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Disulfide Formation within the Regulatory Light Chain of Skeletal Muscle Myosin†

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ABSTRACT: Thiol–disulfide exchange reactions between myosin and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) lead to the formation of 5-thio-2-nitrobenzoic acid (TNB)-mixed disulfides as well as to protein disulfide bonds. After incubation with DTNB, myosin was treated with an excess of *N*-ethylmaleimide (NEM) before electrophoretic analysis of the protein subunits in sodium dodecyl sulfate (SDS) without prior reduction by dithiothreitol (DTT). Without NEM treatment, thiol–disulfide rearrangement reactions occurred in the presence of SDS between the residual free thiols and DTNB. In the absence of divalent metal ions at 25 °C, DTNB was shown to induce an intrachain disulfide bond between Cys-127 and Cys-156 of the RLC. This intrachain cross-link restricts partially the unfolding of the RLC in SDS and can be followed as a faster migrating species, RLC′. Densitometric evaluation of the electrophoretic gel patterns indicated that the stoichiometric relation of the light chains (including RLC and RLC′) remained unchanged. The two cysteine residues of the fast migrating RLC′ were no more available for reaction with [^{14}C]NEM, but upon reduction with DTT, the electrophoretic mobility of the RLC′ reverted to that of unmodified RLC and of the RLC modified with two TNB groups. Ca^{2+} or Mg^{2+} was able to prevent this disulfide formation in the RLC of myosin by 50% at a free ion concentration of 1.1×10^{-8} and 4.0×10^{-7} M, respectively, at 25 °C and pH 7.6. Intrachain disulfide formation of RLC never occurred in myosin at 0 °C. Incubation of isolated RLC with DTNB always resulted in intrachain disulfide formation, irrespective of temperature or the presence or absence of divalent metal ions.

All double-headed myosin species seem to contain two types of light chains (LC),[1] one of each associated noncovalently with the heavy chain (HC) in each head portion. Not much is known about the function of the alkali-type LC (LC-1 and LC-3) in fast skeletal muscle myosin. The second type of LC in the 19 000-Da molecular mass region is often referred to as DTNB-LC, as P-LC, or as regulatory light chain (RLC). These different trivial names derive from some of its properties: (i) part of this LC may be removed by treatment of myosin with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of EDTA at elevated pH; (ii) this LC can be reversibly phosphorylated at its Ser-15 in vitro and in vivo (Perrie et al., 1973); and (iii) homologous protein subunits serve as

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[1] Abbreviations: Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HC, myosin heavy chain; LC-1, myosin light chain 1; LC-3, myosin light chain 3; NEM, *N*-ethylmaleimide; [^{14}C]-NEM, *N*-ethyl[^{14}C]maleimide; [^{14}C]NES, *N*-ethyl[1-^{14}C]succinimide; PAGE, polyacrylamide gel electrophoresis; RLC, myosin regulatory light chain; SDS, sodium dodecyl sulfate; TNB, 5-thio-2-nitrobenzoic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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proper regulatory LC by switching on contraction in the myosin of some specific muscles as well as of some nonmuscle cells.

Phosphorylation of the RLC is a prerequisite for triggering contraction in myosin of vertebrate smooth muscles and in many vertebrate nonmuscle cells [for reviews, see Adelstein and Eisenberg (1980) and Kendrick-Jones and Scholey (1981)]. In vertebrate sarcomeric muscles (skeletal and heart muscles), reversible phosphorylation of the homologous RLC may play a modulatory role, but it does not represent the on–off switch for contraction (England, 1984). In the myosin of the striated adductor muscle of the molluscan scallop, the homologous RLC is called EDTA-LC (Szent-Györgyi et al., 1973). It is involved in myosin-linked regulation of contraction and has been shown to be located in the neck region, where the two heads come close together, joining the rod portion (Hardwicke et al., 1983; Vibert et al., 1985). All these homologous types of RLC belong to a superfamily of intracellular Ca^{2+} binding proteins whose common feature is the presence of domains which are structurally related to the EF-hand conformation of carp parvalbumin (Kretsinger, 1980). Such an EF domain comprises a Ca^{2+} binding loop flanked by an α-helix on either side as determined by X-ray crystallography (Kretsinger & Nockolds, 1973). The different types of RLC are able to bind one divalent metal ion in their EF domain 1 while the other three EF domains exhibit sequence deviations apparently incompatible with the binding function [see Bagshaw and Kendrick-Jones (1979)].

In view of the regulatory function of the RLC types in various muscles and their localization in the neck region of the myosin molecule [for a review, see Vibert and Cohen (1988)], the function of the RLC in skeletal muscle myosin is a subject of long-standing interest. Partial extraction and reconstitution of the RLC in skinned fibers of rabbit psoas muscle suggest that this LC modulates the crossbridge kinetics affecting the maximum shortening velocity rather than isometric tension (Moss et al., 1982). Binding of divalent metal ions to the RLC is known to protect the head–rod junction from proteolytic digestion (Weeds & Pope, 1977). Under these latter conditions, the RLC gets cleaved by chymotrypsin between Phe-18 and Ser-19 while in the absence of divalent metal ions the cleavage occurs between Phe-53 and Ala-54.

The RLC of myosin from rabbit fast skeletal muscle contains two free thiols, Cys-127 and Cys-156 (Matsuda et al., 1977). Preliminary observations that treatment with DTNB may induce an intramolecular disulfide bond suggest these two thiols may be localized close together in myosin (Katoh & Lowey, 1987). In the present work, we explored the induction of intrachain protein disulfide bonding in the RLC in situ in the myosin molecule as well as in its isolated state for chemical probing of the relation between the two thiols. Thiol–disulfide exchange reactions are rapid, specific, and virtually free of side reactions (Creighton, 1986). Unambiguous evaluation of DTNB-induced disulfides in the RLC was possible after fixing the modified protein immediately in a stable form by blocking rapidly and irreversibly the remaining free thiols with *N*-ethylmaleimide (NEM). The results indicate that the 2 thiols in the RLC, though separated by 28 amino acid residues in the primary structure, are close to each other in native myosin and that their reactivity is affected specifically by temperature and by divalent metal ions.

Materials and Methods

Myosin was prepared from fast skeletal rabbit muscles (Schaub et al., 1975) and stored until use in 100 mM phosphate buffer, pH 7.1, 5 mM EDTA, and 3 mM NaN_3 at 4 °C. The degree of RLC phosphorylation varied between 40 and 60% as judged from electrophoresis in urea. Fully phosphorylated and fully dephosphorylated myosins were obtained by making use of the endogenous myosin light chain kinase and phosphatase present in the early stage of myosin preparation (Cardinaud, 1986). RLC was isolated from myosin (Weeds & Lowey, 1971) at low ionic strength (100 mM KCl) and concentrated by ammonium sulfate precipitation at 75% saturation. Before use, the RLC was incubated in 500 mM urea, 100 mM NaCl, 5 mM EDTA, 5 mM DTT, and 20 mM Tris-HCl, pH 8.0, for 20 min at 25 °C followed by dialysis with three changes against the same solution except for pH 7.6 and 0.5 mM DTT, and further dialysis with two changes against the same solution without DTT and urea.

Incubation of myosin (8–12 mg/mL) and isolated RLC (0.5–0.7 mg/mL) with DTNB ranging from 0 to 4 mM was performed in 50–150 mM KCl or NaCl and 50 mM Tris-HCl, pH 7.6, at various temperatures for 20–60 min as specified under Results. The DTNB incubation was done either in 5 mM EDTA or in 1 mM EDTA plus 5 mM $CaCl_2$ or $MgCl_2$. Controlled free metal ion concentrations were generated by an EDTA buffer system (5 mM EDTA throughout). The actual metal ion concentrations were calculated with the following values of logarithms of association constants taken from Sillen and Martell (1971): $EDTA^{4-}/H^+$, 10.26; $HEDTA^{3-}/H^+$, 6.15; H_2EDTA^{2-}/H^+, 2.67; H_3EDTA^-/H^+, 1.99; $EDTA^{4-}/Ca^{2+}$, 10.6; $HEDTA^{3-}/Ca^{2+}$, 3.5; $EDTA^{4-}/Mg^{2+}$, 8.7; $HEDTA^{3-}/Mg^{2+}$, 2.3. The association constants for Na^+ and K^+ to EDTA and those for Ca^{2+} and Mg^{2+} to Tris are negligibly small (Good et al., 1966). Distilled and deionized water was used throughout with a contamination by Ca^{2+} and Mg^{2+} below 1 μM. The samples destined for electrophoresis were treated with 5 mM NEM for 2–3 h at 20 °C or for 4–6 h at 4 °C immediately after the incubation with DTNB if not otherwise stated in the text. Treatment with *N*-ethyl[1-^{14}C]maleimide was done with 1.2 mM for 3 h followed by further addition of 4 mM cold NEM for another 2–4 h.

Electrophoresis (SDS–PAGE) was performed on 7.5% polyacrylamide gels (column dimensions, 6 × 85 mm) in SDS, 100 mM Tris, and 100 mM Bicine, pH 8.3, for better resolution in the myosin LC region (Weeds, 1976) with additional 5% glycerol and 0.1 mM EDTA. All samples were taken up in 25 mM Tris–Bicine, 15% glycerol, and 2% SDS with neither addition of DTT nor heating and stored at −20 °C until use. Samples which were reduced with DTT and heated before electrophoresis are mentioned under Results. In this system, LC-1, RLC, and LC-3 displayed apparent molecular masses of 25 000, 20 650, and 18 400, respectively, based on calibration with proteins of known molecular masses. Electrophoresis in 6 M urea with 10% glycerol was performed according to Perrie et al (1973) for estimation of the degree of phosphorylation of the RLC in myosin. Gels were, in general, heavily loaded (60–90 μg of myosin and 5–6 μg of isolated RLC) in order to yield unambiguous gel patterns in the LC region. Fixation, staining with Coomassie brilliant blue R-250, and destaining were standardized (Sobieszek & Bremel, 1975). Destained gels were scanned densitometrically, and subunit stoichiometry was calculated by assuming all protein bands stained with equal specific intensity. For this calculation, the following chemical molecular weights were used: HC, 200 000; LC-1, 20 700; RLC and RLC′, 19 000; LC-3, 16 500 (Weeds & Lowey, 1971; Frank & Weeds, 1974). For assessment of radioactivity incorporated into the electrophoretically resolved subunits, the gels were sliced and dissolved in 30% hydrogen peroxide for 20 h at 70 °C (Gray & Steffensen, 1968) before

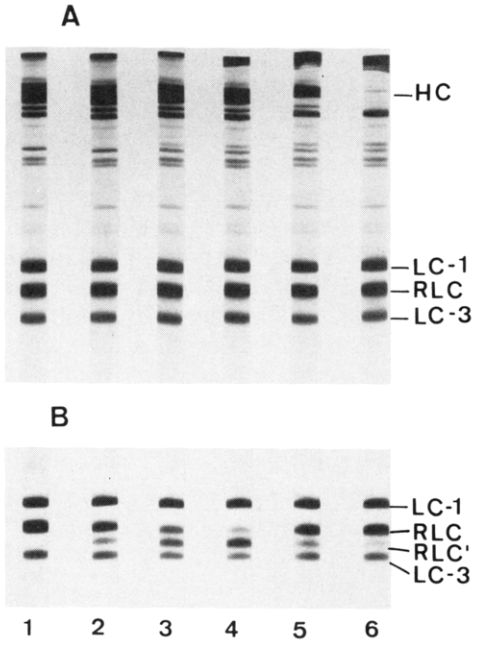

FIGURE 1: SDS–PAGE in the absence of DTT after incubation of myosin with DTNB in the presence of 5 mM EDTA at pH 7.6 followed by treatment with NEM (for details, see Materials and Methods). (A) DTNB incubation for 60 min at 0 °C; (B) DTNB incubation for 20 min at 25 °C (only the LC region is shown in this case). The numbers indicate DTNB (μM) concentrations of (1) 0, (2) 20, (3) 60, (4) 120, (5) 500, and (6) 2000.

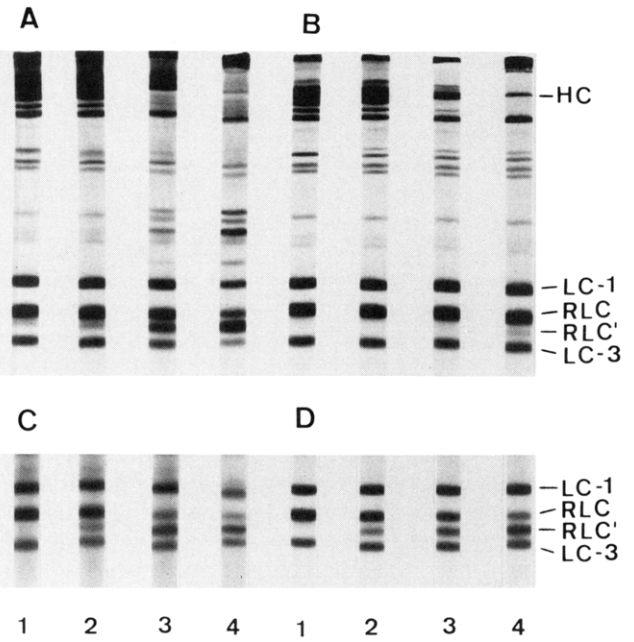

FIGURE 2: SDS–PAGE in the absence of DTT after incubation of myosin with DTNB in the presence and absence of divalent metal ions for 20 min at pH 7.6 and 25 °C (for details, see Materials and Methods). (A and B) DTNB incubation in the presence of 5 mM $MgCl_2$; (C and D) DTNB incubation in the presence of 5 mM EDTA (only the LC region is shown in this case); (A and C) samples were not treated with NEM after DTNB incubation; (B and D) with NEM treatment after DTNB incubation. The numbers indicate DTNB (μM) concentrations of (1) 0, (2) 10, (3) 50, and (4) 100.

scintillation counting (Schaub et al., 1978). Recovery of radioactivity in the gels sliced immediately after the electrophoretic run was always around 100%. After the fixation, staining, and destaining processes, the recovery was reduced in all protein bands by 10–15% except for the LC-3 band where it was reduced by 15–20%.

The reaction of myosin with varying concentrations of DTNB under the conditions specified above was also followed colorimetrically (Ellman, 1959). After DTNB incubation, myosin was diluted 4 times with ice-cold 20 mM KCl, 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 1 mM EDTA and centrifuged, and the production of TNB groups was monitored in the supernatant by measuring the absorbance at 412 nm (ϵ_{412nm} = 14 150 M^{-1} cm^{-1}) or at 450 nm (ϵ_{450nm} = 6970 M^{-1} cm^{-1}) (Riddles et al., 1979). The protein-bound TNB groups were assessed subsequently in the pelleted myosin after it was washed twice with acetone, dissolution of the protein in NaOH, readjustment of the pH to 7.6, and treatment with 5 mM DTT for 20 min at 22 °C (Walser et al., 1981).

Protein concentrations were determined by the biuret reaction or after Bradford (1976) standardized by ultramicro Kjeldahl estimation of nitrogen (Strauch, 1965). All buffer solutions were prepared at the temperature of use.

Results

DTNB-Induced Modification of the RLC in Myosin. Incubation of myosin with DTNB in varying concentrations (0–2 mM) leads to the formation of 5-thio-2-nitrobenzoic acid (TNB)-mixed disulfides as well as to protein disulfide bonds. If not stated otherwise in the text, myosin was treated with NEM in excess immediately after incubation with DTNB in order to block the remaining free thiols irreversibly. Subsequently, SDS–PAGE was performed without prior reduction with DTT. Under these conditions, the stoichiometry of all three types of LC remained unaltered after incubation with DTNB in the presence of EDTA at 0 °C and pH 7.6 (Figure 1A). If the incubation with DTNB was, however, performed at 25 °C, the RLC gradually disappeared and reappeared as the DTNB concentration increased (Figure 1B). Concomitantly, a new band appeared transiently underneath the RLC. Its transient appearance seems to occur at the expense of the RLC. In fact, the stoichiometry of all three types of LC remained unaltered as in Figure 2A when the new band dubbed RLC′ was included with the RLC. Densitometric evaluation of the gel patterns yielded a stoichiometry of 1.21:1.98:0.81 for LC-1 to RLC to LC-3, respectively. It was the same as found after complete reduction of the samples with DTT and heating prior to electrophoresis. The HC did not readily enter the gels in SDS–PAGE without prior reduction and disappeared almost completely with progressing DTNB concentration (Figure 1A). In SDS–PAGE with prior reduction by DTT, the HC entered the gels normally (not shown).

Figure 2 displays SDS-PAGE of myosin after incubation with 0–100 μM DTNB in the presence of either $MgCl_2$ or EDTA at 25 °C with and without subsequent NEM treatment. No changes occurred in the LC bands, and their stoichiometric relationship remained unaltered when NEM treatment was applied after incubation of myosin with DTNB in the presence of $MgCl_2$ (Figure 2B). Without NEM treatment, however, the RLC exhibited partial transformation into the RLC′ band (Figure 2A). Furthermore, the stoichiometric relationship deviated from normal with increasing DTNB concentrations; all three types of LC became reduced irregularly, and, in addition, some RLC′ band as well as some new protein bands appeared between the LC and the HC regions. In the case of $MgCl_2$, the NEM treatment prevented the formation of RLC′ completely. In the presence of EDTA, again, the LC seemed to disappear partially without NEM treatment (Figure 2C), while after alkylation with NEM the stoichiometric relationship remained unaltered even though a portion of its RLC changed into RLC′ (Figure 2D). With EDTA, the

Table I: Effect of DTNB Incubation on Intrachain Disulfide Formation in the RLC of Intact Myosin in the Absence of Divalent Cations and on Subsequent Radioactive Incorporation of *N*-Ethyl[1-^{14}C]succinimide[a]

[DTNB] (μM)	% RLC	% RLC′	% radioact.[b] in RLC	% radioact.[b] in RLC′	% radioact.[c] in RLC	calcd free thiols per RLC
0	100	0	100		100	2.00
40	51	49	40	6	45	0.87
120	2	98	3	2	7	0.11
400	39	61	3	0	2	0.05
2000	98	2	0	0	0	0.00

[a] Myosin was incubated with DTNB in 5 mM EDTA at pH 7.6 and 25 °C for 30 min and subsequently treated with 720 μM [^{14}C]NEM. % RLC and % RLC′ were densitometrically evaluated from SDS–PAGE without DTT. Unmodified free thiols after DTNB incubation were calculated from incorporated radioactivity. For further details, see Materials and Methods. [b] Radioactivity from SDS–PAGE without DTT. [c] Radioactivity from SDS–PAGE after reduction with DTT where the RLC′ species disappears and migrates in the RLC band.

formation of RLC′ did occur with and without NEM treatment; its formation was, however, precipitated without NEM treatment (Figure 2C). The variable reduction of all three types of LC, the formation of RLC′ under all conditions, and the new bands between the LC and the HC regions in SDS–PAGE suggest that without NEM treatment thiol–disulfide rearrangement reactions must occur between DTNB and the free thiols still present, which lead to disulfide cross-links between different LC and between LC and HC, as soon as the incubation samples were transferred to SDS. These artifactual reactions occurred immediately in SDS whether subsequent electrophoresis was performed without delay or after several days of storage at −20 °C. These artifacts were furthermore characteristic for incubation of myosin with DTNB in the low concentration range of 50–200 μM. At higher DTNB concentrations (above 1 mM), fewer artifacts were observed, probably, because most protein thiols have become blocked by TNB groups. Thus, blocking of persisting free thiols with NEM in excess after incubation with DTNB proved to be essential to preserve the DTNB-induced modifications in the LC subunits.

With NEM treatment, RLC′ was not observed when myosin was incubated either with DTNB in the presence of $MgCl_2$ or $CaCl_2$ at 0 or at 25 °C or in the presence of EDTA at 0 °C. On the other hand, RLC′ occurred invariably after incubation of myosin in 2–10 mM EDTA at 25 °C. Time course studies in the presence of EDTA at 25 °C showed that the RLC′ band appeared within 10–20 min; longer incubation with DTNB did not further enhance it.

DTNB-Induced Intrachain Disulfide Bond in the RLC of Myosin. In order to assess the extent of modification of the two RLC thiols by DTNB, myosin was subsequently treated with [^{14}C]NEM. The incorporated radioactivity was then counted in the RLC and RLC′ bands after electrophoretic resolution. Only free thiols which have not reacted with DTNB can become blocked by an *N*-ethyl[1-^{14}C]succinimide group ([^{14}C]NES). After incubation of myosin with DTNB in the presence of $MgCl_2$ for 30 min at 25 °C or in the absence of divalent metal ions (5 mM EDTA) for 60 min at 0 °C, the radioactivity decreased in the RLC band as the DTNB concentration increased. This indicates that when no RLC′ occurs both thiols became progressively blocked by TNB groups. Furthermore, the doubly modified RLC species with two TNB groups or with one TNB and one [^{14}C]NES group or, finally, with two [^{14}C]NES groups did not change their electrophoretic mobility in SDS–PAGE (compare Figures 1A and 2B). In fact, these modified species comigrated with the native RLC (not shown).

A similar experiment after incubation of myosin with DTNB in the presence of 5 mM EDTA for 30 min at 25 °C, where a RLC′ band developed transiently, is shown in Table I. In the absence of DTNB, the 100% radioactivity in the RLC was twice that recovered in either LC-1 or LC-3 on a molar basis. This reflects the fact that the RLC contains two thiols while LC-1 and LC-3 contain only one each. Calculated from the radioactive recovery in the respective electrophoretic bands, the stoichiometry amounted to 1.22:1.97:0.81 for LC-1 to RLC to LC-3, respectively. The conversion of RLC to RLC′ in SDS–PAGE without DTT reached 98% at 120 μM DTNB and subsequently reverted almost completely at 2.0 mM DTNB as determined densitometrically (Table I). At 40 μM DTNB, about half the RLC was converted to RLC′. The radioactivity on the residual RLC band has dropped to about half too. This remaining half of the RLC has then retained its specific radioactivity unchanged and, therefore, has not been modified by DTNB at all. Its other half which has been converted to RLC′ displays almost no radioactivity; therefore, its two thiols have been modified by DTNB. With 120 μM DTNB, almost all RLC has been converted to RLC′, and the radioactivity in the RLC band is negligible. This loss of radioactivity corresponds to the diminished protein staining in the RLC band. With higher DTNB concentrations, the radioactivity of the RLC band approached zero despite its protein staining reverting to its original intensity. The reappearing protein in the RLC band was not radioactively labeled and did not therefore react with [^{14}C]NEM. Its two thiols must have been blocked by the reaction with DTNB by two TNB groups. After reduction with DTT prior to SDS–PAGE, however, the RLC′ species had disappeared, and the protein staining of the original RLC band remained constant. Nevertheless, the radioactivity shows exactly the same dropping as in the absence of DTT when taken together from the RLC and the RLC′ bands.

It can be concluded that under conditions of incubation of myosin with DTNB, where no RLC′ species is occurring, the RLC becomes gradually modified by two TNB groups. The RLC′ species only occurs in EDTA at 25 °C with low DTNB concentrations (10–500 μM). At higher DTNB concentrations (above 1 mM), the RLC reappears at its original electrophoretic position with its two thiols blocked by TNB groups. The DTNB-induced modification leading to the faster migrating RLC′ indicates an apparent lowering of the molecular mass in SDS–PAGE. The original RLC displays an apparent molecular mass of 20 650 (see Materials and Methods) and the RLC′ species 18 400. The fact that the RLC′ band disappears upon reduction by DTT prior to SDS–PAGE and reverts to its original migration position is suggestive that the RLC′ represents a species with an intrachain disulfide bond which affects its electrophoretic mobility.

Release of TNB by Thiol–Disulfide Exchange Reactions in Myosin. The liberated and protein-bound TNB was followed colorimetrically on incubation with substoichiometric amounts up to 10 mol of DTNB per myosin under conditions where the RLC′ species was formed or was suppressed. The amount of liberated TNB was always significantly higher with EDTA at 25 °C where the RLC′ is occurring than in all other

Table II: Released and Protein-Bound TNB Groups on Incubation of 2 mol of DTNB/mol of Myosin under Various Conditions and Calculated Number of DTNB-Induced Protein Disulfide Bridges per Myosin[a]

metal ion	incubation time (min)	temp (°C)	released TNB/myosin	protein-bound TNB/myosin	protein disulfide bonds/myosin
EDTA[b]	20	25	3.1	0.6	1.3
Mg	20	25	2.1	1.1	0.5
Ca	20	25	2.0	1.2	0.4
EDTA	20	0	1.3	0.2	0.6
EDTA	60	0	1.7	0.8	0.5
Mg	20	0	1.1	0.4	0.4
Mg	60	0	1.5	0.6	0.5
Ca	20	0	1.2	0.4	0.4
Ca	60	0	1.7	0.5	0.6

[a] Myosin (18–21 μM) was incubated with 40 μM DTNB at pH 7.6. Released TNB and protein-bound TNB, after treatment with DTT, were determined colorimetrically as described under Materials and Methods. [b] The only condition where an intrachain disulfide occurs in the RLC.

cases. The proportion of protein-bound TNB increases rapidly toward higher DTNB concentrations. With 2 mol of DTNB per myosin, i.e., equimolar DTNB with the RLC in myosin, around 40% of the RLC becomes transformed into RLC′ in EDTA at 25 °C.

Table II summarizes the results on liberated and protein-bound TNB under various experimental conditions after incubation of 18–21 μM myosin with 40 μM DTNB. The amount of liberated TNB (3.1 per myosin) was highest with EDTA at 25 °C. It was lower in all other cases, ranging from 1.1 up to 2.1. The amount of protein-bound TNB has its molecular counterpart among the number of liberated TNB. After subtraction of this portion from the total, the remaining liberated TNB results entirely from thiol–disulfide exchange reactions whereby both TNB moieties become released from each DTNB. The number of DTNB molecules from which two TNB were released during incubation of myosin under varying conditions is given in the last column of Table II. Again, this number is highest for EDTA at 25 °C where RLC′ formation occurs. In all other cases, it was lower, ranging between 0.4 and 0.6 per myosin.

These results indicate that a number of protein disulfide bridges are induced by incubation of myosin with DTNB under various conditions where no RLC′ is formed. These bridges must therefore be located in the HC moiety of the molecule. In EDTA at 25 °C, however, over twice as many protein disulfide bonds are formed together with the occurrence of RLC′. This finding corroborates the suggestion that the RLC′ species contains an intrachain disulfide bond between its two thiols.

Influence of Divalent Cations and Temperature on the Intrachain Disulfide Formation of RLC in Myosin. The presence of $MgCl_2$ or $CaCl_2$ did prevent formation of RLC′ though the thiols of the RLC still became blocked by TNB groups. The DTNB incubation was therefore subjected to varying concentrations of free metal ions, either Mg^{2+} or Ca^{2+}, in an EDTA buffer system. The extent of RLC′ formation was then densitometrically evaluated from SDS–PAGE and plotted versus the free metal ion concentration (Figure 3). The extent of RLC′ formation in 5 mM EDTA was taken as 100%. Ca^{2+} or Mg^{2+} was able to prevent RLC′ formation in myosin by 50% at free ion concentrations of 1.1×10^{-8} and 4.0×10^{-7} M, respectively. From these data, the apparent affinity constants were calculated for Ca^{2+} ($K = 9.1 \times 10^{7}$ M^{-1}) and for Mg^{2+} ($K = 2.5 \times 10^{6}$ M^{-1}). The higher affinity for Ca^{2+} and the lower for Mg^{2+} fall well into the ranges reported for direct binding studies on myosin (Watterson et al., 1979; Holroyde et al., 1979). High-affinity binding of divalent metal ions to the RLC in myosin then causes a change in the reactivity of their two thiols with DTNB when compared with the metal-free state.

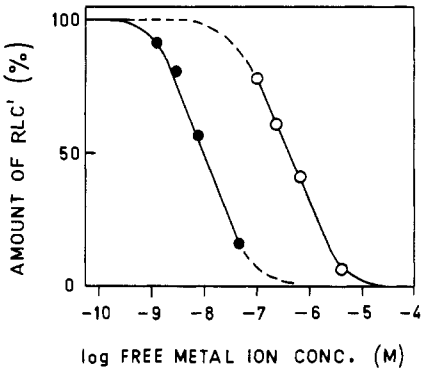

FIGURE 3: Extent of intrachain disulfide formation of the RLC in myosin after incubation with 150 μM DTNB in 5 mM EDTA plus various amounts of divalent metal ions for 20 min at 25 °C and pH 7.6 followed by treatment with NEM was plotted versus free metal ion concentration (for details, see Materials and Methods). (●) Ca^{2+}; (○) Mg^{2+}; averages of two experiments each. Intrachain disulfide formation was evaluated densitometrically from the RLC and RLC′ bands in electrophoretograms.

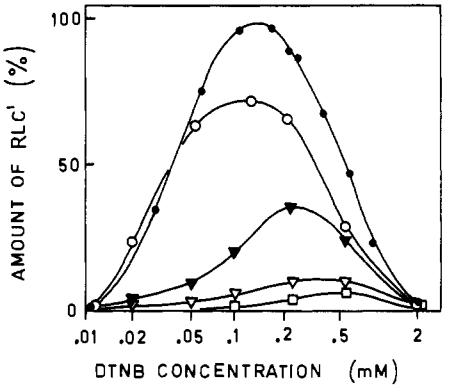

FIGURE 4: Intrachain disulfide formation of RLC in myosin after incubation with increasing DTNB concentrations (log scale) at various temperatures. Incubations were carried out in 5 mM EDTA at pH 7.6 for 20 min followed by treatment with NEM. (●) 25 °C; (○) 20 °C; (▼) 15 °C; (▽) 11 °C; (□) 5 °C; averages of 2–12 experiments at each temperature. The extent of disulfide formation at 25 °C was taken as 100%. At 0 °C, no disulfide formation was apparent. Intrachain disulfide formation was evaluated densitometrically from the RLC and RLC′ in electrophoretograms.

A series of incubations of myosin with varying DTNB concentrations in the presence of 5 mM EDTA at different temperatures revealed a gradual loss of RLC′ formation as 0 °C is approached (Figure 4). The reaction rate of DTNB with the thiols of the RLC was slowed down also at lower temperatures, and the peak of the residual extent of RLC′ formation tended to shift toward higher DTNB concentrations, but no RLC′ was formed at 0 °C even when incubated for 60 min. Experiments with [^{14}C]NEM indicated that both thiols

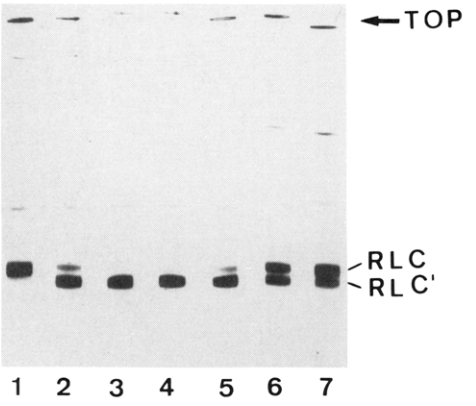

FIGURE 5: SDS–PAGE in the absence of DTT of isolated RLC after incubation with DTNB followed by treatment with NEM. DTNB incubation was performed in the presence of 5 mM $CaCl_2$ at pH 7.6 for 60 min at 0 °C (for details, see Materials and Methods). Numbers indicate DTNB (μM) concentrations of (1) 0, (2) 30, (3) 60, (4) 100, (5) 200, (6) 1000, and (7) 4000. Top of the gels is marked.

became modified quantitatively with TNB groups at DTNB concentrations of 500 μM or higher. Thus, low temperature seems to induce in the RLC of myosin in the presence of EDTA a similar change in thiol reactivity against DTNB as does binding of divalent cations at 25 °C.

In order to test the stability of the RLC′ induced by DTNB in EDTA at 25 °C, a number of immediately following second incubations under different conditions were performed before the NEM treatment was applied: addition of 2 mM DTNB for further 20 min at 25 °C, addition of 8 mM $MgCl_2$ or $CaCl_2$, addition of 500 mM NaCl, lowering the pH to 4.0 or increasing it to 8.6, and lowering the temperature to 0 °C for 20 h; all these additional incubations did not alter the extent of RLC′ formation which occurred within the first 20 min in EDTA at 25 °C. So once formed, the RLC′ species was quite stable. Furthermore, the extent of RLC′ formation was the same with myosin with its RLC fully phosphorylated, fully dephosphorylated, or partially phosphorylated.

DTNB-Induced Intrachain Disulfide Bond in Isolated RLC. Aliquots of isolated RLC with intact thiols were subjected to treatment with DTNB under various conditions. After DTNB incubation, the RLC was also treated with excess NEM, like myosin, prior to SDS–PAGE. Formation of RLC′ in the example given in Figure 5 occurred at 0 °C in the presence of $CaCl_2$. In contrast to the situation in myosin, the isolated RLC developed under all incubation conditions, in the presence and absence of divalent metal ions and at high or low temperature, with DTNB up to 100% RLC′. Furthermore, unlike in myosin, 30–50% RLC′ persisted in the isolated RLC even when incubated with 1–4 mM DTNB (Figure 5). Subsequent incorporation of [^{14}C]NES indicated that the protein in the RLC band, as long as it persists at low DTNB concentrations, still contains two free thiols and that the RLC band, which reverts partially to its original position at high DTNB concentrations, contains the protein whose thiols are both blocked by TNB groups. NEM treatment was also necessary in experiments with isolated RLC in order to prevent formation of interchain disulfide bonds between RLC whose thiols have not yet been modified by DTNB as soon as the samples were transferred to SDS. Without NEM treatment, a large portion of the RLC material appeared in electrophoretic bands of higher molecular masses.

Discussion

DTNB was used to probe the reactivity of the two free thiols in the RLC of intact myosin and of the isolated RLC. In both cases, incubation with DTNB in moderate concentrations (10–200 μM) induces the faster migrating RLC′ in SDS–PAGE. At higher DTNB concentrations (above 1 mM), this RLC′ species reverts to the original migration position completely in myosin and only partially in the isolated RLC. Only the RLC in its original electrophoretic position after incubation with low DTNB concentrations still has two free thiols which may become blocked by [^{14}C]NES. Free thiols are neither available in the RLC′ species nor in the protein at the original RLC position after incubation with high DTNB concentrations.

The two thiols of the protein migrating in SDS–PAGE at the position of the native RLC must have been blocked by TNB groups. These two TNB groups are negatively charged. Charge differences, however, do not affect electrophoretic mobility in SDS–PAGE (Dunker & Rueckert, 1969).

In the RLC′ species, the two thiols are also blocked. Its electrophoretic mobility in SDS–PAGE corresponds to a position of apparent molecular mass which is 11% lower than that of the native RLC. In our SDS–PAGE system, the native RLC migrates at a molecular mass position of 20 650 which is slightly higher than its chemical molecular mass of 18 824 (Matsuda et al., 1977, 1978). The fact that the modified RLC′ migrates faster, together with the observation that after reduction with DTT its electrophoretic mobility reverts back to that of its native counterpart, indicates that its two thiols cannot be blocked by TNB groups but must have formed an intrachain disulfide bond. The electrophoretic mobility in SDS–PAGE is a function of the unfolded length of the polypeptide chain and therefore proportional to its molecular mass (Dunker & Rueckert, 1969; Reynolds & Tanford 1970; Fish et al., 1970). The intrachain disulfide bond between Cys-127 and Cys-156 restricts the complete unfolding of the RLC′ in SDS by cutting out a chain stretch of 28 amino acid residues from its length. This explains why RLC′ in SDS–PAGE appears at a position of lower apparent molecular mass.

Treatment of DTNB-modified myosin or isolated RLC with an excess of NEM proved to be essential to prevent further disulfide rearrangement reactions upon introduction of SDS (Creighton, 1986). Without NEM treatment, such rearrangement reactions resulted in myosin in the formation of aggregates between different LC and between LC and HC, and in isolated RLC to the formation of dimers and trimers. Nevertheless, even with NEM treatment, interchain disulfide formation must have occurred between the HC since, after incubation with higher DTNB concentrations, the aggregates did no more enter the gels in SDS–PAGE (Figures 1A and 2B). The occurrence of DTNB-induced interchain disulfide bonds between the HC in the myosin rod (Stewart, 1982) and in the head portion (Wells & Yount, 1980; Chaussepied et al., 1986; Huston et al., 1988) has been described previously. The stoichiometric relationship among the LC, including the RLC′ species in myosin, was never affected by incubation with DTNB when followed by NEM treatment. The DTNB-induced formation of disulfide bonds in the HC moiety of myosin was corroborated by measuring the liberated amount of TNB dianions colorimetrically. With incubation of 2 mol of DTNB/mol of myosin, an average of 0.5 disulfide bond was formed under all conditions where no RLC′ occurred (Table II). The additional 0.8 disulfide bond found in the presence of EDTA at 25 °C corresponded to the amount of RLC′ as assessed densitometrically from SDS–PAGE. Under these conditions, around 40% RLC′ occurred, i.e., 0.8 mol of RLC′/mol of myosin. This presents additional evidence that the RLC′ species contains an intrachain disulfide. Further-

more, densitometric evaluation of the RLC′ species in SDS–PAGE allows reliable quantification of intrachain disulfide formation in the RLC in myosin. Thus, DTNB and its type of modification of the thiols in the RLC of intact myosin may serve as a chemical probe to sense changes in the microenvironment of these thiols and, therefore, to report on conformational changes involving the RLC.

Temperature and divalent metal ion binding are both of interest in the present context. Both affect the conformation of the RLC in native myosin as shown herein. Ca^{2+} and Mg^{2+} are effective in concentrations which correspond to their high-affinity binding to the first EF domain in the RLC (Bagshaw & Kendrick-Jones, 1979; Kretsinger, 1980). In isolated RLC, they have no effect on the DTNB-induced RLC′ formation at all. Yet, the isolated RLC is still able to bind Ca^{2+} or Mg^{2+} ions, albeit with affinities that are almost 3 orders of magnitude lower (Alexis & Gratzer, 1978) than in intact myosin (Watterson et al., 1979; Holroyde et al., 1979). The isolated RLC is therefore assumed to adopt a conformation in solution where its two thiols are close together. Cys-127 is the last amino acid residue of the EF domain 3 and Cys-156 the second one in the second α-helical portion of domain 4 (Matsuda et al., 1977). The closeness of the two thiols in the isolated RLC indicates that most of the EF domain 4 running toward the C-terminus cannot lie in linear extension but that the end of domain 3 must line up with the beginning of the second α-helix of domain 4.

In going from the isolated RLC to intact myosin, its conformation seems to remain preserved at least as far as the closeness of its two thiols is concerned. However, lowering the temperature or binding of divalent metal ions prevents intrachain disulfide formation completely as if the two free thiols were further separated from one another. In myosin, unlike in isolated RLC, the binding of divalent metal ions to the EF domain 1, near the N-terminus, induces a conformational change in the C-terminal portion of the RLC affecting the reactivity of its two thiols. It has to be postulated that this flux of information requires the interaction of the RLC with the HC. Further evidence for mutual interaction between HC and RLC is based on limited proteolysis as conformational probing. The binding of divalent metal ions to the RLC in myosin is known to protect the head–rod junction from proteolytic attack (Weeds & Pope, 1977; Yamamoto & Sekine, 1980; Miller & Reisler, 1985). Also, changing temperature affects the proteolytic susceptibility of the myosin HC in that region (Redowicz & Strzelecka-Golaszewska, 1988) where the RLC is thought to be located (Waller & Lowey, 1985; Winkelmann & Lowey, 1986; Katoh & Lowey, 1987). Work is in progress to follow conformational changes in the RLC of actomyosin systems and in isolated myosin subfragment 1 retaining its RLC.

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Interaction between Stretch of Residues 633-642 (Actin Binding Site) and Nucleotide Binding Site on Skeletal Myosin Subfragment 1 Heavy Chain†

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ABSTRACT: Using a complementary sequence or antipeptide to selectively neutralize the stretch of residues 633-642 of skeletal myosin heavy chain, we recently demonstrated that this segment is an actin binding site operating in the absence as in the presence of nucleotide and that this stretch 633-642 is not part of the nucleotide binding site [Chaussepied & Morales (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7471-7475]. In the present study, we determined that the covalent cross-linking of the antipeptide to the stretch 633-642 [induced by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide] does not alter the overall polypeptide conformation since no changes were observed on the far-ultraviolet CD spectra and thiol reactivity measurements. The presence of the antipeptide did not influence significantly the enhancement of tryptophan fluorescence induced by ATP·Mg^{2+} or ADP·Mg^{2+} binding to the myosin head (S1) nor did it on the ATP·Mg^{2+}-induced tryptic proteolysis of S1 heavy chain. Moreover, fluorescence quenching studies, using acrylamide and the analogue, 1,N^6-ethenoadenosine 5′-triphosphate, indicated that the nucleotide bound to antipeptide–S1 complex has an accessibility to the solute quencher close to that observed when it is bound to native S1. Additionally, neutralization of the stretch 633-642 of the S1 heavy chain by the antipeptide did not influence the stabilization of the Mg^{2+}·ADP·sodium vanadate–S1 complex. On the other hand, experiments using antipeptide-induced protection against the cleavage of the S1 heavy chain by Arg-C protease demonstrated that the presence of Mg^{2+}·ADP·sodium vanadate in the S1 nucleotide site did not affect the interaction of the antipeptide with the stretch of residues 633-642. Together, these results show that the occupancy of the stretch of residues 633-642 by the antipeptide does not affect the overall structure or the ATP binding and hydrolysis properties of skeletal myosin head and that the reactivity of the stretch 633-642 is not significantly dependent on the presence of nucleotide in the active site. Because the stretch 633-642 seems available to actin interaction in the presence of nucleotide and because blocking it strongly decreases actin interaction with S1–nucleotide complex, it is proposed that the stretch of residues 633-642 represents an essential constituent of the so-called "weak" actin–S1 interface.

Muscle contraction, as well as cell movement processes based on the actomyosin system, take place through a cyclic interaction of actin and ATP with the myosin globular head (S1)[1] (Huxley, 1963). Although actin and ATP binding sites are structurally separated in the myosin molecule (Barany & Barany, 1959), these two ligand binding sites are known to be strongly interdependent. Thus, the binding of the polyphosphate moiety of ATP to S1 weakens the interaction between S1 and actin (Szent Gyorgyi, 1947), and the binding of actin to S1 alters the interaction between S1 and nucleotide, resulting in an activation of the S1 ATPase activity (Eisenberg & Moos, 1968; Highsmith, 1976).

Together, these experimental data led Morales and Botts (1979) to postulate that energy transduction in the myosin molecule is based on structural communication between the actin and the ATP binding sites. This hypothesis has recently been strengthened by kinetic evidence in which the ATP binding and hydrolysis described as a several-step process (Lymn & Taylor, 1971; Trentham et al, 1976) are associated with a two-step actin binding process (Eisenberg & Hill, 1985; Geeves et al., 1984; Coates et al., 1985). In the first step, actin is bound weakly to S1 (possibly bound to ATP·S1 or ADP·P_i·S1 intermediates), and during the second step, the acto–S1 complex isomerizes to reach finally the "rigor" acto–S1 complex (containing either bound ADP or no bound nucleotide).

Unfortunately, none of these observations have yet been interpreted in terms of structure, since neither actin nor ATP binding sites are definitively located in the myosin head. With the exception that the N-terminal part of the alkali light chain A1 can, under certain conditions, interact with actin (Henry et al., 1985), both binding sites have been located on the S1 heavy chain (Wagner & Giniger, 1981; Sivaranakrishnan &

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[1] Abbreviations: S1, subfragment 1; S1(A2), isoenzyme of S1 with alkali light chain 2; Vi, sodium vanadate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DTE, dithioerythritol; ε-ATP, 1,N^6-ethenoadenosine 5′-triphosphate; $NaDodSO_4$, sodium dodecyl sulfate; M_r, molecular weight; kDa, kilodalton; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid.

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