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Articles

On the Mode of the Alkali Light Chain Association to the Heavy Chain of Myosin Subfragment 1. Evidence for the Involvement of the Carboxyl-Terminal Region of the Heavy Chain†

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ABSTRACT: Evidence is presented that the removal of the alkali light chain subunit from myosin subfragment 1 results in the exposure of a site (or sites) at the carboxyl-terminal region of the heavy chain that is rapidly digested by both trypsin and α-chymotrypsin. In the case of trypsin digestion, cleavage at this site proceeds at a much higher rate than cleavages at the two other sensitive regions located in the interior of the primary structure of this chain. This initial cleavage is responsible for the generation, on further digestion with trypsin, of a carboxyl-terminal fragment about 3000 daltons smaller than the corresponding fragment formed by digestion of subfragment 1. The ability of the heavy chain to reassociate with alkali light chain at 4 °C in the presence of MgATP is essentially abolished by cleavage at this exposed site by either trypsin or chymotrypsin. These observations indicate that the alkali light chain is binding to, or is capable of perturbing, a region of the heavy chain adjacent to the subfragment 1/subfragment 2 "hinge" region and support recent proposals that both the DTNB light chain and the alkali light chain may be interacting and may be modulating this flexible region of the cross bridge.

Previous studies have shown that under certain solvent conditions the stability of the subunit interactions in myosin and myosin subfragment 1 (SF1)[1] is sufficiently labile to heat that dissociation can be detected (Higuchi et al., 1978; Wikman-Coffelt et al., 1979). However, in the presence of the MgATP substrate no subunit dissociation appears to occur at

† From the Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106. *Received January 12, 1983.* Supported by grants from the National Institutes of Health (NS-15319-03A1) and National Science Foundation (PCM-8007876) and by the Muscular Dystrophy Association. Preliminary accounts of some aspects of this work were presented at the 12th International Biochemical Congress, Perth, Australia, and at the Biophysical Society Meeting, San Diego, CA, 1983.

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[1] Abbreviations: TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; SF1 and SF2, myosin subfragments 1 and 2, respectively; SF1(A1) and SF1(A2), myosin SF1 containing A1 and A2 light chains, respectively; T1 and T2, trypsin digestion sites on the SF1 heavy chain, with T1′ near the carboxyl-terminal end; TF1, TF2, and TF3, tryptic fragments of SF1 heavy chain numbered in sequential order from the carboxyl-terminal end of the subfragment 1 heavy chain; STI, soybean trypsin inhibitor; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)-aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; ATPase, adenosinetriphosphatase.

0006-2960/83/0422-3046$01.50/0

37 °C, since no free light chain was detected in the supernatant of myosin rapidly salted out by $(NH_4)_2SO_4$ precipitation (Dreizen & Richards, 1972). Recent studies from our laboratory on the stability of myosin subfragment 1, and of myosin, have shown that at 37 °C in the presence of MgATP considerable exchange between free and bound alkali light chains occurs in these proteins (Sivaramakrishnan & Burke, 1981; Burke & Sivaramakrishnan, 1981) despite the fact that no evidence for progressive subunit dissociation could be detected (Burke & Sivaramakrishnan, 1982). It was proposed, on the basis of these observations, that the subunits in myosin subfragment 1 may exist in a rapid, reversible equilibrium between their associated and dissociated states, and support for this proposal was obtained by the demonstration that the heavy chain could be isolated by subjecting subfragment 1 to ion-exchange chromatography at 37 °C in the presence of MgATP (Sivaramakrishnan & Burke, 1982).

In the present study we have attempted to obtain further information about the heavy chain[2] and the mode of its interaction with the alkali light chain. This has been studied by examining the course and the extent of tryptic and chymotryptic digestions on the isolated heavy chain and comparing these results to those known to occur by digesting subfragment 1 with the two enzymes. The results obtained from these studies show that (i) the removal of the alkali light chain exposes a site (or sites) close to the carboxyl-terminal region of the heavy chain, (ii) this site (or sites) is rapidly digested by both proteases and in the case of trypsin cleavage occurs here prior to digestion at the two other protease-sensitive regions in the interior of the primary structure of the heavy chain, and (iii) the ability of the heavy chain to reassociate with alkali light chain requires the integrity of the COOH-terminal region of the heavy chain. These studies, together with the recent observation by Sutoh (1982) that the alkali light chain of SF1 can be cross-linked to actin in acto-SF1, suggest that the alkali light chain can perturb sites close to the SF2/SF1 junction and to the actin binding domain and may play a role in the ability of the molecule to communicate between the SF2/SF1 junction and the actin binding domain.

Materials and Methods

Distilled water was purified to reagent grade by a Millipore QTM system and used throughout. [^{3}H]-*N*-E α-chymotrypsin, soybean trypsin inhibitor, and TPCK were obtained from the Sigma Chemical Co., and TPCK–trypsin was a product of Worthington. [^{3}H]-*N*-Ethylmaleimide (specific activity 55 Ci/mmol) was purchased from New England Nuclear. All other reagents were of analytical grade.

Preparation of Proteins. Myosin was prepared by the method of Godfrey & Harrington (1970). SF1 was prepared by digestion of myosin with chymotrypsin and separated into its isoenzymes by the procedure of Weeds & Taylor (1975). The free alkali light chains were obtained by denaturation of myosin by treatment in 6 M guanidine hydrochloride followed by ethanolic precipitation of the myosin heavy chain as described by Holt & Lowey (1975). The light chains were fractionated by ion-exchange chromatography as described by those authors. Heavy chain preparation from the SF1(A2) isoenzyme followed the procedure described recently by us (Sivaramakrishnan & Burke, 1982). When the temperature of the ion-exchange column was raised to 39 °C, an improved yield of the heavy chain was obtained. Protein concentrations were obtained by absorption employing $E^{1\%}_{280nm}$ values of 5.5, 7.5, and 2.0 for myosin, SF1, and the alkali light chains, respectively. Alternatively, the Bradford (1976) micromethod employing Coomassie Brilliant Blue R-250 was used with calibration curves constructed from the respective purified proteins. The calibration curve for SF1(A2) was used for determining the concentration of the SF1 heavy chain.

Trypsin and Chymotryptic Cleavage of Heavy Chain and SF1. Tryptic and chymotryptic cleavages of SF1(A2) and heavy chain (each at 0.1 mg/mL) were done in 0.093 M imidazole, 10 mM ATP, 14 mM $MgCl_2$, and 0.1 mM dithiothreitol, pH 7.0 at 25 °C, by employing a protease protein ratio of 1:50. In the case trypsin digestions, aliquots for subsequent gel electrophoresis in sodium dodecyl sulfate were removed at specified time intervals, immediately made 1% in sodium dodecyl sulfate and 2-mercaptoethanol, and placed straight away in a boiling water bath for 3 min. For samples to be used to examine the influence of tryptic digestion of the heavy chain on its ability to reassociate with free alkali light chain, aliquots were removed from the digestion mixture and added to small volumes of soybean trypsin inhibitor (weight ratio of 2 to 1 for STI to trypsin), and the samples were immediately cooled to 0 °C. Similar procedures were used in the case of chymotrypsin digestion except TPCK was used as the inhibitor in the samples to be used for the alkali light chain reassociation experiments. The final concentration in TPCK was 0.3 mM. In the case of the sequential digestion study, the heavy chain was predigested with chymotrypsin (heavy chain to protease ratio of 100:1) for 3 min at 25 °C and the digestion terminated by the addition of TPCK to make the final solution 0.3 mM in TPCK. Trypsin was then added in a ratio of 50:1 (heavy chain to trypsin), the samples were digested for various periods, and aliquots were then removed and denatured by boiling in sodium dodecyl sulfate (1%) and 2-mercaptoethanol (1%) as described above prior to examination by gel electrophoresis in the presence of sodium dodecyl sulfate.

Studies on Reassociation of Alkali Light Chain to Heavy Chain. Reassociation of heavy chain to free alkali light chain was done in 0.093 M imidazole, 10 mM ATP, and 14 mM $MgCl_2$, pH 7.0 at 0 °C. The heavy chains or predigested heavy chains (0.1 mg/mL) were incubated with a 6-fold molar excess of the A2 light chain and allowed to incubate at 4 °C from 1 to 2 h before being run on gel electrophoresis under nondenaturing conditions. Preparation and isolation of reconstituted subfragment 1 proceeded by incubating 1.6 mg (0.8 mg/mL) of heavy chain with a 6-fold molar excess of A2 as described above, but this sample was allowed to incubate at 4 °C for 16 h. The sample was then dialyzed exhaustively against 0.05 M imidazole and 0.1 mM dithiothreitol, pH 7.0 at 4 °C, to remove the nucleotide. This mixture was passed through a DEAE-cellulose column (DE-52, 5 cm × 1 cm, flow rate 40 mL/h) equilibrated at 4 °C with the same buffer. Under these conditions residual free heavy chain does not bind and is eluted from the column. Application of 0.12 M NaCl in the column buffer eluted the reconstituted SF1(A2) (Wagner & Weeds, 1977) as a single peak and the excess A2 light chain was subsequently eluted from the column by application of a 0.5 M NaCl step. The eluted proteins were checked by gel electrophoresis both under nondenaturing conditions and in the presence of sodium dodecyl sulfate.

Labeling of the SF1(A2) at the SH1 Thiol. Modification of the SH1 thiol was done at low ionic strength as described earlier (Burke & Reisler, 1977). A 11.5-mg sample of SF1-(A2) (10.5 mg/mL) in 30 mM KCl and 25 mM Tris-HCl,

[2] Hitherto the heavy chain refers to the segment of the myosin heavy chain in the SF1 region.

pH 7.0, was reacted with 200 μL of [^{3}H]-*N*-ethylmaleimide (specific activity 2.2 Ci/mmol) for 2 h at 4 °C. Dithiothreitol was added to give a 4 molar excess over the *N*-ethylmaleimide, and the solution was dialyzed against 50 mM imidazole and 0.1 mM dithiothreitol, pH 7.0 at 4 °C. Samples of the labeled SF1(A2) to be used for the heavy chain isolation were diluted with a 2 molar excess of unlabeled SF1(A2) before being subjected to the "thermal" isolation procedure. Samples to evaluate the site of modification were dialyzed into 0.1 M $KHCO_3$, pH 8.0, digested in this solvent with trypsin at 25 °C as described by Mornet et al. (1979), and subsequently examined by gel electrophoresis in the presence of sodium dodecyl sulfate.

Gel Electrophoresis under Nondenaturing Conditions and in the Presence of Sodium Dodecyl Sulfate. Polyacrylamide gel electrophoreses under nondenaturing conditions were done as described earlier (Burke & Sivaramakrishnan, 1981). Sodium dodecyl sulfate gel electrophoreses were done by the method of Laemmli (1970) except in some cases gradient gels from 8 to 15% polyacrylamide were used for the separating gel. Proteins to be examined by sodium dodecyl sulfate electrophoresis were concentrated by passage through Sephadex G-10 columns employing a modification of the Penefsky method (1977). The protein concentration was increased by about 8-fold, and control studies with diluted samples of tryptic-digested SF1 showed the same pattern on concentration as the undiluted samples. The gels ran in the presence of sodium dodecyl sulfate were subsequently stained and destained as described by Weeds (1976). Molecular weights were estimated on the basis of the mobilities of the following standard proteins: myosin, phosphorylase *a*, bovine serum albumin, actin, carbonic anhydrase, chymotrypsinogen, soybean trypsin inhibitor, myoglobin, and lysozyme. For visualization of the proteins separated by gel electrophoresis under nondenaturing conditions, the silver staining method of Morrissey (1981) was used, except that 0.1% sodium dodecyl sulfate was used in the first incubation solution. For detection of the ^{3}H label in the polypeptide bands separated by sodium dodecyl sulfate gel electrophoresis, the Coomassie Brilliant Blue stained bands were carefully excised and each treated with 200 μL of 30% hydrogen peroxide in miniscintillation vials at 40 °C for 20 h. On complete solubilization of the gel 4 mL of Hydromix (Yorktown Research) was added, and the contents of its vial were mixed and then subsequently counted in a liquid scintillation counter. Slices of the gels where no protein stain was evident were also treated in the same way to estimate the background level in the counting experiments, and these background counts were subtracted from the counts obtained from the protein bands.

Results

Digestion by Trypsin. The course and extent of tryptic digestion of free heavy chain were analyzed by sodium dodecyl sulfate gel electrophoresis, and the results are presented in Figure 1. The data shown here are paired comparisons of free heavy chain and SF1(A2) as a function of digestion time under identical conditions. The pattern of cleavage obtained in the case of SF1(A2) is consistent with the previous findings of Mornet et al. (1979, 1981) and by Yamamoto & Sekine (1979a,b). As shown in Figure 2, the heavy chain in SF1(A2) is cleaved initially at the T1 site resulting in the formation of a 21 000-dalton C-terminal (TF1) and a 75 000-dalton N-terminal (TF2-TF3) fragment. The latter is subsequently cleaved at the T2 site producing a 50 000-dalton middle segment (TF2) and a 27 000-dalton N-terminal (TF3) fragment. Cleavage of the isolated heavy chain shows a similar pattern

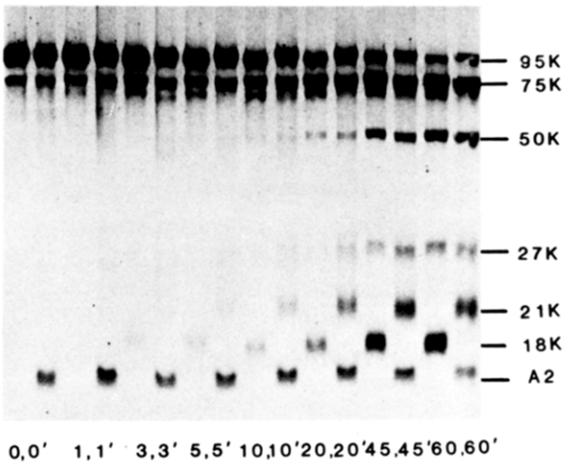

FIGURE 1: Sodium dodecyl sulfate gel electrophoretograms of the time course of trypsin cleavage of heavy chain and of SF1(A2). The unprimed and primed numbers represent the digestion times for heavy chain and SF1(A2), respectively. Both proteins at 0.1 mg/mL were digested with trypsin by using a trypsin to protein weight ratio of 1:50 at 25 °C in a solvent comprised of 0.093 M imidazole, 10 mM $MgCl_2$, 10 mM ATP, and 0.1 mM dithiothreitol. 12.5% polyacrylamide gel.

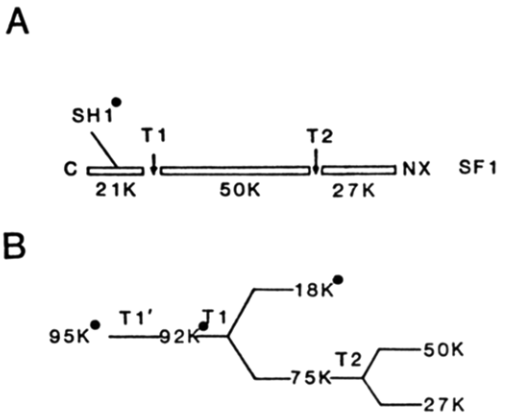

FIGURE 2: Schematic representation of trypsin cleavage of the heavy chain subunit in (A) associated SF1(A2) state and in (B) free state. The locations of the T1 and T2 sites are indicated. The closed circle represents the site of the radioactive label attached to the SH1 thiol.

except that an 18 000-dalton segment is formed apparently in place of the 21 000-dalton species formed in the case of SF1(A2). These data suggest that tryptic cleavage of the free heavy chain proceeds in an analogous fashion as that for SF1(A2) except that an additional site (or sites) in the C-terminal 21 000-dalton segment is also cleaved due to the prior removal of the alkali light chain. To examine this possibility the following experiment was done. SF1(A2) was labeled at its SH1 thiol with [^{3}H]-*N*-ethylmaleimide employing conditions described elsewhere (Burke & Reisler, 1977). The specificity of the modification was examined by cleaving the labeled protein with trypsin and following the disposition of the label with relation to the fragments separated by sodium dodecyl sulfate gel electrophoresis. The results of this experiment are shown in Table I. It is clear that initially 95% of the label was incorporated in the uncleaved heavy chain, but on digestion to form the various TF fragments the label was confined to the 21 000-dalton (TF1) fragment which is known to contain the SH1 (and SH2) thiols (Balint et al., 1978; Gallagher & Elzinga, 1980). The ^{3}H-labeled undigested SF1(A2) was next diluted with unmodified SF1(A2) and the heavy chain isolated from this mixture as described under Materials and Methods. This labeled heavy chain was next cleaved with trypsin, and the disposition of the label among

Table I: Distribution of Radioactivity in Peptides Derived from Tryptic Digestion of [^{3}H]-NEM-SH1, SF1(A2), and Heavy Chain[a]

protein	digestion time (min)	radioactivity in peptides (%) 95K[b]	75K[b]	50K[b]	25-27K[b]	21K[b]	18K[b]	A2	cpm
SF1(A2)	0	95	4	0				1	3804
SF1(A2)	5	65	5	0	3	24		3	4847
SF1(A2)	45	13	4	0	2	78		3	3936
SF1(A2)	90	2	2	1	3	87		5	3506
heavy chain	0	96	4						1572
heavy chain	5	66	0				34		1280
heavy chain	45	8	4	0	0		88		1553
heavy chain	90	0	0	0	0	0	100		1390

[a] The lower counts per gel electrophoretogram of the free heavy chain resulted from the dilution of the ^{3}H-labeled SF1(A2) with unmodified SF1(A2) prior to isolation of the heavy chain and its digestion by trypsin. [b] Molecular masses in daltons.

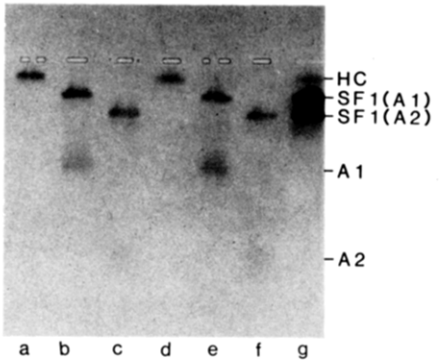

FIGURE 3: Gel electrophoretograms under nondenaturing conditions. (a) Heavy chain; (b) heavy chain incubated with A1; (c) heavy chain incubated with A2; (d) [^{3}H]-*N*-ethylmaleimide-labeled heavy chain; (e) [^{3}H]-*N*-ethylmaleimide-labeled heavy chain incubated with A1; (f) [^{3}H]-*N*-ethylmaleimide-labeled heavy chain incubated with A2; (g) mixture of heavy chain, SF1(A1), and SF1(A2). Details of the reassociation conditions are presented under Materials and Methods.

the fragments produced was again examined as described above for SF1(A2). These results are also presented in Table I. It is clear that on digestion the ^{3}H label was found only in the 18 000-dalton fragment. This, therefore, demonstrates that the 18 000-dalton species contains the SH1 thiol and that it must arise from the 21 000-dalton C-terminal region of the heavy chain.

Further support that the 18 000-dalton fragment results from the exposure of a site (or sites) unmasked by the removal of the alkali light chain is provided by the following experiment. Here, undigested free heavy chain was mixed at 0 °C with free alkali light chain A2, resulting in reconstitution of SF1(A2) as shown in Figure 3. It is clear that addition of either A1 or A2 to the isolated heavy chain results in the formation of species with the same electrophoretic mobility as native SF1(A1) or SF1(A2), respectively. On isolation of the reconstituted SF1(A2) by ion-exchange chromatography at 4 °C on DEAE-cellulose (Wagner & Weeds, 1977), it was digested with trypsin, and this was compared with a sample of native SF1(A2) digested in the same fashion. These results are shown in Figure 4. It is immediately apparent that digestion of the reconstituted SF1(A2) resulted in the formation of a 21 000-dalton fragment identical in terms of electrophoretic mobility on sodium dodecyl sulfate gel electrophoresis with that produced with native SF1(A2). It is clear, therefore, that reassociation of the alkali light chain to the heavy chain restores the protective effect to the heavy chain and prevents formation of the 18 000-dalton fragment.

An examination of the digestion pattern of the free heavy chain (Figure 1) suggests that the 18 000-dalton fragment arises without the formation of a 21 000-dalton precursor, since there is no indication of a band corresponding to the latter even

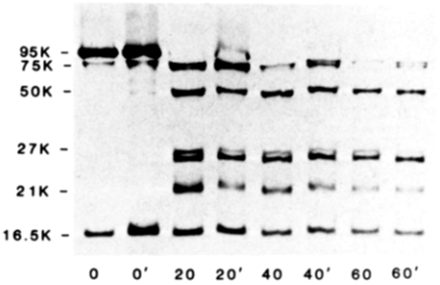

FIGURE 4: Sodium dodecyl sulfate gel electrophoretograms of trypsin digestion of native SF1(A2) and reconstituted SF1(A2). Digestions were done on both proteins at 0.1 mg/mL at 25 °C in 0.05 M imidazole and 0.1 mM dithiothreitol, pH 7.0, for the times indicated. The primed and unprimed numbers on the abscissa refer to the time of digestion in minutes for reconstituted SF1(A2) and native SF1(A2), respectively. 12.5% polyacrylamide gel.

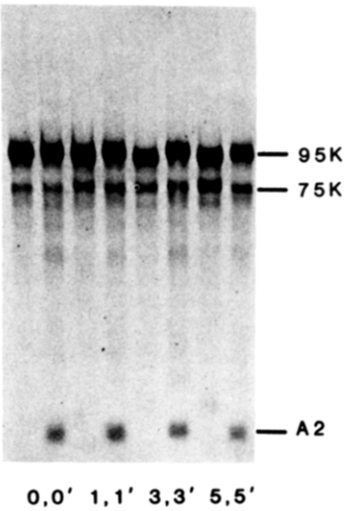

FIGURE 5: Sodium dodecyl sulfate gel electrophoretograms of the early stages of trypsin digestion of heavy chain and SF1(A2). Conditions as described in the legend for Figure 1 except the polyacrylamide content of the gel was 5%. The numbers on the abscissa represent the time of trypsin digestion in minutes.

at early stages of digestion. A possible clue to the genesis of the 18 000-dalton fragment is provided by a closer examination of these electrophoretograms and especially of the band corresponding to the undigested heavy chain. In the case of SF1(A2) little, if any, change can be detected in the mobility of the residual heavy chain with increasing times of digestion. On the other hand, in the case of the free heavy chain, it is apparent that there is a rapid change in the mobility of the heavy chain band so that it moves slightly faster, even at the earliest stages of digestion, and before any evidence for cleavage at the T1 or T2 sites can be discerned. These differences in the behavior of the heavy chains are more apparent

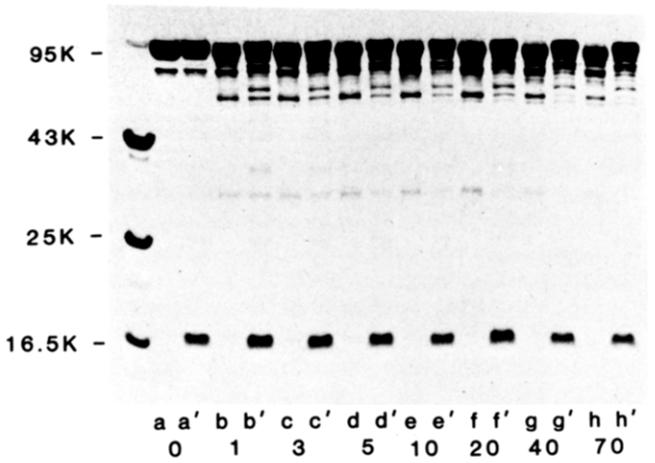

FIGURE 6: Sodium dodecyl sulfate gel electrophoretograms of the time course of α-chymotrypsin digestion of the heavy chain and SF1(A2). Digestions were done under the same solvent conditions described in the legend of Figure 1 except that a 50:1 weight ratio of protein to α-chymotrypsin was used here. Polyacrylamide gradient gel of 8–15% was employed. The unprimed and primed letters correspond to heavy chain and SF1(A2), respectively, and the numbers along the abscissa represent the digestion times in minutes.

in the sodium dodecyl sulfate gel electrophoretograms shown in Figure 5. When this more porous gel is used, a conservative estimate of the loss in weight corresponding to the gain in the electrophoretic mobility of the heavy chain can be made. It appears that this weight loss is in the range of about 2000–3000 daltons and suggests that tryptic cleavage is occurring initially at one or both ends of the heavy chain. Since subsequent cleavage of the isolated heavy chain produces fragments (with the exception of the 18 000-dalton species) with molecular masses very close to if not identical with the 50 000- and 27 000-dalton fragments, corresponding respectively to the middle and N-terminal segments of the heavy chain obtained by digestion of SF1(A2), where no initial cleavage of the residual heavy chain band can be detected, it appears likely that the major if not entire portion of the 2000-3000-dalton molecular mass loss arises from the C-terminal end. This interpretation would be consistent with the experimental data which fail to show any indication of the formation of a 21 000-dalton precursor for the 18 000-dalton fragment.

Digestion with α-Chymotrypsin. Because of the high specificity of trypsin for digestion at the carboxyl peptide bonds of lysyl and arginyl residues, it was of interest to examine whether similar conclusions could be reached by digesting SF1(A2) and the free heavy chain with a protease of different specificity. Figures 6 and 7 show $NaDodSO_4$ gel electrophoretograms following the time course of digestion of these two proteins with α-chymotrypsin. An inspection of these data shows that, under similar conditions of digestions to those employed in the earlier studies with trypsin, the heavy chain both in the SF1(A2) protein and in isolated form is relatively insensitive to cleavage as can be discerned by the absence of any accumulation of significant amounts of intermediate fragments even on prolonged digestion. However, in both gel systems (8–15% gradient and 5% acrylamide) it is clear that in the case of the isolated heavy chain, chymotryptic digestion results in the residual heavy chain moving with a higher mobility, consistent with a rapid cleavage at one or both ends of the heavy chain. Moreover, in the case of digestion of SF1(A2) no such change in the mobility of the residual heavy chain band is apparent in the early stages of digestion. Subsequent tryptic cleavage of a chymotryptic predigested free heavy chain produced the fragmentation pattern shown in

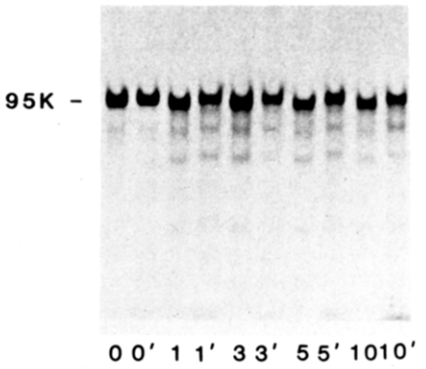

FIGURE 7: Sodium dodecyl sulfate gel electrophoretograms of the early stages of α-chymotrypsin digestion of free heavy chain and SF1(A2). Conditions as described in the legend for Figure 6 except the polyacrylamide content of the gel was 5%. The A2 light chain present in the electrophoretograms of the SF1(A2) samples (primed number) is at the bottom of the gel.

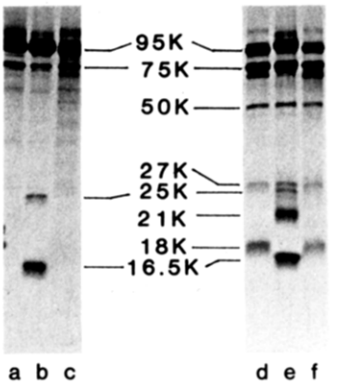

FIGURE 8: Sodium dodecyl sulfate gel electrophoretograms of sequential digestion of heavy chain by chymotrypsin and then trypsin. (a) "Native" heavy chain; (b) SF1(A2) showing a small proportion of SF1(A1); (c) heavy chain predigested with chymotrypsin (heavy chain to chymotrypsin 100:1) for 3 min at 25 °C and made 0.3 mM in TPCK; (d, e, and f) samples shown in (a), (b), and (c), respectively, after digestion by trypsin (protein to trypsin ratio of 50:1) for 30 min at 25 °C.

Figure 8 together with that for SF1(A2) and for trypsin-digested heavy chain. This sequentially digested sample yielded fragments of 27 000 and 50 000 daltons indistinguishable from fragments produced by tryptic cleavage of SF1 or of isolated heavy chain alone. It is known that the 27 000-dalton fragment contains the blocked amino terminus of the heavy chain (Lu et al., 1978), and therefore, it is possible to conclude that the initial attack of chymotrypsin on the free heavy chain is confined predominantly, if not exclusively, at the COOH-terminal end, although the loss of a few residues at the amino-terminal end of the heavy chain would not be detected by the electrophoretic method. This is supported by the fact that tryptic digestion of the prechymotryptic digested heavy chain produces a species of 18 000 daltons very similar to that formed by tryptic digestion alone. This finding suggests that the site of the initial chymotryptic cleavage resides closer to the carboxyl-terminal end than the trypsin-sensitive site, since otherwise a fragment of molecular mass smaller than 18 000 daltons should have been formed by the sequential digestion.

Studies on the Effect of Cleavage at the Carboxyl-Terminal End of the Heavy Chain on the Ability To Reassociate with the Alkali Light Chain. Since the studies with the heavy chain described in the previous sections indicate that the removal of the alkali light chain does expose a segment of the heavy chain near the carboxyl-terminal region and renders it vulnerable to proteolytic attack, it was of interest to determine

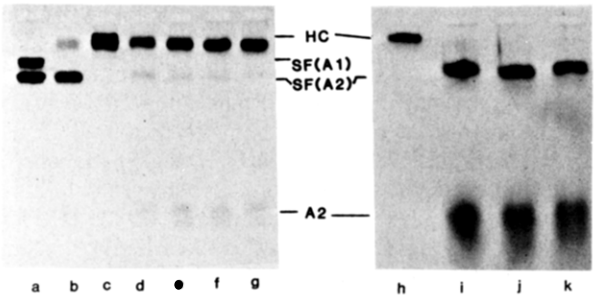

FIGURE 9: Gel electrophoretograms under nondenaturing conditions of the effect of trypsin digestion of the heavy chain on its ability to reassociate with A2 light chains. Digestions were done under the conditions described in the legend for Figure 1. At different times of digestion the samples were cooled to 0 °C and a 6-fold molar excess of the A2 light chain was added. (a) SF1(A1) and SF1(A2) standards; (b) undigested heavy chain exposed for 40 min at room temperature prior to incubation at 0 °C with A2; (c) undigested heavy chain; (d–g) same as (b) but heavy chain incubations with A2 after trypsin digestions for 1, 3, 5 and 10 min, respectively; (h–k) controls done with another heavy chain preparation (h) to examine whether reassociation of the heavy chain with A_2 (i) is influenced by the presence of soybean trypsin inhibitor (j) or the soybean trypsin inhibitor–trypsin complex (k). Other details under Materials and Methods. Silver-stained gels.

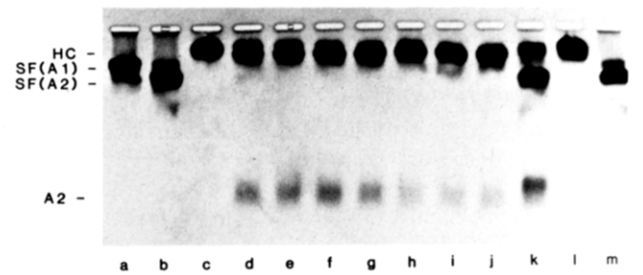

FIGURE 10: Gel electrophoretograms under nondenaturing conditions of the effect of chymotryptic digestion on the ability of the heavy chain to reassociate with A2 light chains. Digestions were done under the conditions described in the legend to Figure 6. (a) SF1(A1); (b) SF1(A2); (c) heavy chain; (d–j) heavy chain incubations with A2 after chymotryptic digestions for 3, 6, 10, 20, 30, 45, and 60 min, respectively; (k) undigested heavy chain maintained for 60 min at 25 °C before incubation with A2; (l) heavy chain; (m) SF1(A2). These are silver-stained gels. Other details under Materials and Methods.

whether heavy chain digested in this way would be capable of rebinding alkali light chain. The results of such experiments are presented in Figure 9 for the tryptic-digested heavy chain. An examination of the electrophoretogram of the heavy chain run on nondenaturing gel electrophoresis reveals that it runs as a broad band which on closer observation appears to be actually two bands moving with similar electrophoretic mobilities (Figure 9c). The reason for this is unclear since some preparations of the heavy chain run electrophoretically as a single band (Figure 9h). Unfortunately because of the different colors associated with the silver-stained electrophoretograms, it is not possible to estimate the amount of protein associated with each band. However, the fact that the heavy chain runs as a doublet here does not seem to interfere with its ability to reassociate with the alkali light chain A2 to form SF1(A2) (Figure 9b,i). Trypsin cleavage of the heavy chain, on the other hand, rapidly reduces the ability of the heavy chain to reassociate to A2, and this occurs within 1 min of adding the trypsin (Figure 9d–g). At these early stages of digestion the only significant change in the free heavy chain is the cleavage(s) that results (result) in the apparent molecular mass loss of 2000–3000 daltons, which we believe is primarily restricted to the carboxyl-terminal segment of the heavy chain (Figure 1). Furthermore, incubation of the heavy chain without digestion at room temperature for periods exceeding the duration of the tryptic digestion has little if any effect on the ability of the heavy chain to reassociate with free A2 (Figure 9b). The failure of the digested heavy chain to reassociate with the alkali light chain does not appear to be caused by the presence of the soybean trypsin inhibitor or its complex with trypsin since the extents of reassociation here with the undigested heavy chain (Figure 9j,k, respectively) appear to be the same as the control (Figure 9i).

Similar results were obtained for the heavy chain subjected to digestion with chymotrypsin. As shown in Figure 10k, prior to digestion the heavy chain is capable of reassociation with free A2 since a species with the same electrophoretic mobility as SF1(A2) is formed by mixing these two subunits. However, digestion with chymotrypsin for just 1 min causes the heavy chain to be no longer capable of binding the A2 light chain (Figure 10d). The only major change occurring in this early stage of the chymotryptic digestion is the cleavage resulting in a slightly higher electrophoretic mobility of the residual heavy chain (Figure 7), consistent with the cleavage at the carboxyl-terminal segment of this subunit.

Discussion

Although a number of interesting relationships between the DTNB light chain and certain sites in the heavy chain have been described (Bagshaw, 1977; Weeds & Pope, 1977; Margossian et al., 1975; Oda et al., 1980), the information concerning the alkali light chain's interaction with the heavy chain is rather scant. Fluorescence energy transfer experiments of the distance between the single thiol of the alkali light chain and the SH1 thiol or the ATPase site of the heavy chain have yielded distances of about 40 Å (Marsh & Lowey, 1980; Moss & Trentham, 1979), but because of the known flexibility of certain regions of the heavy chain (Highsmith et al., 1979) and in particular at the SH1 and SH2 region (Burke & Reisler, 1977; Wells & Yount, 1979; Wells et al., 1980), the significance of such measurements is not at all clear. In the case of scallop myosin Szentkiralyi (1982) has shown that both the regulatory light chain and the SH light chain (the alkali light chain equivalent) can be isolated as a complex with a 14 000-dalton fragment of the heavy chain, although the location of this fragment relative to the COOH-terminal region of this chain in the SF1 segment was not specified. Furthermore, in this invertebrate myosin there is additional strong evidence that the two classes of light chains are in very close proximity and probably interacting (Wallimann et al., 1982).

The primary purpose of this study was to examine whether a comparison of the pathways of tryptic cleavage of the heavy chain subunit in free or SF1-associated states would provide information about the nature of the alkali light chain interaction with the heavy chain. Our choice for employing limited trypsin proteolysis to probe this question was based on the following considerations. First, the cleavage pattern obtained from the heavy chain subunit when associated with the alkali light chain in SF1 has been well characterized by a number of laboratories (Balint et al., 1975; Mornet et al., 1979, 1981; Yamamoto & Sekine, 1979a,b) and is shown schematically in Figure 2. Second, in SF1 the cleavages at the T1 and T2 sites of the heavy chain are sensitive to actin (Mornet et al., 1981; Yamamoto & Sekine, 1979a,b) and to nucleotide perturbation (Muhlrad & Hozumi, 1982), respectively.

The present study has shown that a region close to the carboxyl-terminal end of the SF1 heavy chain is perturbed by the presence or absence of the alkali light chain. Removal of the alkali light chains exposes these sites to very rapid cleavage either by chymotrypsin (Figures 6 and 7) or by trypsin (Fig-

ures 1 and 5), and this is responsible for the generation of an approximately 18 000-dalton carboxyl-terminal fragment containing the SH1 thiol on further digestion with trypsin and cleavage at the T1 site (Figures 1 and 8 and Table I). The fact that reassociation of the alkali light chain results in the protection of these sites to proteolytic attack (Figure 4) indicates that their cleavage in the free heavy chain is a consequence of light chain removal and not to irreversible changes associated with its isolation.

The failure to detect a significant perturbation of the cleavage pathway within the TF2 and TF3 segments is suggestive that the alkali light chain interaction with these regions of the heavy chain is minimal in SF1. This interpretation would be consistent with the finding that the ATPase site (or part thereof) resides in the TF3 region (Szilagyi et al., 1979) and is functional in the absence of alkali light chain (Wagner & Giniger, 1981; Sivaramakrishnan & Burke, 1982). It is unlikely that this failure can be attributed to the proteolytic specificity of trypsin, since chymotrypsin also showed little if any tendency to attack the free heavy chain at these regions.

This conclusion is difficult to reconcile with the recent report by Labbé et al. (1981) that the alkali light chain in SF1 can apparently be cross-linked to the TF3 segment of the SF1 heavy chain. Their conclusion was based on two-dimensional gel electrophoretic analyses of tryptic-cleaved cross-linked SF1, showing the presence of an off-diagonal 27 000-dalton fragment after thiolysis of the disulfide-bearing cross-linking reagent prior to electrophoresis in the second dimension. This assignment, however, must be viewed as tentative since it was not demonstrated that trypsin cleavage of the cross-linked sample would proceed in the same fashion as the un-cross-linked SF1 protein. Should this assignment be sustained, it would indicate that the TF2 and TF3 segments of the heavy chain may be inaccessible to proteolytic attack whether or not the alkali light chain is bound. Additionally, it would indicate that the alkali light chain may be capable of interacting with both the carboxyl-terminal and amino-terminal segments of the heavy chain, the latter known to be part of the ATPase site (Szilagyi et al., 1979). However, because of the estimated hydrodynamic length of the free alkali light chain (Alexis & Gratzer, 1978), it is not possible to ascertain whether or not portions of these noncontiguous segments are in close proximity in the heavy chain.

Of particular interest, we feel, is the observation that the ability of the heavy chain to reassociate with the alkali light chain is lost after cleavages at these exposed sites by either trypsin or chymotrypsin (Figures 9 and 10). These results suggest that the integrity of the carboxyl-terminal region of the SF1 heavy chain is crucial for this binding. Whether or not this means that this region is directly involved in the binding of the alkali light chain cannot be assessed at present. It should be noted that this (these) cleavage(s) does (do) not appear to have affected adversely the other functional properties of this subunit, since we have observed that the truncated heavy chain, even with cleavages at the T1 and T2 sites, exhibits ATPase activities for its unmodified and SH1-modified forms similar to those for the corresponding SF1 species (Burke & Kamalakannan, 1983).

These studies also have interesting implications relating to the role of the DTNB light chain in myosin function. Substantial evidence exists that this light chain can perturb both the SF2/SF1 junction and the actin binding domain in myosin (Bagshaw, 1977; Weeds & Pope, 1977; Oda et al., 1980; Margossian et al., 1975; Kuwayama & Yagi, 1977; Kardami & Gratzer, 1982). However, the fact that these perturbations have been only observed in preparations containing the bound alkali light chain raises the possibility that the alkali light chain may also be involved in these processes. The possibility of a cooperative role of the two light chains in the Ca^{2+} regulation of the actomyosin interaction has been proposed by Wallimann & Szent-Györgyi (1981) based on their elegant studies of the subunit structure and function in scallop myosin (Wallimann & Szent-Györgyi, 1981; Wallimann et al., 1982). Evidence that the DTNB light chain and the alkali light chains may be interacting in skeletal myosin subfragment 1 has been provided by Hozumi et al. (1979). The observation by Sutoh (1982) that the alkali light chain can be cross-linked to actin in acto-SF1 and the present study clearly indicate that the alkali light chain is capable of either spanning the TF1 segment in myosin or at least perturbing the actin binding site and the carboxyl-terminal end of the TF1 segment of the heavy chain. These results, although far from proving the case, favor the possibility that the DTNB light chain and the alkali light chain in skeletal muscle myosin function together in enabling events at the SF1/SF2 function of the molecule to be transmitted to the actin binding domain of the heavy chain.

Acknowledgments

We are thankful to Dr. William F. Harrington and Dr. Emil Reisler for their reading of the manuscript and for valuable suggestions.

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Tropomyosin–Troponin and Tropomyosin–Actin Interactions: A Fluorescence Quenching Study†

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ABSTRACT: Rabbit skeletal $\alpha\alpha$-tropomyosin was specifically labeled at Cys-190 with the fluorescent probe *N*-(iodoacetyl)-*N'*-(1-naphthyl-5-sulfo)ethylenediamine (1,5-IAEDANS). The fluorescence decay of the resultant AEDANS-labeled $\alpha\alpha$-tropomyosin (Tm*) was monoexponential with a lifetime of 13.55 ns. When acrylamide was used as the quencher, the apparent Stern–Volmer quenching constant K_{sv}' for Tm* was measured to be 5.78 M^{-1} and the quenching rate constant k_q to be 3.20×10^8 M^{-1} s^{-1}. The presence of troponin reduced the magnitude of K_{sv}' to 4.14 M^{-1} and induced the appearance of a second decay component. This second component had an amplitude of ~20% of the total intensity, a lifetime of ~20 ns, and a k_q of 4.5×10^{-7} M^{-1} s^{-1}. Similarly, the presence of F-actin induced the appearance of a minor longer lived decay component with a decreased k_q. On the basis of the increase in the lifetime and the decrease in k_q, the appearance of the long-lived decay component was interpreted to be due to troponin or actin interacting with Tm* near the Cys-190 site in both cases. Our results further suggest that the label was capable of equilibrating between an exposed hydrophilic environment on the surface of Tm* and a buried hydrophobic environment at the troponin–Tm* or actin–Tm* interaction interfaces.

It is now well established that tropomyosin (Tm)[1] in conjunction with troponin (Tn) mediates the regulation of mammalian skeletal muscle contraction by calcium ions (Ebashi & Endo, 1968). The molecular mechanism of this regulatory process is as yet not fully understood. It is, therefore, important to obtain information on the interactions between Tm and the other contractile proteins. It has been inferred that Tn binds to Tm near the Cys-190 site of Tm from electron microscopy studies (Cohen et al., 1972; Ohtsuki, 1974; Stewart, 1975) and from sequence analysis studies (McLachlan & Stewart, 1976a). More recently, evidence suggesting that the Tn binding site in Tm is more extensive was reported (Mak & Smillie, 1981). Repeated regions of sequence similarity in

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[1] Abbreviations: Tm, tropomyosin; Tn, troponin; S1, myosin subfragment 1; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(1-naphthyl-5-sulfo)-ethylenediamine; Tm*, 1,5-IAEDANS-labeled $\alpha\alpha$Tm; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Nbs_2, 5,5'-dithiobis-(2-nitrobenzoate); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-3053$01.50/0