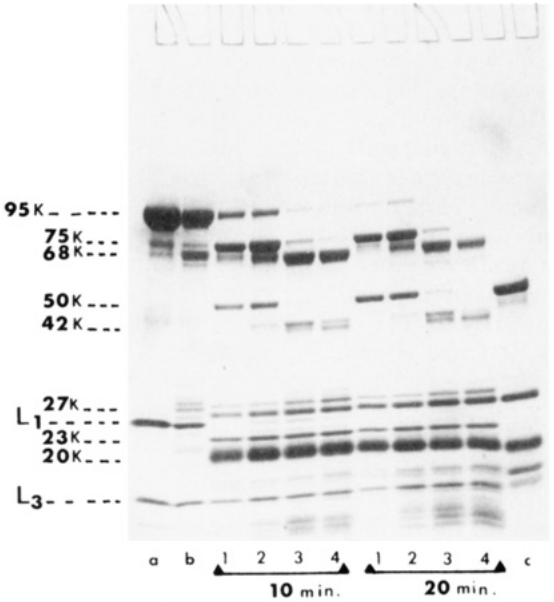

FIGURE 3: Cleavage of DTNB-modified S-1 by trypsin. S-1 (A1 + A2) was treated with DTNB employed at a 2 (1), 3.5 (2), 5 (3), and 10 (4) molar excess, respectively, under the conditions reported in Figure 1; the protein samples were isolated and digested by trypsin for 10 and 20 min under the conditions specified under Materials and Methods. Controls were (a) native S-1 (A1 + A2), (b) S-1 (A1 + A2) treated with a 10-fold excess of DTNB and subsequently reduced by DTE, and (c) trypsin-split (27K–50K–20K)-S-1.

75K fragment and its COOH-terminal 20K counterpart together with the release of the 27K and 50K fragments. In addition, there was formation of a limited amount of a new band with a mass of approximately 68K. This species was chiefly produced from TNB–S-1 obtained by treating S-1 with a 5-fold excess of reagent; no production of the 75K peptide was apparent in the digest of this derivative. TNB–S-1 formed in the presence of a 10-fold excess of DTNB was disgested quite similarly. Furthermore, the proteolysis of the two latter samples did not yield the conventional 50K peptide, but instead, a doublet band migrating at the 42K–40K position was observed; the absence of any changes in the release of the 27K and 20K fragments suggested that this new pair of peptides is essentially resulting from a cleavage of the 50K fragment. It should be noted that, after reduction with DTE, the heavy chain of TNB–S-1 was digested by trypsin similarly to that of the normal S-1 even when it derives from a reaction of S-1 with a 10-fold excess of DTNB (data not shown).

The 68K entity was also produced in the digests of AEDANS–S-1 and pPDM–S-1 after they were treated with DTNB but not before this treatment. A quantitative densitometric estimation was carried out for the 68K species released by trypsin from all the isolated TNB–S-1 derivatives in order to correlate its production with the events of thiol modification promoted by DTNB in S-1 (Table I). For native S-1, the extent of 68K increased progressively with the incorporation of the TNB groups as mixed disulfides; it amounted to 30% in S-1 containing about one TNB group together with the SH_1–SH_2 cystine disulfide, and it reached a maximal value of approximately 95% in S-1 treated with a 5-fold excess of DTNB with the incorporation of three TNB groups. For AEDANS–S-1, the production of 68 K was also extensive upon binding of only two TNB groups produced by reaction with a 5-fold excess of reagent. However, for pPDM–S-1, the highest amount of 68K released was near 85%, and this value was reached after the incorporation of at least three TNB groups per S-1.

Selective Cleavage of the 50K Segment in TNB–S-1 with Thrombin. For a better structural characterization of the new cleavage site promoted by DTNB in the 50K region of S-1 and to probe the influence of the proteolytic reaction on its enzymatic activities, the TNB–S-1 was subjected to digestion with thrombin. This protease does not cut the two connector segments joining the 50K fragment to the 27K and 20K peptides of S-1 heavy chain. Because it has a conformation-dependant activity on protein substrates, the use of this protease would be convenient to probe the change in S-1 conformation upon thiol modification. Figure 4A shows that, in contrast to the normal S-1, TNB–S-1 (formed by reaction of S-1 with a 5-fold excess of DTNB) was readily attacked by thrombin in a highly selective manner. The heavy chain was split into apparently two fragments with masses of about 68K and 30K whereas the two alkali light chains were unaffected. The mobility of the former peptide was similar to that of the tryptic species reported above. The thrombin digestion was abolished upon reduction of TNB–S-1 with DTE; also, thrombin-cut DTNB–S-1 recovered all its thiols upon subsequent reaction with DTE as will be demonstrated in the accompanying paper. Consequently, ATPase assays were performed during the course of the thrombic reaction after treatment of the protein samples with DTE. The results are shown in Figure 4B. The thrombic digestion led to a progressive and concomitant inactivation of the K^+-, Ca^{2+}-, and actin-stimulated Mg^{2+}-ATPase of S-1. The extent of the ATPase loss could be correlated with the amount of digested S-1 (Figure 4B). The observed selective thrombic digestion is actually occurring only within the 50K heavy chain region. This was confirmed by analyzing the time course of the thrombic digestion of TNB–(27K–50K–20K)-S-1 (obtained by reaction of trypsin-cut S-1 with a 5-fold excess of DTNB). The results reported in Figure 4C show that the 50K fragment was progressively and entirely converted into the 42K–40K peptides whereas the 27K and 20K fragments remained unchanged. We have not investigated whether the 10K difference peptide remained intact or was cleaved into small pieces during the thrombic digestion.

The digestion of TNB–S-1 with chymotrypsin resulted also in the inactivation of S-1; native S-1 is refractory to this protease (Wagner & Weeds, 1976; personal observation). As shown in Figure 5A, the TNB–S-1 heavy chain was cut by chymotrypsin into two new fragments with masses near 67K

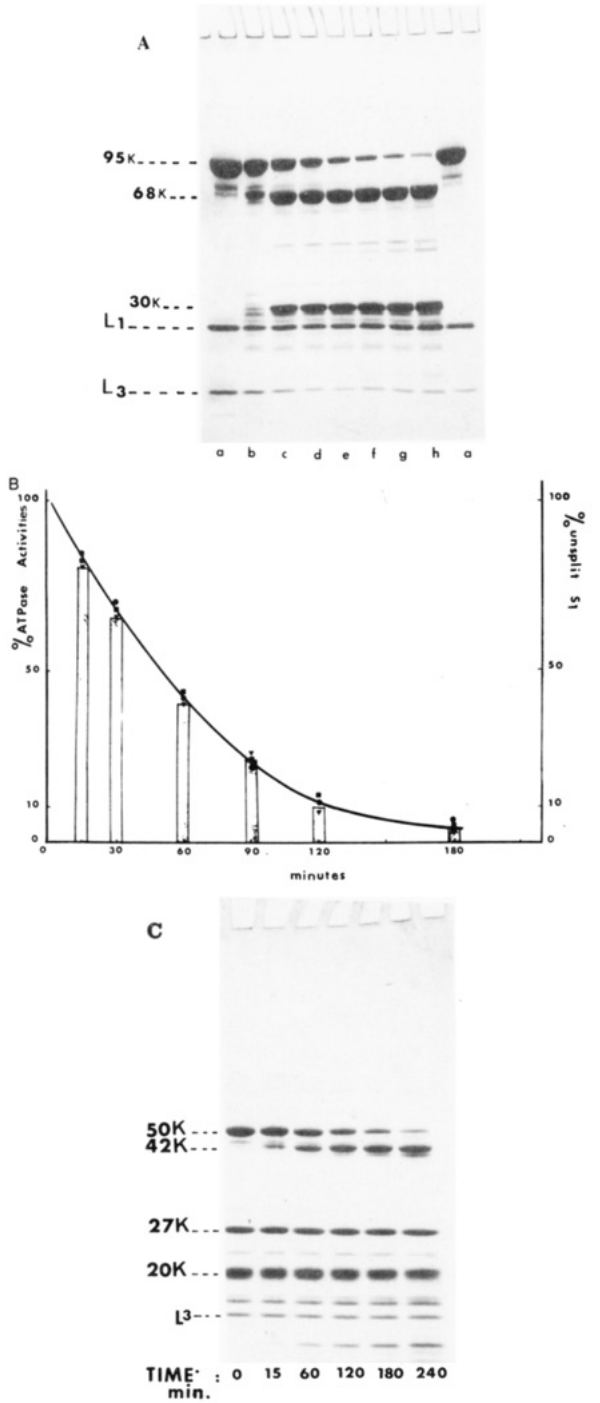

FIGURE 4: (A) Time course of the limited digestion of DTNB–S-1 by thrombin. S-1 (A1 + A2) was modified by reaction with a 5 molar excess of DTNB. The purified DTNB enzyme was digested with thrombin as reported under Materials and Methods; at various time intervals [(b) 0, (c) 30, (d) 60, (e) 90, (f) 120, (g) 150, and (h) 180 min], aliquots of the digest were analyzed by gel electrophoresis. (a) Native S-1 (A1 + A2). (B) Time course of the change in ATPase activities during the digestion of DTNB–S-1 by thrombin. Samples of DTNB–S-1 (A1 + A2) treated with thrombin as indicated in (A) were reduced by DTE and subjected to ATPase assays as specified under Materials and Methods. (●) K^+-EDTA–ATPase; (■) Ca^{2+}-ATPase; (▼) actin-activated Mg^{2+}-ATPase; the bars represent the percent of residual unsplit S-1 that was determined by densitometric measurements of the 95K heavy chain present on the electrophoregram of the thrombic digest. (C) Time course of thrombic cleavage of the 50K heavy chain segment within DTNB–(27K–50K–20K)-S–1. Trypsin-split (27K–50K–20K)-S-1 (A1 + A2) was reacted with a 5 molar excess of DTNB; the isolated modified derivative was subjected to digestion with thrombin as indicated under Materials and Methods. At the times indicated, samples of the digest were subjected to slab gel electrophoresis.

and 29K; the size of these peptides was very close to those of the peptides released by thrombin. In addition, both the 30K and 29K materials were fluorescent when the original S-1 was labeled with IAEDANS (Figure 5B); the subsequent addition of trypsin to the thrombic and chymptryptic digests led to the breakdown of the 30K and 29K peptides and to the production of a fluorescent 20K fragment in both cases (results not shown). The digestion of TNB–AEDANS–S-1 with trypsin produced directly the fluorescent 20K peptide together with its 22K precursor (Figure 5). The results clearly indicate that the 68K and 67K fragments generated by thrombin and chymotrypsin, respectively, represent the N-terminal portion of the heavy chain whereas the 30K and 29K fragments are their respective C-terminal counterparts. They are also consistent with the idea that the new cleavage site is located mainly within the 50K region.

Identification of Lys^{560}*-Ser*^{561} *as the Site of Primary Cleavage of TNB–S-1 Heavy Chain with Thrombin.* To throw further light on the structural relationship between the thrombin-generated 68K and 30K peptides, NH_2- and COOH-terminal sequence analyses were carried out on the two purified fragments. A unique NH_2-terminal sequence was obtained for the 30K element in agreement with its gel pattern, which shows a homogeneous single band (results not shown). This sequence (Ser-Asn-Asn-Phe-Gln-Lys-Pro-Lys-Pro-Ala) is identical with that of a peptide stretch located toward the COOH-terminal portion of the 50K heavy chain region and comprising residues 561–570 (M. Elzinga, personal communication). Thus, the 30K fragment derives from a thrombic hydrolysis of the peptide bond between Lys-560 and Ser-561. The 68K preparation was analyzed by digestions with carboxypeptidases B and A. Lys (2.0 mol) was released first followed by Gly (0.9 mol), Asn (0.9 mol), Leu (0.5 mol), Phe (0.4 mol), and His (0.25 mol). The removal of two lysine residues suggested the occurrence of two kinds of peptides; the band pattern of the 68K peptide indicated indeed that the material migrates as a doublet (data not shown). The data of the carboxypeptidases degradations could then be easily interpreted in light of the known 50K sequence that is adjacent to Ser-561 (M. Elzinga, personal communication). For one peptide, the COOH-terminal sequence would be ...His^{557}-Leu^{558}-Gly^{559}-Lys^{560}; it represents the intact NH_2-terminal 68K fragment of the S-1 heavy chain. For the other 68K entity, the COOH-terminal could be ...Phe^{550}-$(CH_3)_3$Lys^{551}-Asn^{552}-Lys^{553}; it is shorter than the latter peptide by the loss of a small stretch of six amino acids between Lys-553 and Lys-560. It results from a secondary thrombin cleavage at Lys^{553}-Tyr^{554}.

DISCUSSION

The intent of this research is to provide a new proteolytic S-1 species useful for examining specific relationships between the substructure of the 50K heavy chain region and the functions of the myosin head. This represents a continuation of our studies on the use of proteolytic approaches for the characterization of the S-1 structures involved in its energy transducing activity (Mornet et al., 1979, 1981a; Chaussepied et al., 1983). We have surmised that selective proteolytic reactions would be directed toward the relatively less stable 50K portion of the heavy chain by blocking of thiols in S-1. Previously, it has been suggested that the chemical substitution of SH groups of S-1 is likely to produce polypeptide distortions in the enzyme molecule that are transmitted to the ATPase and actin binding sites (Botts et al., 1979). Consequently, such thiol blocking dependent structural perturbations could reflect themselves by a definite alteration of the proteolytic suscep-

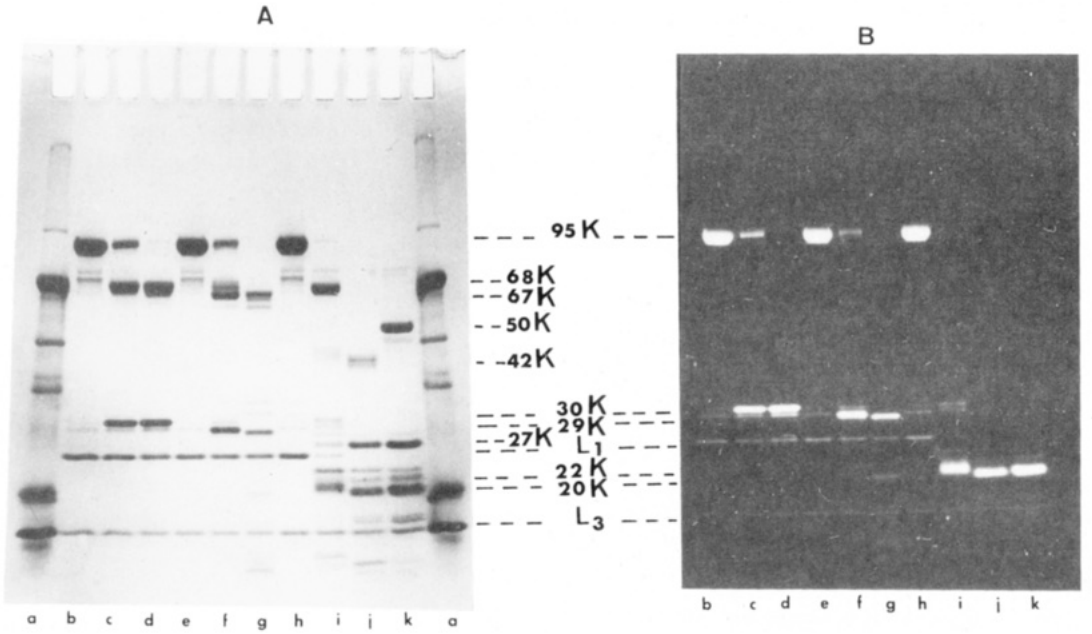

FIGURE 5: Comparison of the reactions of thrombin, chymotrypsin, and trypsin with DTNB–AEDANS–S-1. Fluorescent AEDANS–S-1 (A1 + A2) was treated with a 5 molar excess of DTNB; the doubly modified S-1 was isolated and submitted to digestions with thrombin for 90 and 180 min (lanes c and d, respectively), with chymotrypsin for 5 and 15 min (lanes f and g, respectively), and with trypsin for 5 and 15 min (lanes i and j, respectively) in the conditions described under Materials and Methods. (a) Protein control markers myosin heavy chain (200K), phosphorylase A (100K), serum albumin (67K), ovalbumin (43K), D-amino acid oxidase (37K), trypsin inhibitor (21K), and light chain 3 (17K); (b, e, and h) triplicate samples of DTNB–AEDANS–S-1 before proteolysis; (k) fluorescent trypsin-cut (27K–50K–20K)-S-1 labeled on its 20K fragment with 1,5-IAEDANS. (A) Gel stained with Coomassie blue; (B) gel viewed under UV light.

tibility of some critical regions of the S-1 heavy chain. As far as the SH_1 and SH_2 groups are the only sites to be modified, no apparent change in the proteolytic sensitivity of the S-1 heavy chain was noticed. However, our present study shows that the blocking of a few other thiols in S-1 leads to the formation of new clip sites that reside essentially in the 50K region, at least for the three proteases we have tested, trypsin, chymotrypsin, and thrombin.

We have selected DTNB as the thiol modifier because the disulfide formation it induces in S-1 is known to be fully reversible (Wells & Yount, 1980); this reversibility is essential for investigating the influence of proteolysis on the S-1 activities. Moreover, the progress of its disulfide exchange reaction can be carefully controlled and conveniently analyzed. Finally, recent studies with the myosin rod and α-tropomyosin have illustrated the usefulness of the DTNB-promoted disulfide bonds for the generation of new proteolytic sites through a local protein unfolding (Lu & Lehrer, 1984; Nyitray et al., 1985). We have extended the reaction of DTNB with native S-1 to the premodified S-1 forms AEDANS–S-1 and pPDM–S-1 in which either the SH_1 site or both the SH_1 and SH_2 sites, respectively, have been specifically masked. This allowed us to show unambigously that the change in the proteolytic pattern of the heavy chain is associated with the incorporation of the TNB groups into a second class of maximally two to three thiols in S-1. The observation that the reaction of the reagent with AEDANS–S-1 leads to the formation of an intramolecular cystine disulfide involving most probably the SH_2 group is of particular interest; it indicates that this critical thiol has a close spatial relationship not only with SH_1 but also with another thiol in S-1. In this regard, Mornet et al. (1985b) have recently described an inter-thiol cross-linking produced by the bifunctional agent dibromobimane between SH_1 and another unknown thiol located in the 50K region; unlike the covalent union of SH_1 and SH_2, this new thiol cross-linking did not result in "trapping" Mg^{2+} nucleotide. Work is in progress in our laboratory to assess the ability of the DTNB-modified AEDANS–S-1 to trap Mg^{2+}-ADP at the active site (Chaussepied et al., 1986a).

Because the specificity of thrombin is linked to very few peptide bonds including selective amino acids (Chang, 1985), we have extensively investigated the (68K–30K)-S-1 resulting from its reaction on TNB–S-1; this derivative is a stable inactive complex of two heavy chain fragments, 68K and 30K, associated with an intact light chain. As indicated by the amino acid sequence data, the proteolytic events have occurred in the 553–560 region of the heavy chain within the 50K segment; the thrombin-susceptible peptide bond is Lys^{560}–Ser^{561} and to a lesser extent Lys^{553}–Tyr^{554}. Most of the isolated thrombin-cut S-1 preparations contained a 68K doublet; the ratio of the two protein species was dependant upon the extent of TNB–S-1 digestion by thrombin. If the proteolysis is terminated before all the 95K heavy chain was digested, then the upper band was much more represented on the gel, as judged from densitometric measurements; under conditions where the heavy chain was totally consumed by thrombin, the two peptides were produced in nearly equal amount. However, it should be emphasized that the degree of ATPase loss was directly correlated with the amount of split heavy chain; this indicates that the primary cleavage of a single peptide bond at Lys^{560}-Ser^{561} is sufficient to induce the observed inhibition of S-1. The fact that trypsin as well as chymotrypsin gives rise to fragments of similar sizes as those produced by thrombin strongly suggests that these proteases are acting on vicinal sites within the TNB–S-1 heavy chain with concomitant abolition of S-1 ATPase activities. The inhibitory influence of this selective proteolytic cleavage of the 50K segment is in contrast with the absence of an effect on the S-1 ATPases observed during the nucleotide-promoted tryptic degradation of the 50K peptide into a 45K peptide (Mocz et al., 1984). We relate the thrombin- and chymotrypsin-induced inhibition of S-1 to the fact that the clip sites were generated at or near a portion of the polypeptide chain whose conformation and degree of exposure are linked with the expression of the functional

properties of native S-1; it is noteworthy that the 553–570 region, which includes the thrombin sites, exhibits an extensive sequence homology with the nematode heavy chain counterpart (Mc Lachlan, 1984). Since this critical region is sensitive to proteolysis not in the normal S-1 but only in TNB-S-1, we anticipate that the peptide bond fission has impaired its proper refolding to a native, active structure during the thiol reduction of the digested TNB-S-1. The clip sites are localized within a peptide stretch that makes part of the preexisting loop segment thought to occur inbetween the 50K and 20K segments of the S-1 heavy chain (Mornet et al., 1985b). Our sequence data make possible the future identification of the thrombin split region in the three-dimensional structure of the S-1 heavy chain, now in progress (Winkelman et al., 1985); this will provide valuable information on its actual conformation. Meanwhile and as illustrated in the accompanying paper, the thrombin-nicked S-1 appears as a new protein species useful for learning more about the structure and function of the 50K region within the myosin head.

Registry No. DTNB, 69-78-3; ATPase, 9000-83-3; protease, 9001-92-7; thrombin, 9002-04-4; chymotrypsin, 9004-07-3; trypsin, 9002-07-7.

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