Localization of a new proteolytic site accessible in oxidized myosin rod

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We have compared the proteolysis pattern of reduced and oxidized myosin rods in which the five pairs of SH-groups were interchain crosslinked by employing $CuCl_2$ or 5,5′-dithiobis-2-nitrobenzoate. In the tryptic digest of oxidized rod three new fragments appeared on SDS–polyacrylamide gel electrophoresis (chain masses of 100, 45, and 25 kDa). Based on the N-terminal sequences of the isolated peptides, it is concluded that oxidation creates a new cleavage site 102 residues away from the N-terminus of the rod, in the vicinity of one of the modified SH-groups (Cys-108). This observation indicates that S-S crosslinking of myosin rod leads to a local unfolding of the coiled-coil structure.

Myosin rod *Coiled-coil structure* *Disulfide crosslinking* *Limited proteolysis*

1. INTRODUCTION

The myosin molecule consists of two globular heads, which have ATPase activity and interact with actin, and of a rod-like coiled-coil α-helical tail, which forms the backbone of thick filaments. The complete rod can be separated from heads by limited proteolysis. Prolonged tryptic digestion of rod produces well-defined S2 and LMM peptides, the positions of which have been established within the rod structure [1,2]. S2 and LMM peptides are derived from the segment immediately adjacent to the heads and from the distal segment, respectively.

The two chains of the rod are in register and it has been demonstrated that disulfide bonds can be formed between the chains [3,4]. Previously, we reported that aging of HMM, likely accompanied by oxidation of SH-groups, changes the tryptic susceptibility of the α-helical portion of HMM [5]. Reisler et al. [6] showed that in oxidized myosin rod filaments a new proteolytic site is 'expressed' probably in the N-terminal portion of these particles. More recently, Lu and Lehrer [7] found that reaction of the coiled-coil S2 with Nbs_2 changes the conformation, resulting in a new pattern of tryptic cleavage. In the case of α-tropomyosin, another coiled-coil molecule, disulfide formation at the unique Cys also increases local unfolding of the helix accompanying changes in tryptic cleavage sites [8]. We report here that S-S crosslinks lead to a local unfolding of the coiled-coil rod about 100 residues away from the head/rod 'swivel'.

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Abbreviations: HMM, heavy meromyosin; LMM, light meromyosin; S2, myosin subfragment-2; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; Nbs_2, 5,5′-dithiobis-2-nitrobenzoate

2. MATERIALS AND METHODS

Myosin was prepared from rabbit skeletal muscles as described previously [5]. Myosin rod was obtained by digestion with chymotrypsin as in [2]. The new S2 peptides, S2-A_x and S2-C_x were prepared by digesting oxidized rod with a trypsin to substrate ratio of 1:120, in a solution containing 30 mM KCl, 20 mM Tris–HCl (pH 8.0), and 1 mM EDTA, for 1.5 min (for preparation of

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S2-A_x) or 65 min (S2-C_x) at 25°C. S2-A_x and S2-C_x were separated from other fragments by gel filtration on Sephadex G-200 (4 × 200 cm) in 0.2 M KCl, 10 mM Na-phosphate (pH 7.0), 1 mM EDTA, and 5 mM 2-mercaptoethanol at 25°C.

Myosin rod was oxidized in its monomeric form, at 3–5 mg/ml in 0.5 M KCl and 10 mM Tris–HCl (pH 8.0) by incubation with either about 5-fold molar excess of $CuCl_2$ for 1 h or 5 mM Nbs_2 for 15 min. The excess reagents were removed by extensive dialysis.

Edman degradation was carried out according to [9]. Dansylated amino acid residues were identified by TLC on polyamide sheets. Solvent systems (v/v) were: (i) 1.5% formic acid, (ii) benzene/acetic acid (9:1), (iii) ethyl acetate/methanol/acetic acid (20:1:1). All samples of the unknown (0.5–1.0 nmol) were chromatographed in quintuplicate.

SDS–PAGE was carried out essentially according to [10] using 12% acrylamide in 150 × 150 × 2 mm slabs [3].

3. RESULTS AND DISCUSSION

Myosin rod was treated with either Nbs_2 or $CuCl_2$ to generate interchain disulfide crosslinks [3,4]. The formation of disulfide bonds between

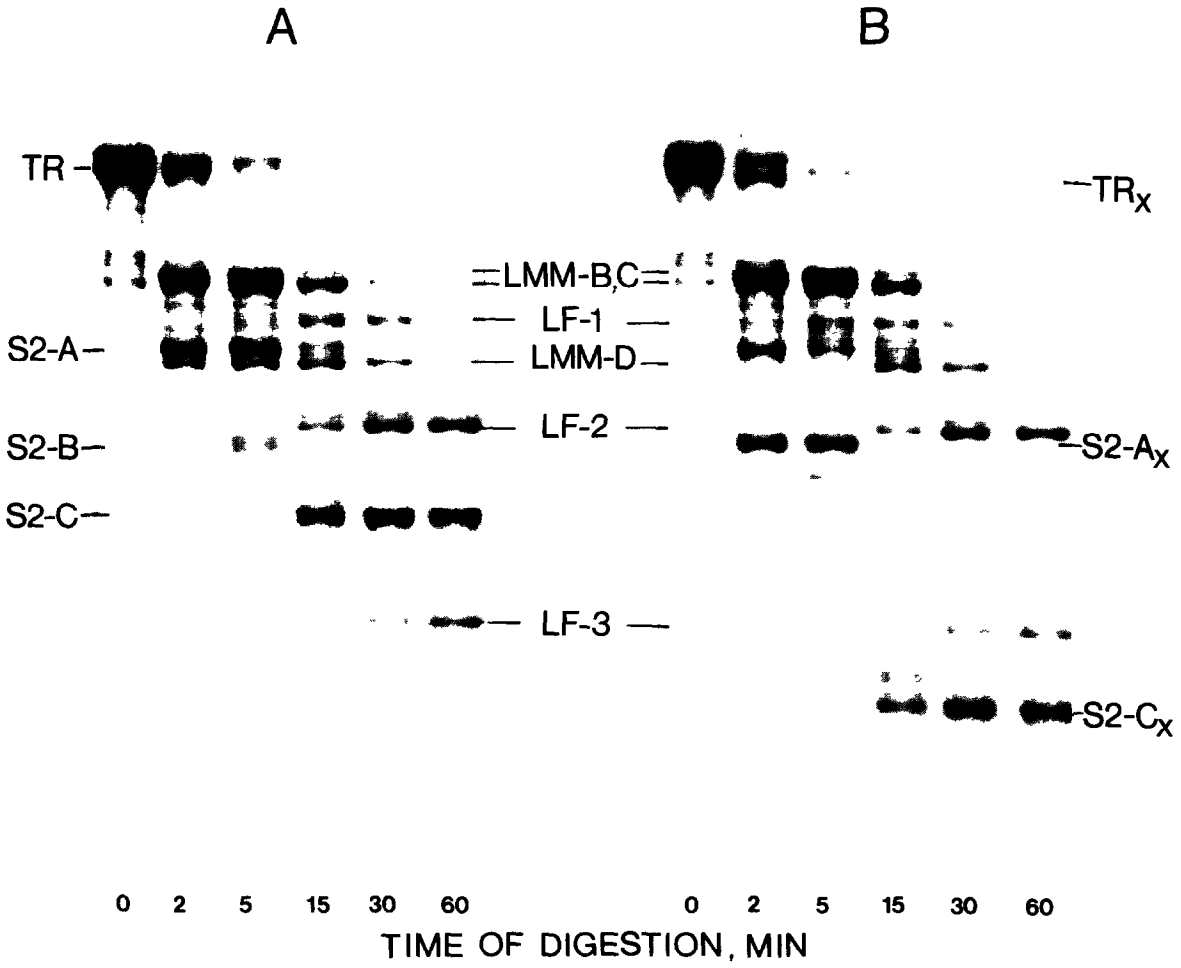

Fig.1. Time course of the tryptic digestion of reduced (A) and oxidized (B) myosin rod. Rods were digested with a trypsin to substrate ratio of 1:120, in 0.5 M KCl, 20 mM Tris–HCl (pH 8.0), 1 mM EDTA. The reduced rod sample contained 5 mM DTT. Aliquots were taken at given times and 25 μg of protein were placed on 12% gel.

chains was essentially quantitative in both cases as judged by Ellman titration of free SS-groups after modification. The $CuCl_2$-oxidized and Nbs_2-oxidized rod contained less than 0.2 and between 0.2 and 0.7 free SH/chain, respectively. There are five SH/chain in reduced rod, three in S2-A and two in LMM.

Reduced and oxidized rods were digested with trypsin at high ionic strength and analyzed on SDS–PAGE (fig.1). The well defined S2 (S2-A, -B, -C) and LMM peptides were formed from reduced rod [3]. S2-A (chain mass of 58 kDa) can be identified as long S2 [11,12], while S2-C (37 kDa) as short S2 [13]. In the digest of oxidized rod, on the other hand, there was no S2-C but three new major bands (100, 45 and 25 kDa) appeared. The 100 kDa band (denoted as TR_x) is a truncated shorter rod, while the 45 kDa ($S2\text{-}A_x$) and 25 kDa ($S2\text{-}C_x$) peptides must be derived from the S2 segment of the rod, because each LMM peptide (LMM-B, -C, -D and LF-1, -2, -3) in the digest of reduced and oxidized rods was identical. These observations suggest that a peptide should be removed from the N-terminal region of oxidized rod when TR_x is formed. If one takes into account the temporal changes in the intensities of the bands in the digest of oxidized rod (fig.1) it appears that $S2\text{-}A_x$ is the precursor of $S2\text{-}C_x$. When reduced or oxidized $S2\text{-}A_x$, isolated from oxidized rod, was digested with trypsin the only fragment which appeared was $S2\text{-}C_x$. On the other hand, prolonged tryptic treatment of oxidized S2-C resulted in complete conversion to $S2\text{-}C_x$, whereas reduced S2-C was only partially degraded (not shown).

To obtain direct information on the localization of the new cleavage site(s) accessible in oxidized rod, $S2\text{-}A_x$ and $S2\text{-}C_x$ were isolated (see section 2) and partial sequence analysis was carried out. The same N-terminal sequence, viz., Lys-Leu-Glu-Asp-Glu... was obtained for both $S2\text{-}A_x$ and $S2\text{-}C_x$. Based on the published amino acid sequence of S2-C [14], the common N-terminus of these two fragments is placed at residue 103. Thus, the new trypsin cleavage site is located in the vicinity of one of the crosslinked SH-groups (Cys-108), indicating that oxidation leads to a local interruption of the stable coiled-coil structure. At present we cannot exclude the possibility that the conformational characteristics of the rod near the other four S-S-crosslinks also change, but if it is so, its effect on the proteolysis pattern examined by SDS–PAGE should be very limited. Fig.2 represents the locations of various S2 peptides, derived from reduced and oxidized rods, along the coiled-coil structure. The fact that S2-C and $S2\text{-}C_x$ differ about 12 kDa in their chain masses (corresponding to about 100 residues) suggests that only a few residues, possibly none, were removed from the C-terminus of S2-C. The same applies to the relationship between S2-A and $S2\text{-}A_x$.

$S2\text{-}A_x$ probably corresponds to the truncated S2 (46 kDa) found by Reisler et al. [6] in the digest of oxidized myosin rod filaments. They assigned the new cleavage site to the N-terminal portion of the rod, in agreement with our results. However, $S2\text{-}A_x$ must differ from S2-B, an intermediate during the course of tryptic degradation of reduced rod, for at least two reasons: (i) S2-B has a slightly higher mobility on SDS–PAGE than $S2\text{-}A_x$ (44 and 45 kDa); (ii) if S2-B was a product of cleavage at the N-terminal region, as in the case of $S2\text{-}A_x$,

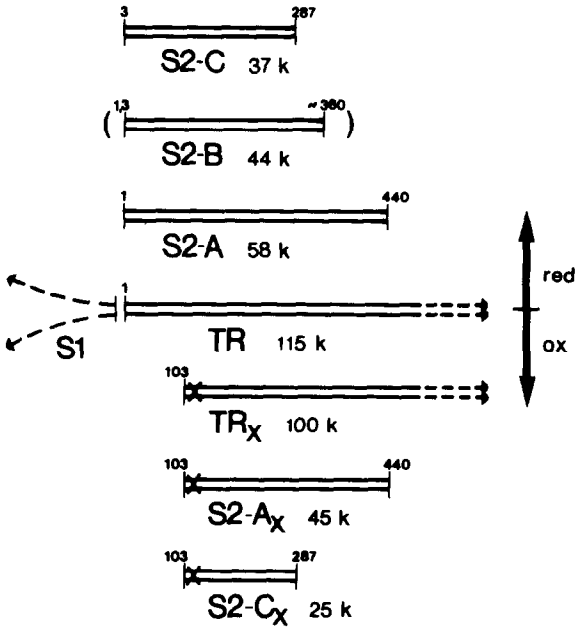

Fig.2. Locations of various kinds of S2 peptides along the rod portion of myosin heavy chain. The numbers indicate N- and C-termini within the rod sequence (taken from [1,7,14]). The lengths of fragments are proportional to their apparent chain masses estimated from SDS–PAGE. X represents the S-S crosslinked Cys-108. The location of S2-B is only tentative.

the gel pattern of reduced rod should contain a short rod as well. S2-C_x may be identical with what Bálint et al. [5,15] referred to as HMM-S3, a smaller helical fragment derived from tryptic digests of either an oxidized S2-C or an aged HMM preparation. Recently, Lu and Lehrer showed that interchain disulfide crosslinks in Nbs_2-modified S2-A can create two new trypsin cleavage sites, at residues 102 and near 360. One of the new peptides (T_2; residues 103–287) corresponds to S2-C_x, while the other (T_1; residues 3–~360), based on its similar chain mass and location within the rod structure, may be very similar or identical to S2-B which, however, appears only in the digest of reduced rod. The apparent differences in the tryptic cleavage patterns of oxidized rod and S2-A could be explained by postulating that the extra coiled-coil polypeptide in the rod (viz., LMM) affects the conformation and consequently the proteolytic sensitivity of the N-terminal S2 segment of the molecule. These changes in the conformation could be connected with accumulation of stress in a long coiled-coil structure [16].

At present nothing is known about the oxidation state of sulfhydryls in myosin rod in vivo. Nevertheless, if thiol-disulfide exchange does exist it can change the conformation of the coiled-coil structure and may play an important role in regulation of force generation.

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