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Intramolecular crosslinking of tropomyosin via disulfide bond formation: Evidence for chain register

(muscle/SH groups/polyacrylamide gel electrophoresis)

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ABSTRACT **Rabbit skeletal muscle tropomyosin can be crosslinked in the native state by the use of 5,5′-dithiobis(2-nitrobenzoate), which forms disulfide bonds between the two subunits. Using polyacrylamide gel electrophoresis in sodium dodecyl sulfate we have shown that this crosslinking goes to completion over a wide range of protein concentration, ionic strength, and reagent concentration. Crosslinks are not formed in denaturing solvents such as sodium dodecyl sulfate and guanidine hydrochloride despite the fact that the same number of SH groups react as in the native state (2.3 ± 0.2). The sedimentation coefficients of crosslinked and non-crosslinked samples are identical. Thus, crosslinks are formed between corresponding cysteines on different chains of the same molecule. This provides strong evidence for a model of chain interaction with both chains in register. Evidence has also been obtained that rabbit skeletal tropomyosin is composed only of $\alpha\alpha$ and $\alpha\beta$ subunits rather than a random mixture of chains.**

Tropomyosin is a rod-shaped molecule of 68,000 daltons whose subunits are made up of two parallel α-helical polypeptide chains of about equal molecular weight (1). It is one of the components of the thin filaments of skeletal muscle, and is involved in the regulation of muscle contraction (1). Recently, considerable progress has been made in characterizing the two subunits (2) and in analyzing the amino-acid sequence (3–5), and it is clear that rabbit skeletal tropomyosin consists of two kinds of chains, α and β, which differ slightly in amino-acid composition and sequence (2–5). The α chain, which has one cysteine, is present in about a 3-fold excess over the β chain, which contains two cysteines (2). Although the precise arrangement of chains is not known, there is some evidence to suggest that tropomyosin is not composed of a random mixture of α and β chains (6). Sodek *et al.* proposed a model for the interacting chains based on the observation that hydrophobic sidechains are predominantly located in two series which repeat every seventh residue in the sequence (7). This appears to allow maximum hydrophobic interaction along a line between the two α-helical chains when they are in register or when one chain is translated 7, 14, 21, . . . residues with respect to the other. Although this model showed favorable contacts when the two chains were in register, the 14-residue staggered model was preferred because it appeared to optimize hydrophobic interactions and account for end-to-end aggregation.

Evidence has accumulated over the years that unless care is taken to prevent heavy metal ion contamination, the SH groups of tropomyosin are readily oxidized to S-S (8–10).

Abbreviations: $(NbS)_2$, 5,5′-dithiobis(2-nitrobenzoate); NbS, 2-nitro-5-thiobenzoate; $NaDodSO_4$, sodium dodecyl sulfate; GuHCl, guanidine hydrochloride.

The work reported below is an extension of these early observations using a different approach. We have found that it is possible to completely crosslink reduced tropomyosin by reaction with 5,5′-dithiobis(2-nitrobenzoate) $(NbS)_2$. This aromatic disulfide causes the formation of S-S bonds between the corresponding cysteines of the two parallel chains within the same molecule by a two-step disulfide exchange process. Since this process requires the close proximity of two SH groups, these studies provide strong evidence for the model in which the chains are in register. In addition, from our consistent observations that only two crosslinked dimer chain species of approximately equal concentration are formed, it appears that the tropomyosin molecule is composed of only $\alpha\alpha$ and $\alpha\beta$ chains.

MATERIALS AND METHODS

Preparation of Reduced Tropomyosin. Tropomyosin was prepared by the method of Bailey (11) as modified by Greaser and Gergely (12). Tropomyosin at about 5 mg/ml was dissolved in buffer (0.05 M Na phosphate, 0.01 M Na acetate, 1.0 M NaCl, 1 mM EDTA) at pH 7.5, containing 20 mM dithiothreitol, filtered through a Millipore HAWP 0.45 μm filter, and warmed to 45° for 1 hr. After cooling, the pH was immediately reduced to 4.6 by addition of a few drops of HCl, and the isoelectric precipitate was collected by centrifugation for 15 min at 15,000 rpm. After discarding the supernatant, the precipitate was homogenized in a large volume of pH 4.6 buffer and centrifuged again. This process was repeated until the supernatant at pH 7.0 did not develop yellow color when $(NbS)_2$ was added (usually 3–4 centrifugations). The final precipitate was dissolved in buffer at pH 7, and passed through a Millipore filter again to yield a final concentration of about 3 mg/ml. This preparation usually gave ratios of SH/protein = 2.1–2.5. Similar results were obtained by incubating with dithiothreitol for 1 hr at room temperature, then overnight at 4°. It was found that these samples retained their SH groups for at least several days at pH $\leq$ 7 if stored in stoppered, N_2-bubbled buffers.

Concentration of Tropomyosin. This was routinely determined by the biuret reaction using bovine albumin (Pentex) as a standard. The concentration determined by this method was found to agree with the results of two spectrophotometric determinations on the same sample. One method measured the absorbance at 295 nm at pH 12.5, where all the tyrosyls are ionized. The concentration was calculated assuming a value $\epsilon_{295}(Tyr^-) = 2480\ M^{-1}\ cm^{-1}$ (13) and 11.1 Tyr/mol (1, 2). The absorbance spectra at pH 12.5 indicated

that no corrections for light scattering were necessary. The other method used the absorbance spectra obtained at neutral pH and $E(277\ nm) = 0.22\ (mg/ml)^{-1}$, which was calculated from $\epsilon_{277}(Tyr) = 1350\ M^{-1}\ cm^{-1}$ (13) and 11.1 Tyr/mol. In order to obtain agreement with the biuret and high pH determination, the light scattering was measured, extrapolated, and subtracted from the 277 nm value (14).

Reaction with $(NbS)_2$ and Measurement of SH Content. A stock solution of $(NbS)_2$ (0.1 M) was made by slowly dissolving solid $(NbS)_2$ (Worthington) into 5 mM Na phosphate and maintaining the pH above about 5 by occasional addition of 1 M NaOH. High pH must be avoided to prevent cleavage of the disulfide bond (15). It was stored at 4° in a dark bottle and was stable for at least 2 months. The reaction of $(NbS)_2$ with tropomyosin was monitored at 412 nm in matched cuvets containing equal concentrations of $(NbS)_2$ and the concentration of SH groups was calculated from $A_{412\ nm}$ using $\epsilon = 13{,}600\ M^{-1}\ cm^{-1}$ (16). The reaction was initiated either by adding a small volume of $(NbS)_2$ first to the reference cuvet and then to the sample cuvet containing the tropomyosin, or by adding a small volume of a stock solution of tropomyosin to the sample cuvet which contained $(NbS)_2$. The data always followed pseudo first-order kinetics, since the $(NbS)_2$ was present in great excess. For reaction in sodium dodecyl sulfate ($NaDodSO_4$) (Schwartz/Mann) and guanidine hydrochloride (GuHCl) (Schwartz/Mann), the reaction was initiated by the addition of $(NbS)_2$, after dilution of the protein into the denaturing solvent and waiting 5 min.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis and Densitometry. All samples were usually treated with 0.02 M iodoacetamide for ½–1 hr at room temperature; then 1% $NaDodSO_4$ was added and samples were dialyzed against 1% $NaDodSO_4$, 5 mM Na phosphate, pH 7.0, *in absence* of reducing agent. Gels were prepared as outlined by Weber and Osborn (17), using chemicals from Canalco, and run at 6 mA per tube. Reducing agents were never used. Gels were stained with a solution of 0.6 g of Coomassie blue in 250 ml of 50% methanol and 9.2% acetic acid for 2 hr, then destained in 50% methanol, 9.2% acetic acid overnight and 5% methanol, 7.5% acetic acid for 24 hr. The gels were stored in 7% acetic acid. Densitometric tracings of the stained gels were obtained with a Joyce Loebl microdensitometer using a yellow filter. The fraction of monomer and dimer chains present was obtained by weighing the cut out areas under the densitometric tracings.

Sedimentation Studies. A Spinco model E ultracentrifuge operating at 52,640 rpm, 20° with Schlieren optics, was used. The crosslinked and untreated samples were run at the same time, with one sample in a wedge cell which displaced its baseline with respect to the other. The sample concentrations were 3.0 mg/ml in 1.0 M NaCl, 1 mM EDTA, 50 mM Na phosphate buffer, pH 7.0. The crosslinked sample contained 2 mM $(NbS)_2$ and both samples contained 0.02 M iodoacetamide. $NaDodSO_4$ polyacrylamide gel electrophoresis showed total conversion to dimer chains for the crosslinked sample, and less than 5% dimer present in the uncrosslinked sample. The sedimentation coefficients were calculated from photographs, measuring the positions of the Schlieren peaks with time with a Gaertner microcomparator.

Determination of Cysteic Acid. Tropomyosin was oxidized with performic acid and analyzed for cysteic acid as outlined by Moore (18). The ratio of cysteine/tropomyosin was calculated using the amino-acid analysis for Asp, Ala, Ile, and Leu determined at the same time as cysteic acid and the known composition (1, 2).

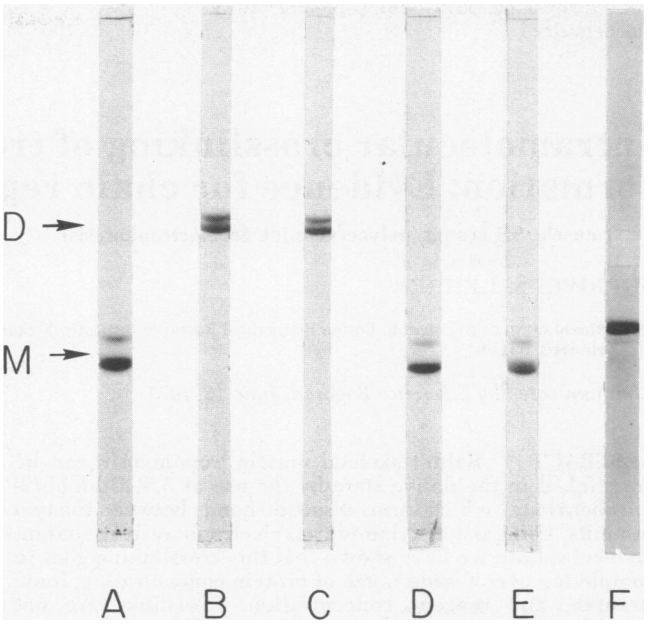


FIG. 1. Products of the reaction of tropomyosin with $(NbS)_2$ in the native and denatured states. 5% $NaDodSO_4$/polyacrylamide gels. M (arrow) = monomer (34,000 daltons); D (arrow) = dimer (68,000 daltons). A, unreacted control; B, in buffer reacted with 2 mM $(NbS)_2$; C, in buffer reacted with 0.2 mM $(NbS)_2$; D, in 5 M GuHCl and buffer reacted with 2 mM $(NbS)_2$; E, in 1% $NaDodSO_4$ and buffer reacted with 2 mM $(NbS)_2$; F, actin gel marker (42,000 daltons). Buffer is 1.0 M NaCl, 1 mM EDTA, 0.05 M Na phosphate, pH 7.4. The reaction with $(NbS)_2$ at room temperature was monitored at 412 nm and allowed to go to completion before treating for electrophoresis (see *Materials and Methods*). Tropomyosin concentration during reaction was 0.35 mg/ml. Amount applied = 3 μg.

RESULTS

In the course of characterizing tropomyosin samples by polyacrylamide gel electrophoresis in $NaDodSO_4$, we frequently noticed bands corresponding to 68,000 daltons instead of the expected 34,000 dalton subunits. This was particularly true when we were not careful to maintain reducing conditions and eliminate heavy metal ions during the preparation and handling. After unsuccessfully attempting to find simple and consistent ways to quantitatively crosslink by air oxidation, we decided to develop a standard procedure to insure that a given tropomyosin solution was fully reduced and then to measure the SH content by the often used $(NbS)_2$ technique (16). In Fig. 1 we show the resulting species produced by the reaction of $(NbS)_2$ with fully reduced tropomyosin after electrophoresis on a 5% $NaDodSO_4$/polyacrylamide gel. As expected, a control sample of reduced, carboxymethylated tropomyosin contains two bands, α and β, in the 34,000 dalton region (17, 2). Tropomyosin samples reduced at different times gave 2.1–2.5 mol of SH per mol of tropomyosin, as measured by the color developed at 412 nm during reaction with $(NbS)_2$. The resulting gel patterns showed that, without exception, quantitative conversion to dimer chains occurs. Examples are shown in Fig. 1. In contrast to the results in the native state, tropomyosin denatured with 5 M GuHCl (19) or 1% $NaDodSO_4$ prior to $(NbS)_2$ addition, contains only monomer bands (Fig. 1), despite the fact that the same number of SH groups had reacted. Treatment with 0.02 M iodoacetamide at 40° for ½ hr, which blocked SH groups, also prevented crosslinking by

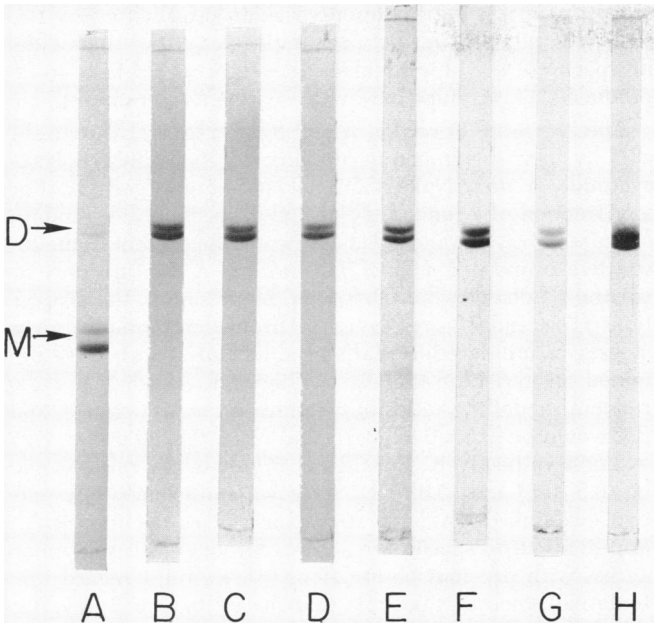


FIG. 2. Products of the reaction of tropomyosin with $(NbS)_2$ at different salt concentration (B-D) and protein concentrations (E-H). 5% $NaDodSO_4$/polyacrylamide gels. A, unreacted control. Tropomyosin in buffer at 0.3 mg/ml reacted with 2 mM $(NbS)_2$ in: B, 1.0 M NaCl; C, 0.1 M NaCl; D, no NaCl. Tropomyosin in buffer and 1.0 M NaCl reacted with 2 mM $(NbS)_2$ at: E, 3 mg/ml; F, 0.3 mg/ml; G, 1 mg/ml; H, 0.1 mg/ml. Buffer is 1 mM EDTA, 0.05 M Na phosphate, pH 7.4; amount applied, 2–5 μg.

$(NbS)_2$ from occurring. Determination of cysteine as cysteic acid by amino-acid analysis on the same preparation of tropomyosin used for all these studies yielded 2.5 ± 0.2 mol/mol of tropomyosin. Thus, $(NbS)_2$ reacts with all of the SH groups of tropomyosin and crosslinks the chains *in the native state*, i.e., prior to denaturation and dissociation by $NaDodSO_4$.

Studies were done to prove that interchain crosslinking occurred within molecules rather than between molecules. Although the reaction rates of $(NbS)_2$ with tropomyosin were dependent upon protein concentration and ionic strength, the same number of SH groups reacted, and quantitative formation of dimer chains always occurred without the formation of higher molecular weight species (Fig. 2). Since it is known that tropomyosin aggregates strongly as the ionic strength drops (11), the invariant gel pattern that was obtained is strong evidence for lack of intermolecular crosslinking. Finally, sedimentation studies were performed on identical samples, one of which had been reacted with 2 mM $(NbS)_2$. After completion of the $(NbS)_2$ reaction, both samples were treated with 0.02 M iodoacetamide. Both samples sedimented identically with s = 2.49 S (3.0 mg/ml, 1.0 M NaCl, 0.05 M phosphate buffer, pH 7.0, 20°). This value agrees quite well with values calculated from Woods' data (20). It would be expected that intermolecularly crosslinked samples would produce species of higher sedimentation coefficient.

Information about the mechanism of the reaction with $(NbS)_2$ can be obtained from the amount of 2-nitro-5-thiobenzoate (NbS) that remains bound to tropomyosin during its reaction. This bound NbS can be freed and estimated by the addition of dithiothreitol. Fig. 3A and B illustrates the results of such an experiment. Tropomyosin was reduced,

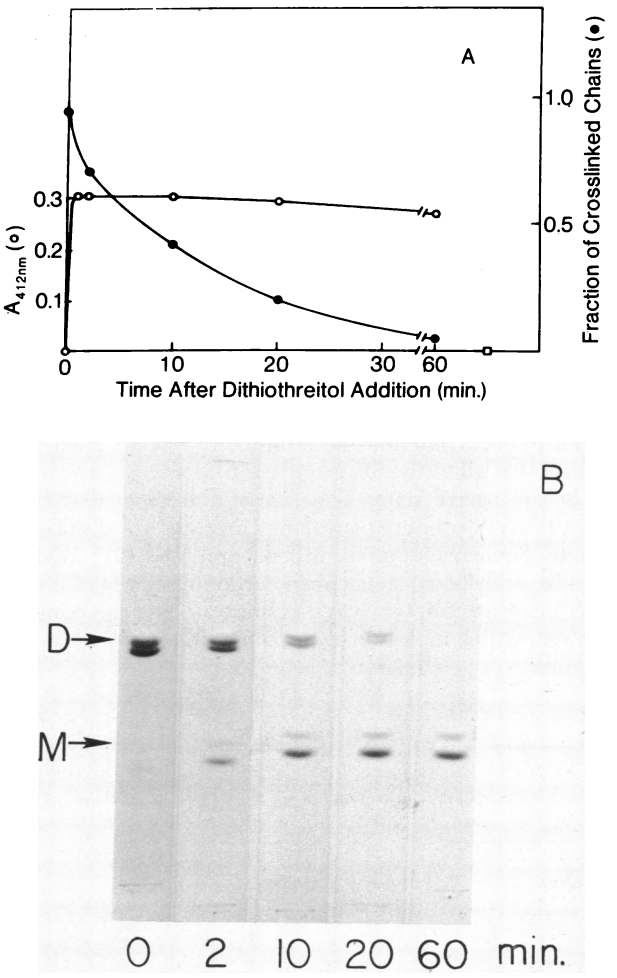


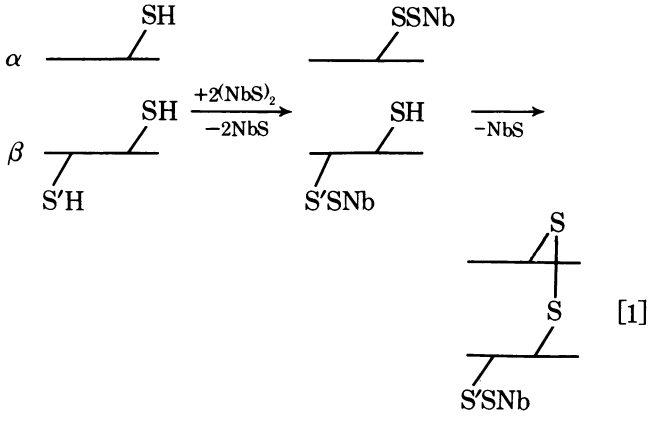
FIG. 3. Comparison of the rate of removal of bound NbS with the rate of cleavage of dimer chains by 20 mM dithiothreitol on $(NbS)_2$-treated tropomyosin (A) ○, rate of color development; ●, rate of cleavage. Reduced tropomyosin (2.8 mg/ml) in buffer at pH 7.4 was reacted with 2 mM $(NbS)_2$ to completion, and the free $(NbS)_2$ was removed by isoelectric precipitation as described in *Materials and Methods*. The tropomyosin was redissolved in buffer at pH 6.9 containing 0.2% $NaDodSO_4$. At zero time, dithiothreitol was added. $A_{412\,nm}$ was measured and at times indicated, samples were withdrawn and added to tubes containing solid iodoacetamide (final concentration = 80 mM). The fraction of dimer chains was measured from densitometric scans of the stained bands. Buffer is 1.0 M NaCl, 1 mM EDTA, 50 mM Na phosphate (B) 5% $NaDodSO_4$/polyacrylamide gels of samples which were removed and carboxymethylated at times indicated.

reacted with $(NbS)_2$, and freed of excess reagent as discussed in *Materials and Methods*. At zero time, 20 mM dithiothreitol was added, and both the development of color due to the release of NbS and the ability of dithiothreitol to convert the dimer chains to monomers were studied with time in the presence of 0.2% $NaDodSO_4$. This was necessary because in the absence of $NaDodSO_4$, $A_{412\,nm}$ did not reach a maximum even after 2 hr. The relative content of dimers and monomers was obtained by $NaDodSO_4$/polyacrylamide gel electrophoresis on samples whose reactions were quenched by the addition of 0.08 M iodoacetamide (Fig. 3B). As can be seen in Fig. 3A, the color developed much more quickly than the conversion of dimer chains to monomers, i.e., bound NbS was removed by dithiothreitol before dithiothreitol acted on the dimer chains. Control studies under identical conditions showed that iodoacetamide blocked the SH

groups of dithiothreitol with a half-time of about 5 min. This means that the upper curve in Fig. 3A should be taken as a maximum rate, since the reaction of dithiothreitol with NbS-tropomyosin proceeded for a few extra minutes after the solutions were mixed with iodoacetamide. For this experiment, from the maximum absorbance at 412 nm it was calculated that 0.53 mol of NbS per tropomyosin were freed by dithiothreitol, which corresponds to 24% of the original SH groups. These data indicate that $(NbS)_2$ reacted with most of the SH groups to form S-S bonds and with the rest to form NbSSR bonds.

DISCUSSION

Our value for the cysteine content of tropomyosin, 2.5 ± 0.2, is in agreement with other recently published values (1, 2). Furthermore, from the agreement between this value and the sulfhydryl content as determined by $(NbS)_2$ reactions, it appears that all of the SH groups of tropomyosin are accessible in the native state. The results also show that $(NbS)_2$ quantitatively produces covalent crosslinks via disulfide exchange. Such reactions have been proposed (21), discussed (22, 23), and shown to occur in certain cases (24, 25). Thus, $(NbS)_2$ can react in two ways, as illustrated by the postulated reaction, with a molecule of tropomyosin composed of α and β chains:



[1]

In this model, the α and β SH groups are oxidized via a double sulfhydryl-disulfide exchange and the β S'H group reacts via a single exchange in the normal manner (16); in both cases NbS is liberated stoichiometrically with the SH content. Evidence for the presence of both kinds of disulfides after reaction of $(NbS)_2$ with tropomyosin was obtained by studies of the action of dithiothreitol. The above experiment (Fig. 3) showed that some NbS was bound, in agreement with the postulated reaction scheme (Eq. 1), and is consistent with the presence of bound NbS on the β chain cysteine of a tropomyosin composed of $\alpha\beta$, which could not form an interchain crosslink.

Since it is known that tropomyosin has a strong tendency to aggregate, the possibility existed that crosslinks are produced between chains of different interacting molecules. The electrophoresis studies do not distinguish between intra- and intermolecular links, since uncrosslinked subunits are dissociated in $NaDodSO_4$. Although it seemed improbable that crosslinks could be produced quantitatively between chains, it was decided to check this possibility. This was done by showing the lack of salt or protein concentration dependence on the extent of reaction, as measured by the total NbS produced and the degree of dimer chain formation. In addition, the sedimentation coefficients of the native and crosslinked samples were identical in the native state. In order to explain the quantitative chain crosslinking by the formation of S-S bonds between molecules rather than within, species of very high molecular weight would have to be produced. This is ruled out by the sedimentation studies.

The two kinds of chains, α and β, are readily distinguishable by their difference in mobility on $NaDodSO_4$/polyacrylamide or urea/$NaDodSO_4$/polyacrylamide gels (2). For any fraction of α and β, f_α and f_β respectively, a random mixture of $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ chains would be present in the respective proportion f_α^2, $2f_\alpha f_\beta$, and f_β^2. A mixture only of $\alpha\alpha$ and $\beta\beta$ chains would, however, be present in the proportions f_α-f_β and $2f_\beta$, respectively. From densitometric scans of gels, assuming equal staining ability of α and β, we obtained an α/β ratio that varied between 3 and 4. For the random model the ratios of crosslinked chains ($\alpha\alpha$, $\alpha\beta$, and $\beta\beta$) would be (0.56, 0.37, 0.07) and (0.64, 0.32, 0.04) for α/β = 3 or 4, respectively. For the $\alpha\alpha$, $\beta\beta$ model the ratios would be (0.5, 0.5) and (0.6, 0.4). Since we consistently saw only two crosslinked bands of approximately equal intensity, it appears that $\beta\beta$ species are absent. Although this suggestion agrees with previous observations (6), we must regard it as tentative, since it is possible that the $\beta\beta$ band may overlap the $\alpha\beta$ band, possibly due to an increased mobility (26) caused by the postulated double crosslink. Information about this question can be obtained from the studies that determined the amount of NbS bound to tropomyosin, if it can be assumed that $\alpha\beta$ is the only species that can bind NbS. For the random model, the fraction of the noncrosslinked SH group (NbS bound) is $f_\alpha f_\beta/(f_\alpha + 2f_\beta)$, as compared to $f_\beta/(f_\alpha + 2f_\beta)$ for the $\alpha\alpha$, $\alpha\beta$ model. For α/β = 3 or 4, the fraction for the random model would be 0.15 or 0.13, respectively; the $\alpha\alpha$, $\alpha\beta$ model gives 0.20 or 0.17, respectively. Although our value of 0.24 more closely agrees with the $\alpha\alpha$, $\alpha\beta$ model, because of the experimental error involved, we must again regard this conclusion as tentative.

Several years ago Woods obtained evidence for S-S bond formation during exposure to air and Cu^{2+} for several hours before dissociation in 8 M urea (8). Although quantitative conversion was not obtained and some oxidation may have occurred after urea denaturation, he did recognize the possibility that intramolecular crosslinks were produced. In contrast to air oxidation, the use of disulfide exchange is an efficient, reproducible, and convenient method for producing S-S bonds between suitably located SH groups of proteins (27). A 0.3 mg/ml of sample of tropomyosin can be quantitatively crosslinked in 20 min at 23°, pH 7.5, by 2 mM $(NbS)_2$. Preliminary experiments with other disulfides indicate that oxidized glutathione and oxidized dithiothreitol can crosslink tropomyosin, although much less efficiently than $(NbS)_2$. The reason for the difference is not clear. Although tropomyosin prepared in the presence of heavy metal chelators appears to be fully reduced (8), the ease with which interchain disulfide bonds are produced by oxygen and disulfide reagents raises the question as to the state of oxidation *in vivo*. Since the ability to form S-S bonds depends upon the distance between corresponding sulfurs on each chain, small movements between chains may control the state of oxidation. Conversely, the presence of crosslinks could control the relative movement between chains. Although there is little or no direct evidence for interchain movement *in vivo*, movements of the tropomyosin filament relative to the actin fiber in the muscle thin filament have been implicated in the regulation of muscle contraction (28). It may be that disulfide exchange plays some role in this regulation.

In conclusion, these studies have shown that, in the native state, S-S bonds can be formed between chains of the same molecule. This must mean that the SH groups of complementary cysteines on adjacent chains must be close to each other. Model building shows that this is only possible for the model in which the chains are in register (7).

Note Added in Proof: After this manuscript was submitted, I learned of the work of P. Cummins and C. Cohen (private communication); Stewart, M. (1975) *FEBS Lett.* **53**, 5–7; and Johnson, P. & Smillie, L. B. (1975) *Biochem. Biophys. Res. Commun.* **64**, 1316–1322. All of these studies used air oxidation to indicate that intramolecular disulfide bonds can be formed.

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1. For recent reviews see articles by C. Cohen *et al.* and R. S. Hodges *et al.* (1973) in "The mechanism of muscle contraction," *Cold Spring Harbor Symp. Quant. Biol.* **XXXVII**, 287–310.
2. Cummins, P. & Perry, S. V. (1973) *J. Biochem.* **133**, 765–777.
3. Hodges, R. S. & Smillie, L. B. (1972) *Can. J. Biochem.* **50**, 330–343.
4. Hodges, R. S. & Smillie, L. B. (1972) *Can. J. Biochem.* **50**, 312–329.
5. Hodges, R. S. & Smillie, L. B. (1973) *Can. J. Biochem.* **51**, 56–70.
6. Yamaguchi, M., Greaser, M. & Cassens, R. G. (1974) *J. Ultrastruct. Res.* **48**, 33–58.
7. Sodek, J., Hodges, R. S., Smillie, L. B. & Jurasek, L. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3800–3804.
8. Woods, E. F. (1967) *J. Biol. Chem.* **242**, 2859–2871.
9. Kominz, D., Saad, F., Gladner, J. A. & Laki, K. (1957) *Arch. Biochem. Biophys.* **70**, 16–27.
10. Drabikowski, W. & Nowak, E. (1966) *Acta Biochim. Pol.* **12**, 61–65.
11. Bailey, K. (1948) *Biochem. J.* **43**, 271–279.
12. Greaser, M. & Gergely, J. (1971) *J. Biol. Chem.* **246**, 4226–4233.
13. Donovan, J. W. (1969) in *Physical Principles and Techniques in Protein Chemistry, Part A*, ed. Leach, S. J. (Academic Press, New York), pp. 101–169.
14. Schauenstein, E. & Bayzer, H. (1955) *J. Pol. Sci.* **16**, 45–51.
15. Brocklehurst, K. & Little, G. (1973) *Biochem. J.* **133**, 67–80.
16. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77.
17. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
18. Moore, S. (1963) *J. Biol. Chem.* **238**, 235–237.
19. Pont, M. J. & Woods, E. F. (1971) *Int. J. Protein Res.* **3**, 177–183.
20. Woods, E. F. (1969) *Biochemistry* **11**, 4336–4344.
21. Diez, M. J. F., Osuga, D. T. & Feeney, R. E. (1964) *Arch. Biochem. Biophys.* **107**, 449–458.
22. Glazer, A. N. (1970) *Annu. Rev. Biochem.* **39**, 101–130.
23. Habeeb, A. F. S. A. (1972) in *Methods in Enzymology*, eds. Hirs, C. H. W. & Timasheff, S. N. (Academic Press, New York), Vol. 25, pp. 457–464.
24. Connellan, J. M. & Folk, J. E. (1969) *J. Biol. Chem.* **244**, 3173–3181.
25. Kleppe, K. & Damjanovich, S. (1967) *Biochim. Biophys. Acta* **185**, 88–102.
26. Griffith, I. P. (1972) *Biochem. J.* **126**, 553–560.
27. Wetlaufer, D. B. & Ristow, S. (1973) *Annu. Rev. Biochem.* **42**, 135–158.
28. Hazelgrove, J. C., Lowy, J., Vibert, P. J. & Huxley, H. E. (1973) in "Mechanism of muscle contraction," *Cold Spring Harbor Symp. Quant. Biol.* **XXXVII**, 341–376.