Flexibility of the Myosin Heavy Chain: Direct Evidence That the Region Containing SH_1 and SH_2 Can Move 10 Å under the Influence of Nucleotide Binding†

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Received April 6, 1988; Revised Manuscript Received July 13, 1988

ABSTRACT: Previous experiments demonstrated that two thiols of skeletal myosin subfragment 1 (SF_1) could be oxidized to a disulfide bond by treatment with a 2-fold excess of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of MgADP [Wells, J. A., & Yount, R. G. (1980) *Biochemistry 19*, 1711–1717]. The resulting characteristic changes in the ATPase activities of SF_1 and the fact that MgADP was stably trapped at the active site [Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970] suggested that the two thiols cross-linked were SH_1 (Cys-707) and SH_2 (Cys-697) from the myosin heavy chain. To verify this suggestion, SF_1, after DTNB treatment as above, was treated with an excess of *N*-ethylmaleimide to block all accessible thiols. The single protein disulfide produced by DTNB oxidation was reduced with dithioerythritol and the modified SF_1 internally cross-linked with equimolar [^{14}C]*p*-phenylenedimaleimide (pPDM) in the presence of MgADP. After extensive trypsinization, the major ^{14}C-labeled peptide was isolated, characterized, and shown to be Cys-Asn-Gly-Val-Leu-Gly-Ile-Arg-Ile-Cys-Arg, in which the two cysteines were cross-linked by pPDM. This peptide is known to contain SH_2 and SH_1 in this order and to come from residues 697–708 in the rabbit skeletal myosin heavy chain [Elzinga, M., & Collins, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4281–4284; M. Elzinga, personal communication]. Parallel experiments with [^{14}C]pPDM and unmodified SF_1 similar to those above gave an identical SH_1, SH_2 tryptic peptide, verifying earlier labeling results [Burke, M., & Knight, P. (1980) *J. Biol. Chem. 255*, 8385–8387]. These combined results demonstrate that SH_1 and SH_2 cross-linked by pPDM (12–13 Å, S to S) or by oxidation with DTNB (2 Å, S to S) can move a minimum of 10 Å under the influence of nucleotide binding. Because these residues are separated by only nine amino acids in the primary sequence, this small section of the heavy chain must possess extraordinary flexibility.

A continuing area of interest in the study of myosin and its proteolytic subfragments has been the role of the kinetically reactive cysteines in the ATPase activity of the enzyme. Alkylation of SH_1,[1] the most reactive thiol (Sekine & Kielly, 1964), inactivates the K^+EDTA-ATPase while simultaneously activating the Ca^{2+}-ATPase activity. A second thiol, SH_2, can be subsequently modified to inactivate the Ca^{2+}-ATPase. The rate of modification of SH_2 is stimulated by the presence of MgADP (Sekine & Yamaguchi, 1963; Yamaguchi & Sekine, 1966; Reisler et al., 1974a; Schaub et al., 1975), suggesting movement of this residue from a buried to an exposed position on the protein. Reisler et al. (1974b) showed that treatment of myosin with the bifunctional reagent pPDM in the presence of nucleotide resulted in the complete inactivation of the enzyme. On the basis of inactivation profiles, the authors concluded that pPDM, with a span of 12–13 Å,[2] was cross-linking SH_1 and SH_2. It was subsequently shown that in the presence of MgADP, F_2DPS, with a span of 10 Å, was an effective cross-linker, suggesting that the pair of thiols moved toward each other upon binding of nucleotide (Burke & Reisler, 1977). Inactivation of SF_1 by chelating a pair of thiols to a Co(III) phenanthroline complex suggested the thiols could approach to within 3–5 Å of each other (Wells et al., 1979). Additionally, treatment of SF_1 with DTNB in the presence of MgADP inactivated the enzyme and led to the formation of a single protein disulfide in which the sulfur-to-sulfur distance was 2 Å (Wells & Yount, 1980). Consistent with the above observations, fluorescence resonance energy transfer measurements by Dalbey et al. (1983) showed that a fluorescent donor on SH_1 and a chromophoric acceptor on SH_2 appeared to move toward each other by 6–7 Å upon addition of MgADP. It appears then, as first suggested by Burke and Reisler (1977), that two thiols do move toward each other upon binding of nucleotide.

The evidence that the various cross-linking reagents act at SH_1 and SH_2 [for review see Wells and Yount (1982)] has

† Supported by grants from NIH (DK-05195) and MDA.

[1] Abbreviations: SH_1 and SH_2, rabbit skeletal myosin heavy chain residues Cys-707 and Cys-697, respectively; pPDM, *N,N′-p*-phenylenedimaleimide; F_2DPS, 4,4′-difluoro-3,3′-dinitrophenylsulfone; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, thionitrobenzoic acid; NEM, *N*-ethylmaleimide; RP-HPLC, reversed-phase high-performance liquid chromatography; IEX-HPLC, ion-exchange high-performance liquid chromatography; TFA, trifluoroacetic acid; SSC, *S*-succinylcysteine; TEAA, triethylammonium acetate; DTE, dithioerythritol; SDS, sodium dodecyl sulfate; dansyl-Cl, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; A_1 and A_2, alkali light chains of myosin; [^{14}C]pPDM-SF_1, SF_1 that has been cross-linked with [^{14}C]pPDM in the presence of MgADP; [^{14}C]pPDM-NEM-SF_1, SF_1 that has been successively oxidized with DTNB, alkylated with NEM, reduced with DTE, and cross-linked with [^{14}C]pPDM in the presence of MgADP.

[2] For ease in comparing sulfur-to-sulfur distances with different cross-linking agents, we quote distances from the center of one sulfur atom to the center of the other. These distances were determined by careful measurement of appropriate Corey–Pauling–Koltun space-filling models. Thus, in a disulfide bond we cite a 2-Å distance, although other workers have called these bonds "zero cross-links" since no atoms of non-protein origin are introduced in the cross-linking reaction. Burke and Reisler (1977) used a value of 7–10 Å for the cross-linking span of F_2DPS and a value of 12–14 Å for pPDM. We find a sulfur-to-sulfur distance of 10 Å for F_2DPS and a 12–13 Å distance for pPDM.

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been largely circumstantial and based primarily on the kinetics of inactivation of the K^+EDTA- and Ca^{2+}-ATPases. In the only definitive paper, Burke and Knight (1980) showed [^{14}C]pPDM labeled SH_1 and SH_2 by isolating a cross-linked peptide having the same composition and N-terminal sequence as the 92 amino acid CNBr peptide known to contain SH_1 and SH_2 (Elzinga & Collins, 1977).

In this paper we demonstrate that the primary site of disulfide formation after DTNB treatment is between SH_1 and SH_2. In addition, we confirm that the primary site of cross-linking with [^{14}C]pPDM is between SH_1 and SH_2. This is the first direct evidence that these two thiols, which are 12–13 Å apart when cross-linked by pPDM, can approach to within 2 Å of each other. Given that SH_1 (Cys-707) is only nine residues away from SH_2 (Cys-697), this small region of the protein backbone must possess an unprecedented degree of flexibility. It is suggested that this flexibility is an essential property of myosin cross-bridges and is the primary reason the amino acid sequence of this region is so highly conserved.

Materials and Methods

Chemicals. [^{14}C]pPDM was synthesized as described (Wells & Yount, 1982) and had a specific radioactivity of 3300 cpm/nmol. DTNB, DTE, and β-mercaptoethanol were all from Sigma, while NEM and TFA were from Pierce. Li_3ADP was from Pharmacia P-L Biochemicals. Ultrapure urea and $(NH_4)_2SO_4$ were from Schwarz/Mann. TPCK-treated trypsin was from Worthington, and soybean trypsin inhibitor was from Sigma. Triethylamine (Baker) was redistilled before use. Dansyl amino acid standards were from Pierce, norleucine was from Sigma, and a standard of *S*-succinylcysteine was prepared by a slight modification of the procedure of Calam and Waley (1963). All other chemicals were of reagent grade. Buffers were prepared by using doubly deionized water.

Protein Preparations and Analytical Procedures. Myosin was isolated from the back and hind legs of rabbits (Wagner & Yount, 1975). Chymotryptic subfragment 1 was prepared by the method of Weeds and Taylor (1975) and assumed to have a molecular weight of 115 000 (Weeds & Pope, 1977) and an $A_{280nm}^{1\%} = 7.5$ cm^{-1} (Wagner & Weeds, 1977). Protein was determined by a dye binding assay (Bradford, 1976), and thiol group analysis was done with DTNB (Ellman, 1959). ATPase activities were measured as described previously (Wells et al., 1979; Wells & Yount, 1980). Radioactivity was measured in aqueous counting scintillant (ACS) from Amersham and counted in either a Beckman LS9000 or LS7500 liquid scintillation counter. The purity of peptides was determined with dansyl chloride (Gray, 1972) and a 2-dimensional TLC separation of dansyl amino acid products on 5 cm × 5 cm micropolyamide sheets (Schleicher and Schuell).

HPLC. The HPLC apparatus consisted of two Model 100A pumps and a Model 420 microprocessor from Altex, a Rheodyne 7120 injector, a Beckman 165 variable-wavelength detector, a Kipp and Zonen BD41 dual pen recorder and a Frac-100 programmable fraction collector (Pharmacia). Reversed-phase separations utilized a Brownlee Aquapore RP-300 semiprep C8 column (25 cm × 7.0 mm) with an attached guard cartridge. Solvents were 0.1% TFA (Pierce) in H_2O (HPLC grade, Burdick and Jackson), and 0.1% TFA in 60% CH_3CN (Burdick and Jackson). Ion-exchange HPLC was performed on a column (200 mm × 4.6 mm) of Vydac TP301 (The Separations Group), packed in this laboratory and equipped with an AX-300 guard cartridge from Brownlee. All chromatography was conducted at room temperature. Prior to use, all solvents were filtered through a Millipore apparatus fitted with a 0.45-μm nylon 66 membrane. All samples were filtered before injection through a centrifugal microfilter (Bioanalytical Systems) by use of a 0.45-μm regenerated cellulose membrane. Pooled HPLC fractions were dried under vacuum on a Speed Vac concentrator (Savant Instruments). Occasionally, HPLC-purified peptides were difficult to dissolve after they had been dried and stored at −80 °C. It was found that if 0.1–0.2 mL of H_2O was added to the peptides immediately after dying and before storage at −80 °C, 85–95% of the peptide(s) remained soluble when thawed later.

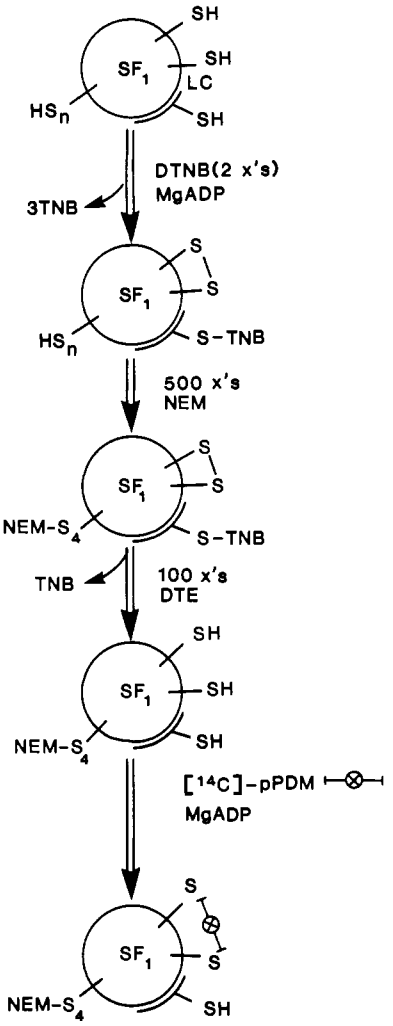
Amino Acid Analysis. Samples were hydrolyzed in 1-mL Teflon-stoppered hydrolysis tubes (Kontes) in 6 N HCl with norleucine as an internal standard. Samples were processed as follows: they were frozen, evacuated, thawed, frozen, evacuated, and purged with N_2 several times with intervening evacuations before the tubes were finally sealed. Hydrolysis was at 110–113 °C for 24, 48, or 72 h. Hydrolysates were dried, dissolved in starting buffer, and analyzed on a Beckman 121C amino acid analyzer. To analyze hydrolysates for radioactivity, the ninhydrin pump was disconnected and the column effluent collected directly into scintillation vials. The difference in the elution times with and without the ninhydrin pump was determined with [^{14}C]aspartic acid as a standard.

Results

Previous work (Wells & Yount, 1980) has shown that treatment of SF_1 with a 2-fold molar excess of DTNB promotes formation of a single protein disulfide, ostensibly between SH_1 and SH_2, and a mixed disulfide between another thiol and TNB. It was suggested that the mixed disulfide occurred between TNB and the single SH group on the A_1 and A_2 light chains. It was important to verify that the third SH group involved in the DTNB inactivation was indeed located on the alkali light chains and not on the heavy chain. To show this, DTNB-modified SF_1 and unmodified (control) SF_1 were treated with a 1000-fold molar excess of [^{14}C]NaCN, which is known to cleave disulfides (Catsimpoolus & Wood, 1966) and mixed disulfides in proteins (Vanaman & Stark, 1970). It was therefore possible to radioactively label one of the SH groups involved in the disulfide (presumably either SH_1 or SH_2) and the thiol involved in the mixed disulfide. As shown in Figure 1A, upon separation of the 95-kDa heavy chain and the A_1 and A_2 light chains by gel filtration HPLC in the presence of SDS, the 95-kDa heavy chain and both alkali light chains were labeled. Labeling of control SF_1 was primarily in the 95-kDa heavy chain with minor labeling of the alkali light chains. The specific labeling of the DTNB–[^{14}C]CN-treated SF_1 is shown in Figure 1B. It was determined that ~54% of the specific counts were in the heavy chains and 46% in the light chains, indicating that the thiol on the alkali light chains is one of the thiols modified by reaction with DTNB.

Since oxidation with DTNB introduces no new atoms into the protein, the following strategy was adopted in order to characterize the site of disulfide formation. Briefly, after treatment of SF_1 with DTNB, all other accessible protein thiols were blocked with NEM. Subsequent reduction of the protein with DTE should regenerate only those thiols modified by reaction with DTNB. These thiols, if they are SH_1 and SH_2, should react rapidly with [^{14}C]pPDM, and the site of covalent incorporation of the label could then be characterized in comparison to [^{14}C]pPDM-modified native SF_1. Burke and Knight (1980) have previously shown [^{14}C]pPDM cross-links SH_1 and SH_2, and here we confirm their work as a reference point from which to study the site of thiol modification by DTNB treatment.

Table I: Reaction Scheme for Preparation of [^{14}C]pPDM–NEM–SF_1[a]

	step	activity (%) K^+	activity (%) Ca^{2+}	SH/SF_1	ΔSH/step
	I	100	100	10.0 ± 0.2	
	II	0	7.6 ± 1.7	7.3 ± 0.4	−2.7
	III	ND	ND	3.4 ± 0.2	−3.9
	IV	45.4 ± 3.8	41.9 ± 1.6	6.1 ± 0.4	+2.7
	V	0	0	4.3 ● 0.5	−1.8

[a] SF_1 was modified as shown here and described in the text. K^+EDTA- and Ca^{2+}-ATPase activities were measured at each step; values are expressed as percent of unmodified control SF_1 activity. The numbers shown are the mean of several determinations plus or minus the standard deviation. Definitions: LC, light chain; S-TNB, the light chain thiol that reacts with DTNB; NEM-S_4-, the four thiols alkylated by NEM treament; ND, not determined. Other definitions are in the text.

Preparation of [^{14}C]*pPDM-SF*$_1$. Subfragment 1 (17 μM) in 50 mM Tris, pH 8.0, 0.1 M KCl, 0.1 mM ADP, and 0.2 mM $MgCl_2$ was treated for 30 min at 0 °C with equimolar [^{14}C]pPDM added dropwise with swirling from a stock solution of 0.7 mM [^{14}C]pPDM in acetonitrile (ϵ_{313nm}^{M} = 942). The reaction was quenched by addition of a 1000-fold excess of β-mercaptoethanol, and approximately 0.95 mol of [^{14}C]-pPDM was incorporated per mole of SF_1. The K^+EDTA-ATPase and Ca^{2+}-ATPase activities were <10% of unmodified controls.

Preparation of [^{14}C]*pPDM–NEM-SF*$_1$. A schematic of the modification protocol is presented in Table I, along with the measured ATPase activities and the results of the thiol analysis at each step. After each step, the modified proteins were carefully purified, a precaution essential to obtain reproducible results. SF_1 (17.8 μM) in 50 mM Tris, pH 8.0, 0.1 M KCl, 0.1 mM ADP, and 0.2 mM $MgCl_2$ was treated with a 2-fold excess of DTNB (in 10 mM KP_i, pH 7.0) (step II, Table I) for 23 h at 0 °C (Wells & Yount, 1980). Such treatment results in rapid incorporation of two TNB groups, one putatively on SH_1 and the other on the A_1 or A_2 light chain single thiol (Wells & Yount, 1980; data in this paper). The TNB group on SH_1 is slowly displaced by another protein sulfhydryl to form a disulfide. Thiol group analysis after DTNB treatment for 23 h showed the loss of 2.7 SH groups, with the release of 2.8 ± 0.1 TNB groups (data not shown). The K^+EDTA-ATPase and Ca^{2+}-ATPase activities were both less than 10%, implicating SH_1 and SH_2 as the thiols oxidized to the disulfide. DTNB-modified SF_1 was precipitated by addition of 2.5 volumes of saturated $(NH_4)_2SO_4$ containing 20 mM EDTA (pH 8.0) and was collected by centrifugation. The resulting pellet was redissolved in 50 mM Tris and 0.1 M KCl, pH 8.0, and passed over a 2.2 × 40 cm column of Sephadex G-50 (fine) that had been equilibrated in the same buffer at 4 °C.

In order to radiolabel specifically the two thiols involved in the protein disulfide bond, it was first necessary to block all other accessible protein thiols. The protein peak collected from the G-50 column was allowed to react for 4 h at 0 °C with a 500-fold excess of NEM (added as 0.5 M reagent in methanol, step III). The modified protein was twice precipitated with saturated $(NH_4)_2SO_4$ containing 20 mM EDTA, pH 8.0, as above, and again passed over the gel filtration column. Alkylation led to the loss of 3.9 additional thiol

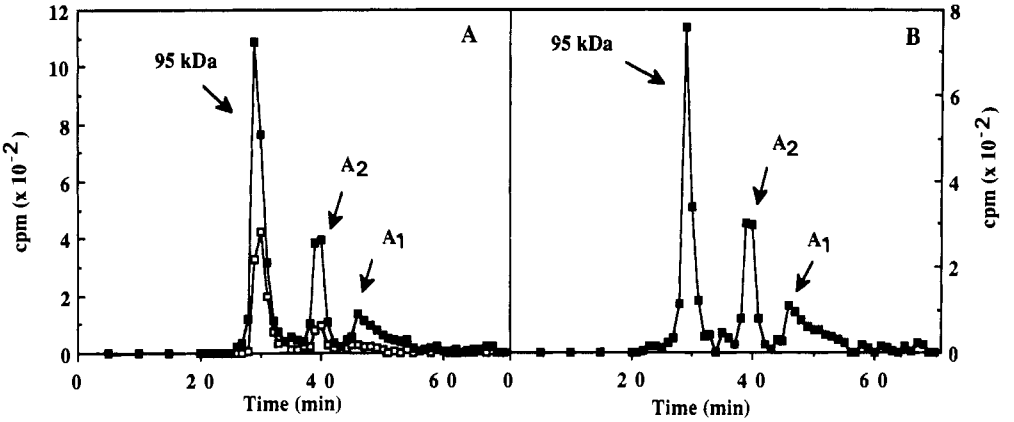


FIGURE 1: Localization of the sulfhydryls reacting with DTNB by cyanylation with [^{14}C]NaCN. SF_1 was inactivated with DTNB and purified as described under Materials and Methods. The purified and inactivated TNB-SF_1 and unmodified SF_1 (control) were treated with a 1000-fold molar excess of freshly prepared [^{14}C]NaCN for 18 h at 0 °C. Excess [^{14}C]NaCN was removed by centrifugation through two consecutive 5-cm^3 columns of Sephadex G-50 equilibrated in 50 mM HEPES and 0.1 M NaCl, pH 7.0 at 4 °C (Penefsky, 1977). Protein and radioactivity were determined as described under Materials and Methods. TNB-SF_1 incorporated 2.2 [^{14}C]CN/SF_1, whereas control SF_1 incorporated 0.7 [^{14}C]CN/SF_1 (specific incorporation = 1.5 [^{14}C]CN/SF_1). Heavy chains and light chains were separated by gel filtration HPLC using two TSK-G3000SW columns (7.5 mm × 60 cm each) linked in series and equilibrated in 20 mM Tris, 0.1 M NaCl, and 0.02% SDS, pH 7.5. Immediately prior to injection, samples were prepared by the addition of SDS to 1%, incubated at 25 °C for 5 min, and centrifuged through a 0.45-μm filter (Bioanalytical Systems). The identification of the heavy and light chains was verified by 12% SDS–polyacrylamide gel electrophoresis. (A) Radioactivity elution profile for control (□) and TNB-SF_1 (■) treated with [^{14}C]NaCN. (B) Radioactivity elution profile for specific incorporation of [^{14}C]NaCN into TNB-SF_1 (TNB-SF_1 − control SF_1).

groups. Approximately 3–4 thiols remained unmodified at this stage; it is presumed that they are not accessible to the alkylating reagent under the conditions employed. Similar labeling results were obtained by Botts et al. (1979), who modified the sulfhydryl groups of native SF_1 with methyl methanethiosulfonate. After alkylation, a 100-fold excess of DTE was added (step IV) and the solution allowed to stand overnight at 0 °C. Reduction resulted in the return of approximately 45% of the ATPase activity. DTE also displaced the TNB group on the alkali light chains (1.3 ± 0.05 TNB released); thiol analysis after reduction showed a gain of 2.7 SH groups per SF_1 molecule. The modified protein was once again subjected to two successive $(NH_4)_2SO_4$ precipitations followed by a desalting step over the G-50 column. Reduced alkylated SF_1 was then treated with a stoichiometric quantity of [^{14}C]pPDM in the presence of MgADP (step V). Again, both K^+EDTA- and Ca^{2+}-ATPase activities were lost, as were approximately 2 thiols.

Prior to addition of [^{14}C]pPDM, approximately 6 thiols groups remained on NEM-alkylated SF_1. Treatment with [^{14}C]pPDM could have cross-linked thiols other than those that earlier were in a disulfide, but this possibility was discounted for the following reasons. A portion of the cross-linked material was run on an SDS–polyacrylamide gel, which was then sliced and the slices were counted. The results (data not shown) indicated that <5% of the radioactivity was associated with the alkali light chain fragments of SF_1, precluding the possibility of the [^{14}C]pPDM reacting with the A_1 or A_2 light chain thiol. Additionally, it is unlikely that thiol groups which were unreactive in the previous step in the presence of a 500-fold excess of NEM would become reactive at this step when treated with a stoichiometric quantity of [^{14}C]pPDM. Finally, the complete loss of ATPase activity after cross-linking parallels the inactivation seen upon disulfide formation, suggesting modification of the same pair of thiols. Thus, it is reasonable that the two thiols cross-linked by [^{14}C]pPDM are the same two thiols that earlier had existed as a disulfide bond.

Preparation and Isolation of [^{14}C]pPDM-Labeled Peptides. In order to characterize the site of cross-linking, extensive enzymatic digestion was utilized. The modified proteins were precipitated by addition of 2.5 volumes of saturated $(NH_4)_2SO_4$ containing 20 mM EDTA, pH 8.0, and pelleted by centrifugation to remove unreacted [^{14}C]pPDM and excess nucleotide. Following centrifugation, the pellet was dissolved in sufficient 50 mM NH_4HCO_3, 15 mM NaCl, and 2 M urea to give a 4 mg/mL solution and equilibrated in a 25 °C water bath. Three additions of TPCK-treated trypsin, each 1/100 (w/w), were made at 0, 20, and 40 min, and the proteolysis was stopped at 60 min by addition of soybean trypsin inhibitor at 3/1 (w/w) over trypsin. The solution was diluted with an equal volume of water and freeze-dried.

The peptides generated by trypsinization were fractionated by passage of the digest over a column of Sephadex G-50 (fine). When the freeze-dried tryptic digest was dissolved in 0.1% TFA, 30–40% of the labeled peptides remained insoluble. This residue was also insoluble in 70% HCOOH, and subjecting it to a second trypsinization did not convert it to a soluble form. This material is most likely trypsin-resistant aggregates of partially digested SF_1. Figure 2A shows a representative elution profile of the soluble tryptic peptides from a digest of [^{14}C]pPDM-SF_1. An analogous profile from a digest of [^{14}C]pPDM–NEM-SF_1 is shown in panel B. The elution profiles of the two different modified proteins are very similar, providing an early indication that the label is attached at the same site in both instances. The radioactive material eluting near the void was present in variable amounts; it appears to be aggregates of partially digested protein. Fractions that contained counts at least 5 times the background level of radioactivity were pooled (cross-hatched areas). Each pooled sample was lyophilized to dryness and stored at −80 °C.

The radioactive pooled samples (Figure 2) were further fractionated by reversed-phase HPLC. The lyophilized samples were dissolved in 0.1% TFA and filtered, and a portion of them was injected onto a Brownlee RP-300 semiprep column (Figure 3). Panel A shows the separation of [^{14}C]-pPDM-SF_1 tryptic peptides and panel B comparable peptides from the [^{14}C]pPDM–NEM-SF_1 digest. The two separations are very nearly superimposable, and in both cases the majority of the radioactivity eluted as a single sharp peak with the same time of elution. This result is a further indication that the same pair of thiols was cross-linked in these two differently treated

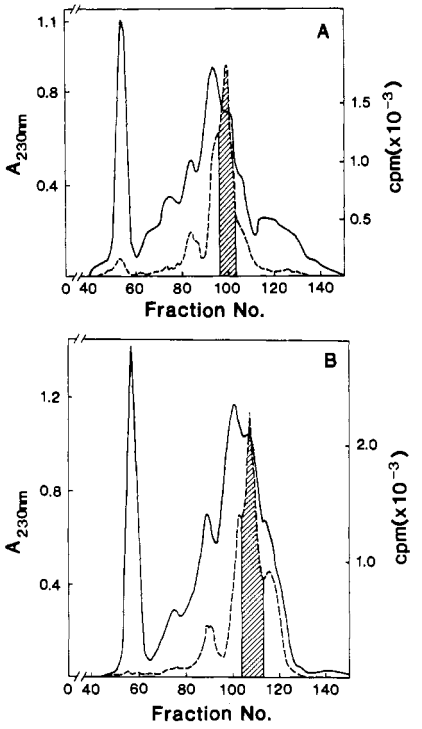


FIGURE 2: Sephadex G-50 chromatography of trypsinized [^{14}C]-pPDM-SF_1 (A) and [^{14}C]pPDM-NEM-SF_1 (B). (A) [^{14}C]pPDM-SF_1 was prepared, trypsinized, and freeze-dried as described in the text. The dried residue was redissolved in 0.1% TFA and 0.01% NaN_3 and applied to a column (2.4 cm × 155 cm) of Sephadex G-50 (fine) that had been equilibrated in the same solvent. The column was run at a flow rate of 25 mL/h, and fractions were collected each 15 min. Fractions were analyzed for protein by absorbance at 230 nm and for radioactivity by counting a 0.1-mL aliquot of each fraction. The fractions indicated by crosshatching were pooled and lyophilized: (—) A_{230}; (---) radioactivity. (B) [^{14}C]pPDM-NEM-SF_1 was prepared, trypsinized, and freeze-dried as described in the text. All chromatographic conditions were identical with those given above.

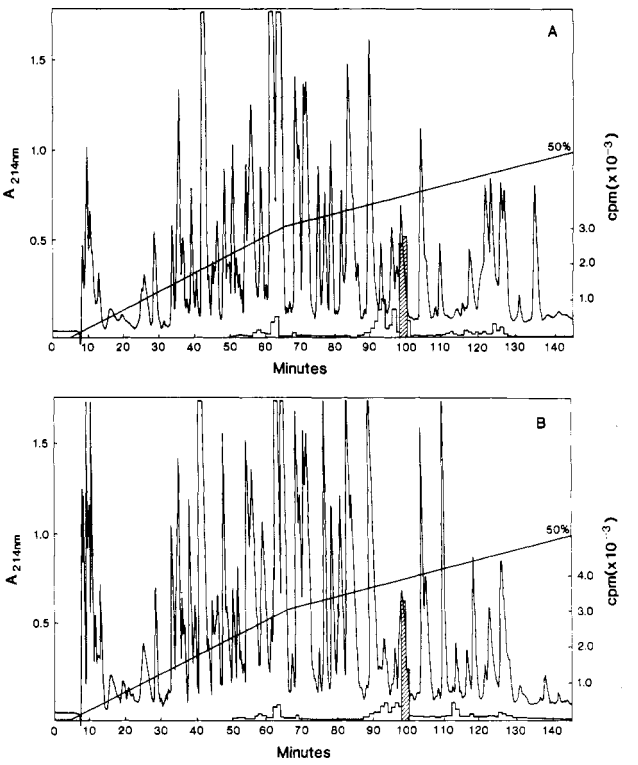


FIGURE 3: Reversed-phase HPLC of tryptic peptides from [^{14}C]-pPDM-SF_1 (A) and [^{14}C]pPDM-NEM-SF_1 (B). (A) The indicated fractions of the G-50 chromatogram shown in Figure 2A were pooled, lyophilized, redissolved in 0.1% TFA, and injected onto a Brownlee Aquapore RP300 semiprep column (0.7 cm × 25 cm). Solvent A: 0.1% TFA. Solvent B: 0.1% TFA in 60% CH_3CN. The flow rate was 1 mL/min, and the temperature was ambient. A two-stage linear gradient was begun 5 min after injection: 0–30% solvent B over 60 min, followed by 30–50% solvent B over an additional 80 min, as shown in the figure. Peptides were detected by their absorbance at 214 nm, 2.0 absorbance units full scale (aufs), and a 50-μL aliquot of each fraction was analyzed for radioactivity. The radioactivity profile is superimposed on the absorbance trace. The fractions shown by crosshatching were pooled and dried under vacuum. (B) The indicated fractions from the G-50 chromatogram shown in Figure 2B were pooled, lyophilized, redissolved in 0.1% TFA, and injected onto a Brownlee Aquapore RP300 semipreparative column. Solvents, gradient, and other conditions were identical with those described in (A).

samples. Use of the semipreparative column allowed 15 mg of peptides, containing as much as 200 000 cpm, to be resolved in a single run. N-Terminal amino acid analysis of the single peaks, however, revealed multiple spots on the TLC plates, and amino acid analysis showed the presence of several amino acids in nonintegral quantities. Repeated attempts to purify the radiolabeled peptide to homogeneity by using a variety of reversed-phase purification steps were unsuccessful.

Further purification of the labeled peptides collected from the RP-HPLC column was accomplished with ion-exchange HPLC, using the solvent system of Dizdaroglu and Krutzsch (1983). These workers were able to separate cationic peptides, from a digest of rat small myelin basic protein, on an anion-exchange column using a CH_3CN/TEAA solvent system. The use of CH_3CN as solvent A presumably suppresses the ionization of basic amino acid side chains sufficiently to allow basic peptides to bind to an anion-exchange column. This suggested that tryptic peptides containing SH_1 and SH_2 would still bind to similar columns, even though the sequence around these thiols shows a predominance of basic amino acids (Elzinga & Collins, 1977). Additionally, this solvent system had the advantage of being completely volatile, unlike most solvents used in ion-exchange separations. Parts A and B of Figure 4 show the IEX-HPLC separation of the pooled radiolabeled peptides from parts A and B of Figure 3, respectively. The peptides had been redissolved in water after first being dried under vacuum. Injecting pooled samples from RP-HPLC separations directly onto the IEX-HPLC column without first removing the TFA/CH_3CN gave unsatisfactory results. The absorbance of the column effluent was monitored at 220 nm because the TEAA solvent absorbed significantly at lower wavelengths. The chromatograms show what is an apparent single major radioactive peptide. The peptide peaks were fairly broad; nevertheless, subsequent amino acid analysis (see below) showed the peptides to be homogeneous. N-Terminal analyses detected dansyl Ile as well as the expected dansyl *S*-succinylcysteine. The dansyl Ile apparently resulted from trypsin cleavage of the Arg-Ile bond known to exist between Cys-697 and Cys-707. The two resulting peptides containing SH_2 and SH_1, respectively, remained covalently linked by pPDM.

The pooled peaks from the ion-exchange HPLC column (Figure 4) were subjected to amino acid analysis (Table II). P1, the labeled peptide isolated from [^{14}C]pPDM-SF_1, and N1, the labeled peptide isolated from [^{14}C]pPDM–NEM-SF_1, have the same amino acid composition. Also shown in Table II are the sequence and composition expected from the smallest tryptic peptide containing cross-linked SH_1 and SH_2 (Elzinga & Collins, 1977). Note that both cysteines will be present as *S*-succinylcysteine after modification by maleimides and acid hydrolysis. The composition of the isolated peptides match the theoretical composition almost exactly. Furthermore, all the radioactivity eluted from the amino acid analyzer

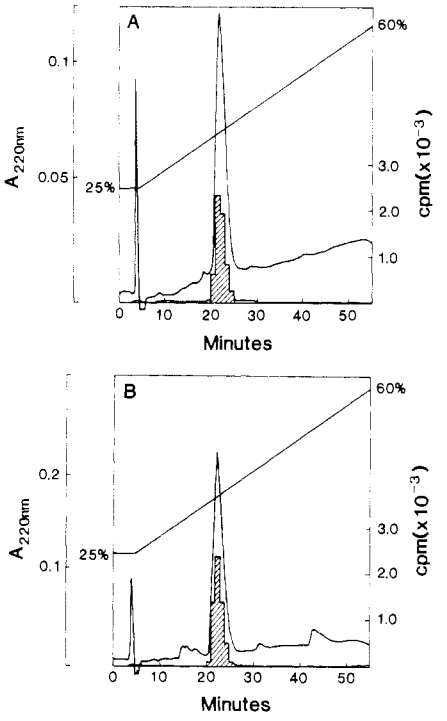


FIGURE 4: Ion-exchange HPLC separation of tryptic peptides from [^{14}C]pPDM-SF_1 (A) and [^{14}C]pPDM–NEM-SF_1 (B). (A) The peak of radioactivity ([^{14}C]pPDM-SF_1) from the reversed-phase chromatogram shown in Figure 3A was pooled, dried, redissolved in water, and injected onto a Vydac TP301 column (0.46 cm × 20 cm). Solvent A: CH_3CN. Solvent B: 10 mM triethylammonium acetate, pH 6.9. The flow rate was 1 mL/min, and the temperature was ambient. Starting 5 minutes after injection, a linear gradient was run from 25 to 60% solvent B over 50 min. Peptides were detected by absorbance at 220 nm (0.2 aufs), and a 0.1-mL aliquot of each fraction was counted to detect radioactivity. The fractions shown by crosshatching were pooled and dried under vacuum. (B) The peak of radioactivity ([^{14}C]pPDM-NEM-SF_1) from the reversed-phase chromatogram shown in Figure 3B was pooled, dried, redissolved in H_2O, and injected onto the Vydac column. All conditions were identical with those in (A) except the detector was at 0.5 aufs.

coincident with standard *S*-succinylcysteine (data not shown). In agreement with earlier reports (Smyth et al., 1961, 1964), *S*-succinylcysteine was most accurately quantitated after 48–72 h of hydrolysis at 110 °C. It is clear from these results that a peptide containing SH_1 and SH_2 has been isolated from each of the modified proteins, and SH_1 and SH_2 are the thiols oxidized to a disulfide by DTNB treatment, as well as the thiols cross-linked by pPDM.

Discussion

The purpose of this study was to establish that the two cysteines in SF_1 cross-linked by DTNB oxidation were, in fact, SH_1 and SH_2. Circumstantial evidence derived from characteristic changes in the ATPase activities and from the observation that MgADP was trapped at the active site strongly implicated these two thiols as the ones cross-linked (Wells & Yount, 1980). However, recent studies by Chaussepied et al. (1986) have shown that if SH_1 is blocked, SH_2 can be cross-linked to a thiol in the 50-kDa tryptic fragment by either pPDM or by oxidation with DTNB. Both reactions give concomitant trapping of MgADP at the active site. In addition, Mornet et al. (1985) had shown earlier that dibromobimane, a fluorescent bifunctional cross-linker, would cross-link SH_1 to a thiol in the 50-kDa fragment, a residue now known to be Cys-522 (Ue, 1987). These observations and the much earlier observation that it was possible to form up to 13 disulfide bonds in myosin and still retain ATPase activity

Table II: Amino Acid Composition of [^{14}C]pPDM Cross-Linked Tryptic Labeled Peptides[a]

amino acid	P1	N1	theor comp
Asx	1.47	1.08	1.0
Thr	0.23	0.04	
Ser	0.08	0.07	
Glx	1.23	1.05	1.0
Gly	2.00	2.00	2.0
Ala	0.26	0.08	
Val	0.85	0.99	1.0
Ile	2.05	1.84	2.0
Leu	1.48	1.07	1.0
Lys	0.26	0.09	
Arg	2.15	1.97	2.0
SSC	1.98	1.97	2.0

Arg-Cys-Asn-Gly-Val-Leu-Glu-Gly-Ile-Arg-Ile-Cys-Arg-Lys
(SH_2) (SH_1)

[a] The peaks of radioactivity from the IEX-HPLC chromatograms shown in Figure 4 were pooled, dried under vacuum, and redissolved in water. A portion of these peptides was processed as described under Materials and Methods and hydrolyzed at 110 °C for 72 h. The numbers reported are nanomoles of each amino acid present in the hydrolysate, normalized to the internal standard Nle and then normalized to 2.0 nmol of glycine. The theoretical composition given is for the tryptic peptide generated by cleavage at the two "T"s enclosed in solid circles. The partial trypsin cleavage site is "T" in a dotted circle. The sequence given is from Elzinga and Collins (1977) and corresponds to residues 696–709 in the heavy chain sequence (M. Elzinga, personal communication).

(Weidner et al., 1978) suggested that many of the thiols of myosin are structurally close to each other and could be involved in the disulfide formation observed.

Because of the above results, it was important to establish the subunit location of the thiols modified by DTNB. For example, it was possible that DTNB reacted first with SH_1 and then SH_2 to form two mixed disulfide bonds. A slower subsequent reaction between a thiol group in the 50-kDa peptide and the TNB-modified SH_2 could give a protein disulfide that would stably trap MgADP as shown by Chaussepied (1986) for other SH_1-blocked SF_1 preparations. Reduction of such a modified protein would regenerate SH_1 and SH_2 that would then be readily cross-linked by [^{14}C]pPDM in the presence of MgADP.

To rule out such a possibility, we made use of the ability of [^{14}C]cyanide to cleave protein disulfides (Catsimpoolus & Wood, 1966) and mixed disulfides (Vanaman & Stark, 1970). We have previously used this approach (Wagner & Yount, 1975) to establish that the purine disulfide ATP analogue 6,6′-dithiobis(inosinylyl imidodiphosphate) modified the single cysteines in A_1 and A_2. In this case, cyanide preferentially displaced the aromatic purine sulfide to yield a thiocyanolated light chain. With simple protein disulfides, cyanide attacks each of the sulfurs in the disulfide bond equally (Catsimpoolus & Wood, 1966). Here, [^{14}C]cyanide treatment of DTNB-inactivated SF_1 was shown to label the 95-kDa heavy chain fragment and the A_1 plus A_2 light chains equally (Figure 1B). This result rules out the possibility that DTNB modifies only thiols in the 95-kDa heavy chain as was suggested above. In addition, it makes it unlikely that a disulfide bond could have occurred between SH_1 (or SH_2) and the thiols in A_1 or A_2. In this latter case, [^{14}C]cyanide treatment would have given 25% of the label in A_1 and A_2 and 75% in the 95-kDa heavy chain, not 46% and 54% as was found. An important aspect to these studies was the use of HPLC gel filtration to separate the ^{14}C-labeled polypeptides. Simply incubating the labeled SF_1 briefly in SDS at 25 °C was sufficient to separate the A_1 and A_2 light chains from the 95-kDa heavy chain, and the mild

Table III: Comparative Myosin Heavy Chain Sequences around SH_1 and SH_2

sequence (SH_2 – SH_1)	species and ref
-C-N-G-V-L-E-G-I-R-I-C-R-	*a*
-C-N-G-V-L-E-G-I-R-I-T-R-	*b*
-C-N-G-V-L-E-G-I-R-I-A-R-	*c*
-Y-L-G-L-L-E-N-V-R-I-R-R-	*d*

[a] Rabbit skeletal; Elzinga and Collins (1977). Rat skeletal; Strehler et al. (1986). Chicken skeletal; Onishi et al. (1986), Molina et al. (1987). Chicken smooth; Yanagisawa et al. (1987). Nematode *Cestrum elegans*; Karn et al. (1983). Rabbit heart; Kavinsky et al. (1984). [b] Slime mold, *D. discoideum*; Warrick et al. (1986). [c] Myosin II, *Acanthamoeba*; Hammer et al. (1987). [d] Myosin IB, *Acanthamoeba*; Jung et al. (1987).

HPLC separation procedure in dilute detergent avoided any loss of counts from the peptides. In contrast, separations on SDS–polyacrylamide gels gave significant and variable losses of radioactivity that did not allow accurate stoichiometries to be determined.

The large size of SF_1 and the large number of cysteines (10–12) in the heads make identification of a specific single disulfide bond difficult. In particular, we wished to avoid conditions that would promote disulfide bond interchange. We chose to attack the problem by first verifying [^{14}C]pPDM cross-linked SH_1 and SH_2 as had been shown earlier by Burke and Knight (1980). Once a protocol to isolate and identify the tryptic peptide containing Cys-697 (SH_2) and Cys-707 (SH_1) cross-linked with [^{14}C]pPDM was developed, then it was deemed possible to repeat this procedure on SF_1 cross-linked by DTNB treatment. In the latter case the strategy was to alkylate with NEM all accessible cysteines that remained after the limited DTNB treatment and then to reduce the single protein disulfide bond with DTE (the mixed disulfide bond between the single cysteine of the A_1 or A_2 light chains and TNB was also reduced) and finally to cross-link NEM-alkylated SF_1 with a stoichiometric addition of [^{14}C]pPDM. Fortunately, all these steps did not affect the subsequent trypsin digestion or the properties of the resulting tryptic peptides in a significant way. Hence, it was possible in each case to isolate and characterize a unique 12 amino acid peptide whose composition corresponded exactly to residues 697–708. The presence of Arg residues at positions 696 and 708 (see Table II) provided convenient trypsin cleavage sites to yield this peptide. In some instances trypsin also cleaved between Arg-705 and Ile-706, but because the [^{14}C]pPDM spanned Cys-697 and Cys-707, the amino acid analyses of the cross-linked peptide(s) were unaffected.

The possibility that [^{14}C]pPDM could have cross-linked thiols other than SH_1 and SH_2 after NEM treatment and DTE reduction seems remote. No radioactivity was found in A_1 or A_2 light chains, and >95% of the counts were associated with the 20-kDa tryptic peptide that contains SH_1 and SH_2. It seems unlikely that stoichiometric amounts of the larger pPDM reagent would react with the 3–4 unblocked thiols remaining in SF_1 that had not reacted previously with a large excess of the chemically similar but much smaller NEM. In addition, we estimate that some 10–15% of the [^{14}C]pPDM label was recovered in the final tryptic peptides analyzed. The major losses occurred because of what appeared to be incomplete trypsinization of insoluble core material. While no other labeled peptides occurred in significant amounts, it is not possible to rule out completely the possiblity that [^{14}C]pPDM may have reacted partially with other thiols. However, the predominant reaction by far in both labeling protocols appears to have been with SH_1 and SH_2.

Two major roles for the SH_1 and SH_2 region have been suggested in the past. These are that (i) SH_1 and SH_2 are involved in some way in ATP binding and cleavage and that (ii) the SH_2, SH_1 region is a primary site for binding to actin (Katoh et al., 1985; Suzuki et al., 1987). The former suggestion, based on early chemical modification studies [Sekine & Kielley, 1964; reviewed by Reisler (1982)], has been ruled out by a variety of subsequent studies. For example, the blocking of SH_1 and SH_2 by smaller reagents (Weidner et al., 1978; Botts et al., 1979) does not prevent ATP cleavage. Fluorescence resonance energy transfer measurements place SH_1 (and SH_2) some 30–40 Å from the active site (Tao & Lamkin, 1981; Perkins et al., 1984), too far for direct interaction with ATP. DNA and protein sequence studies of myosin heavy chains, while indicating general conservation of the sequence between SH_1 and SH_2 (see Table III), show SH_1 is replaced by threonine in slime mold myosin (Warrick et al., 1986) and by alanine in myosin II from *Acanthamoeba* (Hammer et al., 1987), demonstrating the nonessential nature of this residue. In the single-headed mysoin IB from *Acanthamoeba*, SH_1 and SH_2 are replaced by arginine and tyrosine, respectively (Jung et al., 1987), further indicating SH_1 and SH_2 are not essential for ATP cleavage or for force generation. Finally, at least two laboratories (Atkinson & Korn 1986; Okamoto & Sekine, 1987) have isolated SF_1 preparations whose ATPase activities are actin activatable even though the COOH-terminal 20-kDa chain peptide containing SH_2 and SH_1 has been removed. These studies make it unlikely the SH_2, SH_1 peptide region is the primary actin-binding site.

An alternative role for this region is suggested by the cross-linking studies exemplified in this paper. That is, one reason the sequence between SH_2 and SH_1 is so highly conserved may be that this region provides needed flexibility in the heads. Ford et al. (1981) have shown that most of the compliance that exists in frog muscle fibers exists in the active cross-bridges or heads of myosin. This observation has been confirmed by chemical cross-linking studies of glycinerated rabbit psoas fibers by Tawada and Kimura (1986). Electron microscopic studies of biotin–avidin complexes linked to SH_1 of myosin place SH_1 in the thick portion of the pear-shaped heads of myosin (Sutoh et al., 1984), about two-thirds the distance from the head–rod junction to the blunt-end of myosin heads. Thus, structural elements within this portion of the head must be remarkably flexible. All myosin heavy chains have at least one and most have two glycines between SH_2 and SH_1 (see Table III). These residues tend to be highly conserved. It may be that these glycines serve as swivels or fulcrums about which polypeptide segments containing SH_2 and SH_1 move. In the future it would be of interest to replace these glycines with bulkier amino acids to see if the resultant myosins are still functional. The recent demonstration (De Lozanne & Spudich, 1987) that it is possible to make specific mutant myosin heavy chains in *Dictostelium discoideum* means it should be possible to test this suggestion directly in the future.

Registry No. DTNB, 69-78-3; pPDM, 3278-31-7; L-Cys, 52-90-4; MgADP, 7384-99-8.

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