Trypsinolysis Promotes Disulfide Formation between 21- and 50-Kilodalton Segments of Myosin Subfragment 1 during Reaction with 5,5′-Dithiobis(2-nitrobenzoic acid)†

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ABSTRACT: The reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with S1 and tryptic S1 has been examined to identify the sites of mixed and intramolecular disulfides formed in the initial and final stages of the reaction in these two forms of S1. With undigested S1, the two mixed disulfide bonds initially formed were found to be in the 21-kDa segment. The intramolecular disulfide bond, formed in the subsequent slow phase of the reaction, was also found to be mainly confined to the 21-kDa segment although a small fraction arose from disulfide formation between the 21-kDa and 50-kDa segments. Only 35% of the light chain was modified in undigested S1 after 24 h. For tryptic S1, the initial reaction also led to the formation of mixed disulfides in the 21-kDa segment. However, in the second slower phase, the formation of the intramolecular disulfide occurred primarily between thiols in the 21-kDa and 50-kDa fragments, and in this case, the light chain was labeled to about 60% after 24 h. The enhanced formation of disulfide links between 21-kDa and 50-kDa domains in tryptic S1 points to an increase in flexibility between the thiol-containing regions of these segments.

It has been previously demonstrated by Wells and Yount (1980) that when myosin subfragment 1 (S1)[1] is modified with DTNB in the presence of MgADP, the reaction proceeds in two stages. The first stage results in the formation of close to two mixed disulfides, and the second stage involves a slower sulfhydryl–disulfide exchange resulting in the formation of about one intramolecular disulfide. On the basis of changes in the ATPase properties accompanying the reaction and the observation that MgADP was trapped in proportion to the amount of intramolecular disulfide formed, it was postulated that the disulfide was formed by exchange of free SH2 with the mixed disulfide on SH1, suggesting that these two thiols could move within chemical bond length when S1 binds MgADP.

Because of the importance of this conclusion with respect to the substructure of S1 and nucleotide-dependent changes, we have sought to obtain more direct evidence to characterize the locations of the mixed disulfides and intramolecular disulfide formed in the initial and final stages, respectively. Furthermore, on the basis of recent reports of alterations in S1 structure and flexibility induced by trypsinolysis (Cheung et al., 1985; Goodearl et al., 1985; Chaussepied et al., 1986b; Eden & Highsmith, 1987; Chen et al., 1987), we were also interested in determining whether limited tryptic digestion of S1 would bring about any changes in this reaction. Previous studies involving modification of S1 and tryptic S1 with DTNB have failed to find significant differences based on the total amount of TNB formed or on its release by dithioerythritol (Chaussepied et al., 1986a).

The present work shows that in the case of undigested S1 the intramolecular disulfide bond was formed primarily by exchange reactions limited within the 21-kDa[2] segment although a small amount of disulfide formation between the 21-kDa and 50-kDa segments could also be detected. In the case of tryptic S1, the initial reaction with DTNB closely resembled that occurring with undigested S1 in the extent and location of mixed disulfide formation. However, the subsequent exchanges occurring in the modified tryptic S1 led to a significant increase in disulfide formation between the 21-kDa and 50-kDa fragments such that this represented the major fraction of the intramolecular disulfide. Thus, the present study shows that thiols in the 50-kDa segment can also come within chemical bond length of mixed disulfides in the 21-kDa segment and that this occurs to a much higher degree in tryptic S1.

Materials and Methods

Materials. Trypsin, chymotrypsin, soybean trypsin inhibitor, DTNB, DFP, and trifluoroacetic acid were obtained from Sigma. Endoproteinase Glu-C (V8 protease) was from Boehringer Mannheim Biochemicals. Acetonitrile was from Polysciences Inc.

Proteins. The details of the preparation of rabbit skeletal myosin, S1, separation of the S1 isozymes, assay of ATPase activities, and measurement of protein concentrations were as given in an earlier publication (Rajasekharan et al., 1987). Tryptic S1 was prepared by the digestion of S1 in imidazole (50 mM), pH 7.0, NaN_3 (0.1 mM), and dithiothreitol (0.1 mM) at 25 °C for 35 min with an S1:trypsin ratio of 100:1 w/w and stopped by soybean trypsin inhibitor. Digestion with V8 protease (Chaussepied et al., 1983) was done in Hepes (50 mM), pH 8.0, with a protease:S1 ratio of 1:50 w/w for 1 h at 25 °C. The digestion was stopped by adding DFP (Caution!) to a final concentration of 1 mM, and the cleaved protein kept at 4 °C for another 12 h was then dialyzed into Tris (0.05 M)/KCl (0.1 M), pH 8.0. Unless otherwise mentioned, all

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[1] Abbreviations: S1, myosin subfragment 1; S1A2, S1 isozyme containing A2 light chain; HPLC, high-performance liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DFP, diisopropyl fluorophosphate; R_T, retention time; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNB, 5-thio-2-nitrobenzoic acid.

[2] The relative molecular masses given for the tryptic fragments of the S1 heavy chain are those based on SDS–PAGE.

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reactions were done with S1A2. Comparisons between intact S1 and cleaved S1 were made by using protein from the same preparation and were repeated by using S1 from different preparations to check reproducibility.

Modification with DTNB. Intact S1 or cleaved S1 (2 mg/mL) in 0.1 M KCl/0.05 M Tris, pH 8.0, was reacted with a 2 molar excess of DTNB in the presence of 1 mM MgADP at 4 °C. At the time intervals of 30 min, 6 h, and 24 h, aliquots (0.5 mL) were centrifugally chromatographed in 3 mL of Sephadex G-50 in the above buffer (Penefsky, 1977) to remove excess of the reagent and nucleotide. Another aliquot was used to measure the amount of TNB groups formed by measuring the absorption at 412 nm (Ellman, 1959). After purification, the concentration of the modified protein and its ATPase activities were assayed. In the case of reactions with intact S1, a portion of the modified S1 was immediately digested with trypsin (trypsin:S1 ratio of 1:25, w/w) at 4 °C for 30 min. Control digestions using S1 had shown that the above proteolytic conditions assured complete digestion of S1 to its tryptic form as evidence by SDS–PAGE and HPLC analyses. The use of a higher protease to S1 ratio, low temperature, and short duration for the proteolysis minimized any sulfhydryl–disulfide exchange during the digestion. The digestions were stopped either by boiling an aliquot (80 μL) with a 5-fold-concentrated Laemmli sample buffer (20 μL) (Laemmli, 1970) or by diluting (100 μL) into 0.1% (v/v) trifluoroacetic acid in water (900 μL) in preparation to the subsequent SDS–PAGE and HPLC analyses, respectively. Reaction with tryptic S1 or V8-cleaved S1 was stopped by diluting the purified protein into 0.1% trifluoroacetic acid.

In order to ascertain whether the prolonged reaction of S1 and tryptic S1 with DTNB resulted in any denaturation, the proteins after 24-h reaction were treated with 10 mM dithiothreitol for 24 h at 4 °C and centrifugally chromatographed twice through Sephadex G-50 in Tris (0.05 M)/KCl (0.1 M), pH 8.0, and the ATPase activities were determined. The recovery of Ca^{2+}- and K^+-EDTA activities for S1 was 92% and 85%, respectively, and for tryptic S1, 102% and 88% compared to the corresponding activities of the unreacted species.

Gel Electrophoresis and HPLC Analyses. Polyacrylamide (12.5%) gels were prepared and run by the procedure of Laemmli (Laemmli, 1970) and stained with 0.1% Coomassie Blue R-250.

HPLC analyses were done using equipment, solvents, mobile phase, column (Synchropak RP-4, Synchrom Inc.), gradient program, and other experiental conditions as described earlier (Rajasekharan et al., 1987). For HPLC separation, 100 μL of protein solution (2 mg/mL) was diluted into 900 μL of 0.1% trifluoroacetic acid in water and immediately loaded onto the column or frozen and stored for subsequent runs. Keeping the modified protein in the presence of acid did not have any ill-effects on the reproducibility of the elution profile monitored both at 214 nm (absorbance units, full scale 0.32) and at 340 nm (absorbance units, full scale 0.005), thus ascertaining the stability of the mixed and intramolecular disulfide bonds at low pH. For the identification of the eluted peptides, the fractions corresponding to the eluted peaks were collected and lyophilized, and the residue was dissolved in 20 μL of 0.1 M Hepes, pH 8.0, and heated with 5 μL of a 5-fold-concentrated Laemmli sample buffer with or without 2-mercaptoethanol as required. Either 10 or 20 μL of the samples thus obtained was loaded onto the gel for SDS–PAGE.

The omission of 2-mercaptoethanol during sample preparation for SDS–PAGE showed that each of the two fractions eluting out between the light chain and 50-kDa segment consisted of a single 80-kDa band, thereby confirming that the 21-kDa and 50-kDa fragments found in these fractions during SDS–PAGE with 2-mercaptoethanol were indeed linked together through disulfide bonds. As a control, S1 and tryptic S1 peptides were separated on HPLC, lyophilized, and dissolved in Laemmli sample buffer without 2-mercaptoethanol and analyzed by SDS–PAGE. The gels showed a band pattern similar to normal runs except for some band broadening. No abnormal mobilities were observed for the constituent peptides of S1 or tryptic S1.

Determination of Amount of TNB-Mixed Disulfides Formed. The analysis was based on first determining the amount of light chain modified. This was done by integration of the peaks associated with the unmodified (R_T 53 min) and modified (R_T 55 min) light chains in the chromatograms monitored at 214 nm. Using unmodified and DTNB-treated A2 light chain, it was separately ascertained that formation of the mixed TNB disulfide on the light chain had no effect on its 214-nm absorption. Since the modified light chain showed high 340-nm absorption, it was assumed that it was all in the form of TNB-mixed disulfide. Integration of the peak area of the modified light chain in the corresponding chromatogram monitored at 340 nm allowed for evaluation of the amount of 340-nm absorption per mole of mixed TNB disulfide. This value was then used to determine the amount of mixed TNB disulfides associated with the other fragments from the chromatogram monitored at 340 nm.

Results and Discussion

The present study was undertaken to characterize the extent and location of the mixed disulfides and intramolecular disulfides formed in the initial and subsequent stages, respectively, when S1 and tryptic S1 (Balint et al., 1975) were reacted with DTNB in the presence of MgADP under conditions similar to those of Wells and Yount (1980). The method of analysis used in the present work allowed for the direct characterization of the location and the amount of mixed disulfide present at different stages of the reaction. The main requirement for the analysis in the case of intact S1 is that the subsequent tryptic fragmentation (required for analysis by HPLC separations) is not altered by the modification and that the digestion can be accomplished under conditions which minimize the extent of the slower intramolecular disulfide exchange reactions. The SDS–PAGE analysis of S1 digested with trypsin at different times of modification with DTNB indicate that these conditions have been met (see below, Figure 2; right panel). The formation of a 42-kDa band in the reacted samples can be attributed to the effect of the mixed disulfide group present in S1 (Chaussepied et al., 1986a), but since this peptide coelutes with the 50-kDa peptide, its presence does not affect the subsequent analysis for the distribution and amount of mixed disulfide among the heavy chain fragments. The results of this analysis have been summarized in Table I for convenience and will be described in more detail as follows.

The data for intact S1, obtained by measuring the TNB produced during the reaction (Table I), indicated that in the initial 30 min about 1.58 thiols had been modified, which is in excellent agreement with the data of Wells and Yount (1980). However, the amount and distribution (Table I) of S–TNB-mixed disulfides obtained from the HPLC analysis subsequent to tryptic cleavage (Figure 1) indicated that 1.43 mol of the total 1.58 mol of thiols reacted in S1 was still associated with TNB groups. Of these mixed disulfides, 1.3 mol was localized in the 21-kDa segment. Thus, at this stage

Table I: Comparison of the Reaction of DTNB with S1 and Tryptic S1[a]

reaction time	mixed disulfides present[b]				TNB produced during reaction[c]	intra- or intersegment disulfide formed
	21-kDa peak	cross-link peak	light chain peak	total		
			Intact S1			
30 min	1.3	0	0.13	1.43	1.58	0.08
6 h	0.84	0.02	0.3	1.16	2.41	0.63
24 h	0.45	0.09	0.35	0.89	2.65	0.88
			Tryptic S1			
30 min	1.10	0	0.34	1.44	1.53	0.05
6 h	0.39	0.21	0.53	1.13	2.55	0.71
24 h	0.1	0.30	0.56	0.96	2.91	0.98

[a] The values are accurate to ±5%. [b] Estimated as given under Materials and Methods on the basis of HPLC analysis monitored at 214 and 340 nm for the protein and the TNB group, respectively. [c] Estimated by the absorbance at 412 nm.

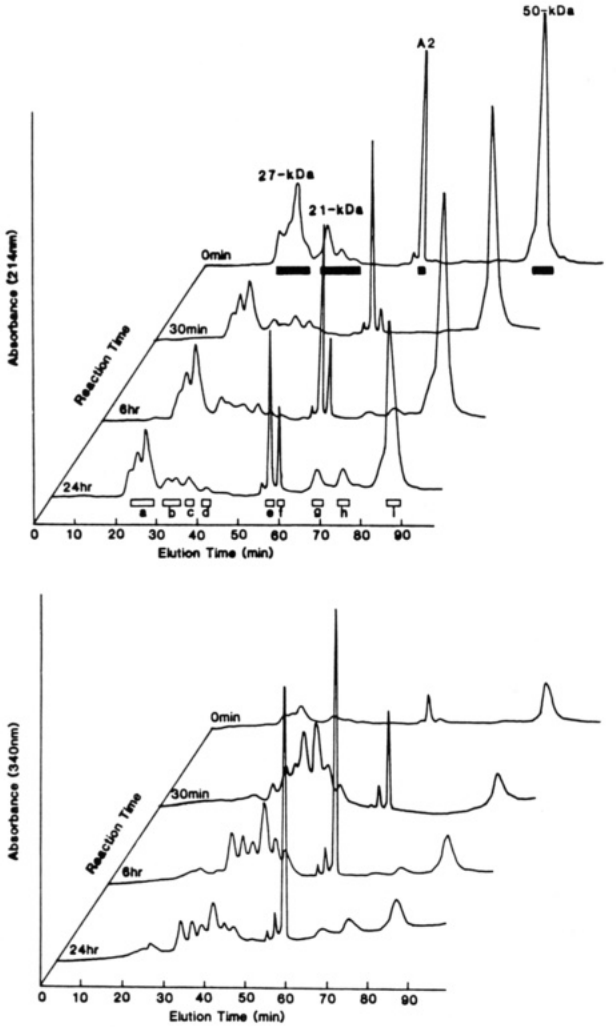

FIGURE 1: Reverse-phase HPLC of S1 modified with DTNB and subsequently digested with trypsin. Upper and bottom panels are the elution profiles monitored at 214 and 340 nm, respectively. The solid bars in the top panel show the pooled fractions, the respective peptide constituent of which are indicated above the corresponding peaks as determined by SDS–PAGE analysis (data not shown). The open bars represent the pooled fractions a–i collected for SDS–PAGE analysis as shown in Figure 2. The topmost trace in the bottom panel (0 min) represents the absorption of the unmodified protein at 340 nm due to the aromatic residues.

of the reaction, about 0.08 mol of intramolecular disulfide had been formed, and the light chain had been only slightly modified (Table I). These results show that more than one thiol in the 21-kDa segment had reacted to form mixed disulfides with DTNB and the reaction was not localized solely to SH1. Since MgADP is known to expose the SH2 thiol

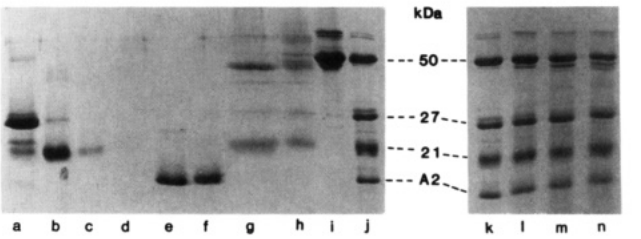

FIGURE 2: SDS–PAGE analysis of the fractions collected during the HPLC separation of S1 reacted with DTNB for 24 h and then digested with trypsin. Lanes a–i of the left panel correspond to the pooled fractions a–i shown in the bottom trace (24 h) of the upper panel of Figure 1. Lane j shows tryptic S1. The right panel shows the SDS–PAGE analysis of S1 digested as described under Materials and Methods subsequent to reaction with DTNB for 0 min (lane k), 30 min (lane l), 6 h (lane m), and 24 h (lane n), respectively.

(Yamaguchi & Sekine, 1966), it is very likely that SH2 was also modified to some extent. The reaction of S1 containing blocked SH1 with DTNB has been in fact suggested (Chaussepied et al., 1986c) to result in the formation of a mixed disulfide at SH2 which subsequently underwent a disulfide exchange with another thiol (Chaussepied et al., 1986a) in S1.

After 24 h of reaction with DTNB, 2.65 mol of thiols in S1 had reacted (Table I), and at this stage of the reaction, 0.88 mol of intramolecular disulfide was formed. The HPLC analysis (Figure 1) indicated that 2.3 mol of thiols in the heavy chain had reacted and that 0.45 mol of mixed disulfide was present in the 21-kDa segment and about 0.1 mol in two new peptide fractions eluting between the alkali light chain and 50-kDa fragment. SDS–PAGE analyses of the fractions from the HPLC analysis of the 24-h modification are shown in Figure 2 from which it could be determined that the new peptide fractions contained both 21-kDa and 42-kDa (fragmented 50-kDa) peptides, suggesting that the above fractions arose from disulfide bridging. SDS–PAGE analysis of these fractions in the absence of 2-mercaptoethanol indicated that each fraction was comprised of a single band. Since the amounts of 50-kDa and 27-kDa peptides seen in the HPLC profile after 24-h reaction (Figure 1) were largely the same as those present before modification, it may be concluded that most of the intramolecular disulfide formation involved exchange between free thiols and S–TNB groups located in the 21-kDa segment in the case of intact S1.

While this work was in progress, Huston et al. (1988) provided evidence that in the case of intact S1, the intramolecular disulfide formed in the second phase of the reaction of S1 with DTNB involved the SH1 and SH2 thiols. However, in the present work with undigested S1, the finding that more than one thiol in the 21-kDa segment was converted into mixed

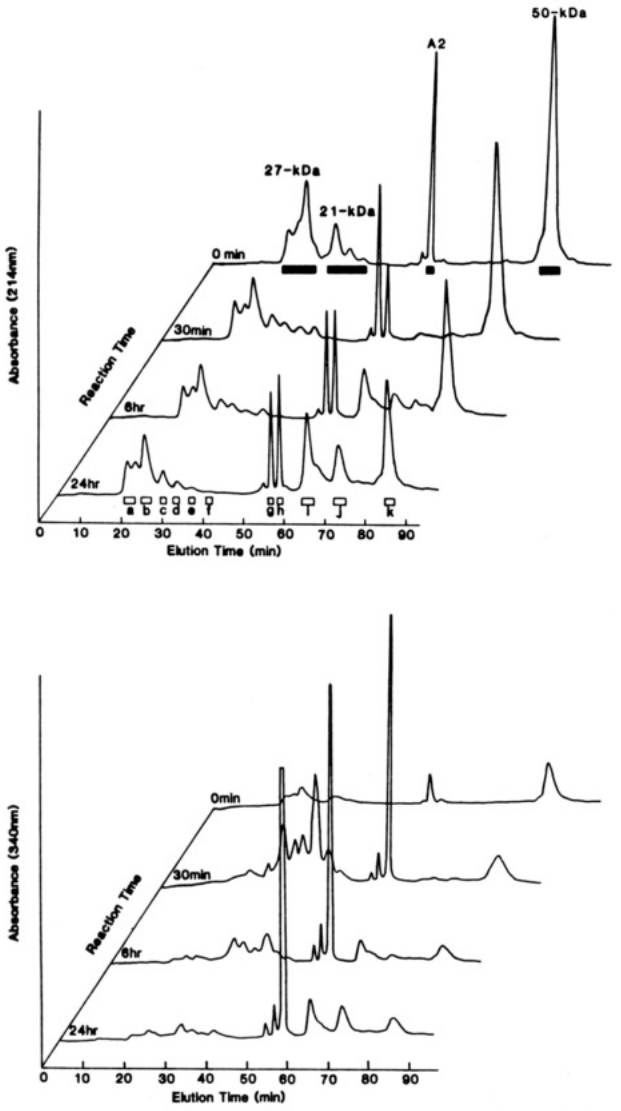

FIGURE 3: Reverse-phase HPLC of tryptic S1 modified with DTNB. Upper and bottom panels are the elution profiles monitored at 214 and 340 nm, respectively. The solid bars in the top panel show the pooled fractions, the respective peptide constituent of which are indicated above the corresponding peaks as determined by SDS–PAGE analysis (data not shown). The open bars represent the pooled fractions a–k collected for SDS–PAGE analysis as shown in Figure 4.

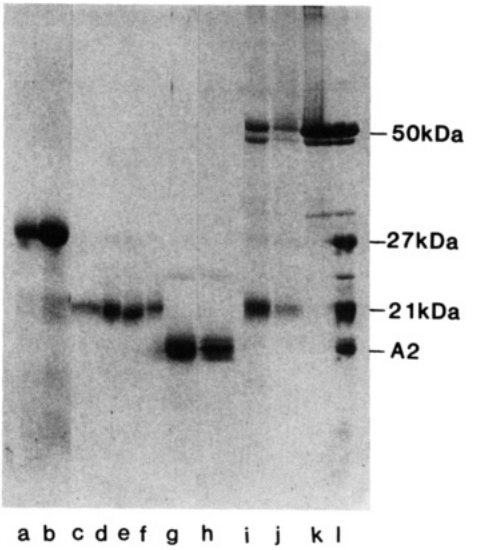

FIGURE 4: SDS–PAGE analysis of the fractions collected during the HPLC separation of tryptic S1 reacted with DTNB for 24 h. Lanes a–k correspond to the pooled fractions a–k shown in the bottom trace (24 h) of the upper panel of Figure 3. Lane 1 shows tryptic S1.

disulfide in the initial stages leaves the possibility open that other thiols in the 21-kDa segment could also be contributing to the intramolecular disulfide formed in the subsequent reaction. Also, in contrast to the observations of Huston et al. (1988), we find that the light chain was not modified stoichiometrically and only 0.35 mol had reacted after 24 h (Figure 1).

In the case of the reaction of DTNB with tryptic S1 under the same conditions used for undigested S1, essentially the same amount of thiols were modifed (1.53) after 30 min of reaction (Table I). Moreover, we observed that the changes in the ATPase properties of tryptic S1 during the modification were the same as those found for undigested S1 and essentially identical with those previously reported by Wells and Yount (1980). The HPLC analysis (Figure 3) of the sample reacted for 30 min with DTNB revealed that 1.2 mol of thiols in the heavy chain was modified and that of these about 0.10 mol had already formed 0.05 mol of intramolecular disulfide. Furthermore, at this stage of the reaction, 1.1 mol of mixed disulfide was present in the 21-kDa segment, and 0.35 mol was in the light chain (Table I).

At the end of 24 h, 2.91 mol of thiols had reacted, of which 2.36 mol was in the heavy chain (Table I), a value similar to that found for undigested S1, and 0.98 mol of intramolecular disulfide was formed. Most of the additional modification observed with tryptic S1 after 24 h could be attributed to the greater modification of the alkali light chain in this preparation. The HPLC analysis of the sample after 24-h reaction (Figure 3) showed that the mixed disulfide remaining in the 21-kDa segment at this time was 0.1 mol and that 0.3 mol was now present in the new fractions eluting between light chain and 50-kDa peptide (Table I). Moreover, the HPLC traces for tryptic S1 showed a marked reduction in both the 50-kDa and 21-kDa fragments with concomitant increases in the two new peaks, indicating that a major proportion of the intramolecular disulfide formation after 24 h arose from exchange between thiols in the 50-kDa and the mixed disulfides in the 21-kDa fragments. The presence of both 21-kDa and 50-kDa peptides in the SDS–PAGE analysis of the two new peptide fractions confirmed that these were disulfide-linked adducts of 21-kDa and 50-kDa segments (Figure 4). Some 45-kDa fragment originally present in the tryptic S1 also appears to have participated in the intersegment disulfide adduct formation. Gel filtration HPLC analyses under nondenaturing conditions on Superose 12 of S1 and tryptic S1 after 24-h reaction with DTNB showed that no intermolecular cross-linking was occurring.

The formation of small amounts of intersegment disulfide adducts in the DTNB reaction with intact S1, as seen by the HPLC and gel analyses (Figures 1 and 2), indicates that cleavage per se is not a necessary requirement for this form of exchange to occur. However, the question which must be addressed is the cause of the higher intersegment disulfide formation occurring in the case of tryptic S1. It is unlikely that differences in the sites of modification in the initial phase of the reaction are responsible, since both S1 and tryptic S1 showed that the mixed disulfide formation was restricted mainly to the 21-kDa segment for both species. In addition, we have also observed essentially identical changes in their ATPase properties over the entire reaction period. Moreover, irreversible denaturation of the tryptic S1 could also be ruled out on the basis of the recoveries of Ca^{2+}- and K^{+}-EDTA ATPase activities of both modified tryptic and undigested S1

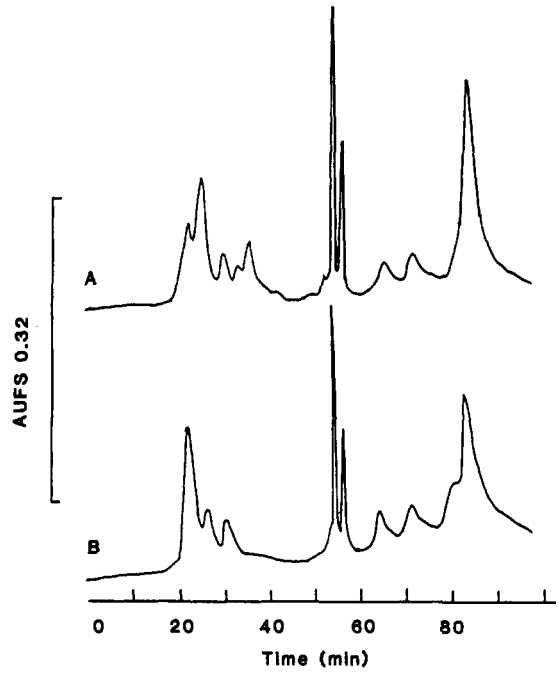

FIGURE 5: Reverse-phase HPLC of S1 modified with DTNB for 24 h and subsequently digested with trypsin (A) and V8 protease cleaved S1 similarly reacted with DTNB (B). The modification conditions were as given under Materials and Methods.

after reaction as described under Materials and Methods.

Alternatively, the enhanced intersegment disulfide formation could arise from an irreversible local conformational change brought about either by peptide bond scission or by the consequent introduction of charge at the cleavage site. This possibility was examined by reacting V8 protease cleaved S1 with DTNB under the same conditions, and these results are presented in Figure 5. This HPLC analysis, together with confirmatory SDS–PAGE data (not shown) of the peptide compositions of the peaks (R_T 63 and 71 min), showed that the amount of 22-kDa and 48-kDa intersegment cross-linking was much lower with V8 protease cleaved S1 (Figure 5) compared to that seen with tryptic S1 (Figure 3). This result suggests that bond scission alone was not responsible for the higher amount of intersegment adduct formation in tryptic S1 but some ambiguity remains in this interpretation because these two proteases cleave S1 differently. No attempt to quantitate the amount of mixed disulfides present by HPLC was made in this case since the V8 protease digestion does not go to completion (Chaussepied et al., 1983).

The most likely explanation for the enhanced disulfide formation between the 21-kDa and 50-kDa segments in tryptic S1 is, therefore, an increase in flexibility which enables a more frequent approach of the thiols of the 50-kDa region to the mixed disulfides in the 21-kDa segment. An increase in the segmental flexibility of S1 upon tryptic digestion has been detected by transient electrical birefringence (Highsmith & Eden, 1987) and has been invoked by Chen et al. (1987) to account for the increased accessibility of the N-terminus of S1 to antibody binding.

The presence of two (21 + 50)-kDa peptide adducts indicates that more than one mixed disulfide in the 21-kDa peptide or more than one free thiol in the 50-kDa segment were involved in the formation of the intersegment disulfide bridge. The thiols involved in each of these products have not been identified as yet.

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