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MgADP-Induced Changes in the Structure of Myosin S1 near the ATPase-Related Thiol SH1 Probed by Cross-Linking†

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ABSTRACT: The structural consequences of MgADP binding at the vicinity of the ATPase-related thiol SH1 (Cys-707) have been examined by subjecting myosin subfragment 1, premodified at SH2 (Cys-697) with *N*-ethylmaleimide (NEM), to reaction with the bifunctional reagent *p*-phenylenedimaleimide (pPDM) in the presence and absence of MgADP. By monitoring the changes in the Ca^{2+}-ATPase activity as a function of reaction time, it appears that the reagent rapidly modifies SH1 irrespective of whether MgADP is present or not. In the absence of nucleotide, only extremely low levels of cross-linking to the 50-kDa middle segment of S1 can be detected, while in the presence of MgADP substantial cross-linking to this segment is observed. A similar cross-link is also formed if MgADP is added subsequent to the reaction of the SH2-NEM-premodified S1 with pPDM in the absence of nucleotide. Isolation of the labeled tryptic peptide from the cross-linked adduct formed with [^{14}C]pPDM, and subsequent partial sequence analyses, indicates that the cross-link is made from SH1 to Cys-522. Moreover, it appears that this cross-link results in the trapping of MgADP in this S1 species. These data suggest that the binding of MgADP results in a change in the structure of S1 in the vicinity of the SH1 thiol relative to the 50-kDa "domain" which enables Cys-522 to adopt the appropriate configuration to enable it to be cross-linked to SH1 by pPDM.

The central problem in the mechanism of muscle contraction at the molecular level is the nature of the force-generating structural change occurring in the myosin subfragment 1 (S1)[1] crossbridge while it is hydrolyzing MgATP and interacting with actin. Although the kinetics of the ATPase cycle in the

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[1] Abbreviations: S1, myosin subfragment 1; pPDM, *p*-phenylenedimaleimide; NEM, *N*-ethylmaleimide; HPLC, high-performance liquid chromatography; SH2-NEM-S1, S1 premodified at the SH2 thiol with *N*-ethylmaleimide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, dinitrophenyl; TFA, trifluoroacetic acid; DTT, dithiothreitol.

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absence and presence of actin have been well characterized (Bagshaw et al., 1974; Sleep & Taylor, 1976; Stein et al., 1979) and although it is known that the conformation of S1 undergoes sequential changes while the nucleotide is processed at the binding site (Morita, 1967; Seidel & Gergely, 1971; Werber et al., 1972; Murphy, 1974), the nature of the accompanying structural changes responsible for force generation is still not well-defined. On the basis of in vitro motility (Toyoshima et al., 1987) and force assays (Kishino & Yanagida, 1988), it is now evident that, together with actin, only the S1 region of myosin is necessary to generate ATP-dependent force or movement and, therefore, it would appear that during the ATPase cycle, when S1 attaches to actin, a movement corresponding to an axial displacement of actin of about 4 nm must occur (Huxley & Kress, 1985). However, to date, no significant change in the shape of S1 has been detected while it interacts with actin or binds Mg-nucleotide (Mendelson et al., 1975; Mendelson & Kretzschmar, 1980; Curmi et al., 1988), but it should be noted that the techniques used are not highly sensitive and would not be able to detect a change of <20% in the structure of the protein. Therefore, the question of the structural basis of contraction remains unanswered, and progress in resolving it must await more detailed information about the folded structure of S1 and how this changes when it binds and hydrolyzes MgATP.

In recent years, significant progress has been made in characterizing the substructure of S1, and this has led to the view that the catalytic heavy chain of S1 is comprised of distinct structural domains on the basis of evidence from limited proteolysis of S1 [reviewed recently by Audemard et al. (1988) and by Mornet et al. (1989) and references cited therein]. On digestion with trypsin, the heavy chain is cleaved at two narrow stretches located at about 27 and 75 kDa from the N-terminus to yield a tryptic S1 comprised of the light-chain subunit and three, interacting protease-resistant heavy-chain fragments of approximately 27, 50, and 21 kDa,[2] arranged in this order from the N-terminus (Balint et al., 1975; Lu et al., 1978). Since a similar cleavage pattern is observed with a number of other proteases (Applegate & Reisler, 1983; Mornet et al., 1984), it is thought that the three heavy-chain fragments are distinct structural domains. If this viewpoint is valid, then it leads to the possibility that force generation may involve relative displacements between these interacting heavy-chain "domains" when S1 binds and hydrolyzes MgATP as discussed by several workers (Hiratsuka, 1986; Botts et al., 1984, 1989).

One region in S1, which is known to be affected by the binding of nucleotide and actin, resides in the 21-kDa C-terminal segment of the heavy chain and encompasses Cys-697 (SH2) and Cys-707 (SH1). The reactivities of these thiols toward N-substituted maleimidyl derivatives is markedly dependent on the state of the bound nucleotide (Yamaguchi & Sekine, 1966; Reisler et al., 1974; Schaub et al., 1975). In a reciprocal fashion, modification at either one of these two thiols results in marked changes in the ATPase properties of the protein (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966), while modification at both results in the abolition of this function (Yamaguchi & Sekine, 1966). In addition to their effects on the ATPase properties, these two thiols are relatively close in the folded structure of S1, and in the presence of MgADP, they can be cross-linked to one another by bifunctional reagents of cross-linking spans ranging from 0.2 to 1.7 nm (Burke & Reisler, 1977; Wells & Yount, 1980; Rajasekharan et al., 1987; Huston et al., 1988). Futhermore, cross-linking of these two thiols causes a huge decrease in the off rate of the bound MgADP, thereby effectively trapping the nucleotide in the protein (Wells & Yount, 1979). Both cross-linking (Burke & Reisler, 1977; Wells et al., 1980) and fluorescence energy transfer analyses (Dalbey et al., 1983; Cheung et al., 1985) indicate that on binding MgADP they move closer to one another.

The manner in which these two thiols affect the binding site is not well understood. Fluorescence energy transfer and EPR data indicate that SH1 and SH2 are well separated from bound nucleotide [see dos Remedios et al. (1987) and references cited therein; Bagshaw & Reed, 1976], but these estimates represent time-averaged values and do not preclude closer approaches (Cheung et al., 1985; Aguirre et al., 1989). Sutoh and Hiratsuka (1988) have recently shown by a cross-linking approach that when the ATP site is unoccupied a thiol, presumably SH2, is quite close (<0.45 nm) from Lys-184 or Lys-189 at the nucleotide binding consensus sequence A of Walker et al. (1982). Occupancy of this site was found to prevent cross-linking to SH2 (Hiratsuka, 1987), suggesting that SH2 is displaced from sequence A in the 27-kDa N-terminal segment by the binding of nucleotide. Moreover, recent photo-cross-linking studies indicate that SH1 is also relatively close to residues in the 27-kDa N-terminal segment (Lu et al., 1986; Rajasekharan et al., 1989). These observations suggest that these two thiols may have a more direct influence on the ATPase function than previously suggested.

In the present study, we have sought to obtain additional information about the structural changes occurring in the vicinity of SH1 when S1 binds MgADP. This has been done by chemical probing to determine whether cross-linking from SH1 can be achieved with pPDM in S1 where the SH2 thiol has been preblocked with NEM. The present results indicate that, while SH1 can be readily modified with pPDM in the presence or absence of MgADP, cross-linking to the 50-kDa middle segment of S1 occurs essentially only in the presence of the ligand. Isolation of the labeled tryptic peptide from tryptic SH2–NEM–S1 reacted with [^{14}C]pPDM and subsequent sequence analysis suggest that the site of cross-linking to the 50-kDa segment is Cys-522. In addition, it was found that the formation of the cross-link results in the trapping of the bound MgADP in the protein.

Materials and Methods

Distilled water further purified through a Millipore QTM system was used throughout. pPDM was purchased from Aldrich. TLCK-treated trypsin, soybean trypsin inhibitor, ATP, and ADP were from Sigma. [^{14}C]pPDM (specific activity 2.44×10^{13} cpm/mol) was prepared as described previously (Burke & Knight, 1980) using [^{14}C]maleic anhydride purchased from Amersham. [^{14}C]ADP was from New England Nuclear. All other reagents were of reagent grade.

Protein Preparation and Modification. The preparations of myosin and S1 and the separation of the S1 isozymes were done as described by Godfrey and Harrington (1970) and Weeds and Taylor (1975), respectively. Protein concentrations were determined by the absorption at 280 nm employing $E^{1\%}$ values of 5.5 and 7.5 for myosin and S1, respectively, or by the Bradford method (1976) using myosin or S1 as standards.

The preparation of SH2–NEM–S1 involved reversible modification at SH1 with FDNB as described by Bailin and Barany (1972) and subsequent modification of SH2 by reaction at pH 7.8 with NEM (1.3 molar excess) in the presence of 1.0 mM $MgCl_2$ and ADP as described previously (Raja-

[2] The weights given for the heavy-chain fragments are the apparent weights based on electrophoretic mobility on SDS–PAGE.

sekharan et al., 1989). Thiolysis of the DNP group from SH1 was done by an overnight incubation at 4 °C in the presence of 0.05 M dithiothreitol. The dithiothreitol was subsequently removed by centrifuging the modified protein twice through Sephadex G-50 as described by Penefsky (1977) prior to modification by pPDM. Tryptic SH2–NEM–S1 was prepared by digesting SH2–NEM–S1 (5.0 mg/mL) with trypsin at a S1:trypsin ratio of 100:1 in 0.05 M Hepes, pH 7.8, for 35 min at 25 °C.

Modification of SH2–NEM–S1 and tryptic SH2–NEM–S1, each at 2.0 mg/mL, by pPDM was done in 0.05 M Hepes, pH 7.8 at 4 °C, using a 1.3 molar excess of pPDM in the presence and absence of 1.0 mM $MgCl_2$ and ADP. At required times, 100 μL of the reaction mixture was removed and made 2.0 mM with respect to 2-mercaptoethanol to terminate the reaction. Aliquots were removed and diluted to 0.2 mg/mL for ATPase measurements by the procedure of Kielley and Bradley (1956). The remainder of the protein was then subjected either to SDS–PAGE by the procedure of Laemmli (1970) or to reverse-phase HPLC on a "new" C-1 column (Synchropack) (see below) using aqueous acetonitrile gradients in 0.1% TFA as described recently (Rajasekharan et al., 1989).

Determination of nucleotide trapping was done by using [^{14}C]ADP (specific activity 2.4 × 10^{13}/mol) during the reaction with pPDM. The reacted protein was precipitated with saturated ammonium sulfate to 66% saturation and collected by centrifugation. The pellet was redissolved in 0.05 M Hepes, pH 7.8 at 4 °C, and subsequently centrifuged twice through Sephadex G-50 (Penefsky, 1977) equilibrated in the same buffer to remove residual untrapped MgADP. Aliquots of these samples were then assayed for radioactivity and for total protein concentration, allowing for the estimation of the amount of nucleotide trapped in the total S1 which includes un-cross-linked and cross-linked SH2–NEM–S1. The amount of cross-linked SH2–NEM–S1 was obtained from densitometric analyses at 550 nm of SDS–PAGE of these samples using a Shimadzu CS-930 TLC scanner with DR-2 data recorder, assuming the same color yields for the cross-linked species and the 95-kDa heavy-chain band. This enabled the fraction of cross-linked SH2–NEM–S1 present in the reacted sample to be evaluated and the amount of [^{14}C]ADP corresponding to the amount of cross-linked S1 to be determined.

Isolation and Sequencing of Labeled, Cross-Linked Tryptic Peptides from Tryptic SH2–NEM–S1 Reacted with [^{14}C]-pPDM in the Presence of MgADP. This was achieved by first collecting the cross-linked 21–50-kDa adduct by reverse-phase HPLC on a "new" C-1 column (Synchropac) as described previously in detail (Rajasekharan et al., 1987, 1989) and then lyophilizing the sample. In the previous work, we employed a C-4 column (Synchropac), but new C-4 columns purchased since that time showed elution behavior corresponding to older C-8 columns. However, newer C-1 columns purchased from Synchropac behaved, with slight modifications to the gradient, like the older C-4 columns previously used by us and were used to separate the light-chain and the tryptic heavy-chain fragments. The cross-linked 21–50-kDa adduct (about 192 μg) was dissolved in 75 μL of 8.0 M urea in 0.05 M ammonium bicarbonate and then diluted to 300 μL with 0.05 M ammonium bicarbonate to 2.0 M urea and digested with 4 μg of trypsin (1.0 mg/mL) at 37 °C for 2 h with occasional shaking. The digestion mixture was then diluted with an additional 300 μL of 0.05 M ammonium bicarbonate and two more additions of trypsin corresponding to 3 μg at 2-h intervals, and the digestion was continued at 37 °C for a total of 8 h. The digestion was terminated by 5-fold dilution of the sample with 0.1% TFA. The tryptic digest was then applied to a new C-4 column (Synchropac) and subjected to elution using water (solvent A) and acetonitrile (solvent B), both in 0.1% TFA. The elution protocol in terms of solvent B was as follows: 0–10 min, 0%; 10–110 min, a linear gradient from 0 to 50%; 110–115 min, linear increase to 60%; 115–135 min, linear increase to 70%. Fractions were collected every 1.2 min using a flow rate of 1.0 mL/min; 100-μL aliquots were removed for scintillation counting. Fractions corresponding to the highest radioactivity were lyophilized and subjected to further fractionation on the new C-4 column using shallower gradients. The purified fractions with the highest radioactivity were then lyophilized and subsequently analyzed on an Applied Biosystems 477A pulse gas-phase protein sequenator with on-line 120A PTH-amino acid analyzer using approximately 100 pmol of peptide.

Gel Electrophoresis. This was done by the procedure of Laemmli (1970) using 12.5% acrylamide gels in the presence of 0.1% SDS and 0.1% 2-mercaptoethanol. The gels were stained with Coomassie Brilliant blue.

Results

Modification of SH2–NEM–S1 with pPDM in the Presence or Absence of MgADP. Previous studies have established the pattern of ATPase activity changes which occur when S1 is modified with N-substituted maleimidyl derivatives at the SH1 and SH2 thiols (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966; Reisler et al., 1974). To form SH2–NEM–S1, we used the two-step procedure in which the SH1 thiol is first modified reversibly with FDNB (Bailin & Barany, 1972) and the SH2 is then subsequently blocked with NEM in the presence of MgADP (Yamaguchi & Sekine, 1966). The changes in the Ca^{2+}- and EDTA-ATPase activities occurring during each step were found to be in good agreement with previous data on these sequential modifications. Modification with FDNB resulted in an activation of the Ca^{2+}-ATPase from 7.7 to about 22 s^{-1} and a decrease in the EDTA/K^+-ATPase from 19 to 3 s^{-1} in agreement with previous reports (Bailin & Barany, 1972; Reisler et al., 1974) and in accord with labeling at the SH1 thiol. Subsequent modification of this S1 with NEM in the presence of MgADP resulted in a fall of the Ca^{2+}-ATPase to 2.8 s^{-1} and a slight decrease in the EDTA/K^+-ATPase to about 0.8 s^{-1}, consistent with labeling at the SH2 thiol (Yamaguchi & Sekine, 1966; Reisler et al., 1974; Rajasekharan et al., 1989). Upon treatment with DTT to remove the DNP group from SH1, the Ca^{2+}-ATPase increased to 10.3 s^{-1} while the EDTA/K^+-ATPase remained at 0.8 s^{-1}, consistent with regeneration of the SH1 thiol (Bailin & Barany, 1972; Reisler et al., 1974).

Modification of SH2–NEM–S1 with a 1.3 molar excess of pPDM resulted in a very rapid inactivation of the Ca^{2+}-ATPase whether or not MgADP was present as shown in Figure 1. Since SH1 is very reactive with *N*-maleimidyl derivatives whether or not nucleotide is present, and as its modification in SH2–NEM–S1 would be expected to abolish the Ca^{2+}-ATPase, the data suggest that SH1 is modified rapidly and independently of the presence of Mg-nucleotide. Consistent with this view were the observations that reverse-phase HPLC on C-1 column of the protein, modified with [^{14}C]pPDM, after tryptic digestion resulted in radioactivity only in the 21-kDa heavy-chain fragment and light chain, consistent with modification at SH1 and some labeling of the single thiol of the associated light chain (data not shown).

Formation of Cross-Links from SH1 by pPDM Modification of SH2–NEM–S1. To examine whether cross-links were formed during the reaction of pPDM with SH2-NEM–S1, the

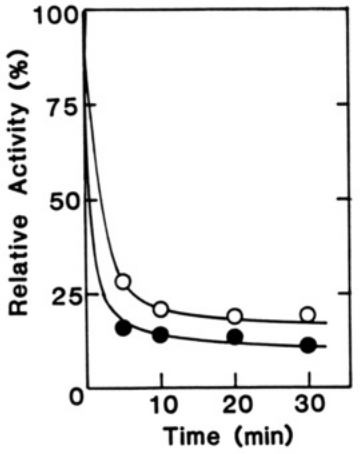

FIGURE 1: Changes in the Ca^{2+}-ATPase activity of SH2–NEM–S1 reacted with a 1.3 molar excess of pPDM at 4 °C in the absence (●) and presence (○) of MgADP.

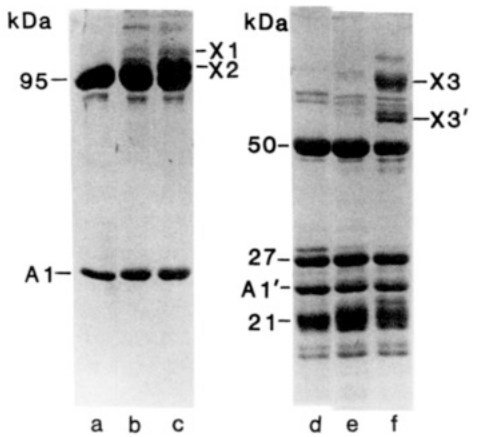

FIGURE 2: SDS–polyacrylamide gel electrophoretograms of SH2–NEM–S1 modified with pPDM at 4 °C for 60 min in the presence and absence of MgADP, Lane a, unmodified SH2–NEM–S1; lane b, modified in the absence of MgADP; lane c, modified in the presence of MgADP. Lanes d–f correspond to lanes a–c, respectively, after tryptic digestion.

peptide composition of the reaction mixture as a function of time was examined by SDS–PAGE as described under Materials and Methods. These results are shown in Figure 2 for modifications done in the presence and absence of MgADP. In the absence of MgADP (Figure 2, lane b), only marginal amounts of cross-linking occurred as evidenced by two very faint bands, designated X1 and X2, of lower mobility than the unmodified heavy chain. The reaction in the presence of MgADP, on the other hand, resulted in an enormous enhancement in the extent of cross-linking as evidenced by the large increase in the amount of X2 (Figure 2, lane c). HPLC gel filtration (TSK-4000 column) under nondenaturing conditions of the sample modified in the presence of MgADP showed no change in the elution volume compared to the unmodified control, thereby indicating that the cross-linking was essentially intramolecular (data not shown). Furthermore, since the amount of alkali light chain remained unchanged, it is possible to rule out that X1 and X2 were generated through cross-linking between SH1 and the single thiol of the light-chain subunit.

Digestion with trypsin of the samples, modified with pPDM in the absence and presence of MgADP, resulted in the electrophoretograms shown in Figure 2, lanes e and f, respectively. For the samples modified in the presence of nucleotide, the digestion resulted in the formation of two major new peptides of M_r about 95 000 (X3) and 63 000 (X3′), and

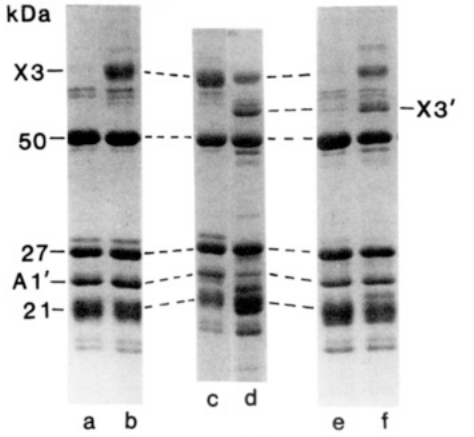

FIGURE 3: SDS–polyacrylamide gel electrophoretograms of tryptic SH2–NEM–S1 modified with pPDM at 4 °C for 60 min in the presence and absence of MgADP. Lanes a and c, modified in the absence of MgADP; lane b, modified in the presence of MgADP; lane d, sample of lane c after digestion with trypsin (protease:protein ratio of 1:10) for 30 min; lanes e and f, SH2–NEM–S1 modified in the absence of MgADP, subsequently digested with trypsin (100:1) for 30 min, and then allowed to incubate for an additional 60 min in the absence and presence of MgADP, respectively.

this was accompanied by a reduction in the amount of 50- and 21-kDa heavy-chain fragments. These observations indicate that the new peptides represent cross-linked species formed through cross-linking from SH1 to the 50-kDa segment. The cross-linked band of M_r 63 000 was shown to be a digestion product of the M_r 95 000 species (see below). Digestion of the sample modified in the absence of MgADP showed almost no trace of these cross-linked peptides.

Modification of tryptic SH2–NEM–S1 with pPDM in the presence and absence of MgADP was also examined by SDS–PAGE as shown in Figure 3. As noted for the undigested S1 species, only modification in the presence of MgADP resulted in significant cross-linking, based on the appearance of a new peptide (X3) with M_r 95 000 (Figure 3, lane b). In a separate experiment, tryptic S1, modified in the presence of MgADP, was redigested with trypsin at a protease to tryptic S1 ratio of 1:10 (Figure 3, lanes c and d), and this led to a reduction of the M_r 95 000 band (X3) and the formation of a new band of M_r 63 000 (X3′). This result confirmed that this latter band, formed on subsequent digestion of native S1 modified in the presence of MgADP (Figure 2, lane f), was a digestion product of the M_r 95 000 species.

In contrast to the results obtained for modification of tryptic SH2–NEM–S1 in the presence of nucleotide, modification in the absence of nucleotide showed little evidence for the formation of cross-links from SH1 in the reaction interval employed in these studies (Figure 3, lane a). However, addition of MgADP, subsequent to modification in its absence, did allow for the formation of cross-links to the 50-kDa segment as shown in Figure 3, lanes e and f. In this experiment, the SH2–NEM–S1 was modified for 30 min at 4 °C with a 1.3 molar excess of pPDM in the absence of MgADP, and the sample was then digested with trypsin for 30 min at 25 °C and terminated by the addition of soybean trypsin inhibitor. The sample was then divided in two parts and incubated for a further 30 min in the absence and presence of MgADP and, subsequently examined by SDS–PAGE as shown in Figure 3, lanes e and f, respectively.

The possibility that nucleotide was trapped by cross-linking from SH1 to the 50-kDa segment was also investigated. The amount of [^{14}C]ADP present in S1, after modification and purification as described under Materials and Methods, was

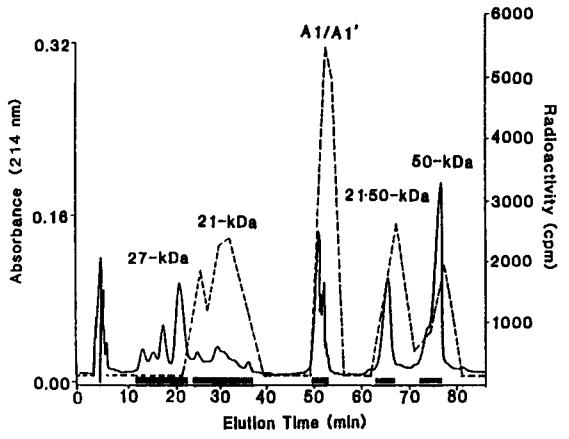

FIGURE 4: HPLC of tryptic SH2–NEM–S1 after 60-min reaction with [^{14}C]pPDM at 4 °C on a "new" C-1 column (Synchropac). See Materials and Methods for details. The solid and dashed plots are respectively the absorbance at 214 nm and the radioactive profile.

obtained from the radioactivity present in a known concentration of the protein. Densitometric analysis of SDS–PAGE electrophoretograms of the modified protein yielded the amount of cross-linked sample present in the modified protein, thereby enabling the amount of trapped nucleotide per mole of cross-linked S1 to be evaluated. These results showed that 0.70 ± 0.10 mol of ADP was trapped per mole of cross-linked S1.

Determination of the Site of Cross-Linking in the 50-kDa Segment from SH1. Since the above experiments indicated that the same cross-linked appeared to be formed from both undigested and tryptic SH2–NEM–S1, the latter was used for identification of the site of cross-linking. The protein modified in the presence of MgADP with [^{14}C]pPDM was subjected to reverse-phase HPLC (on a new C-1 column), and the resulting elution profile is shown in Figure 4. The profile shows a radioactive peak eluting between the position of the alkali light chain and the 50-kDa fragment. This peak was pooled, and after lyophilization, it was digested with trypsin and subjected to reverse-phase HPLC on a new C-4 column, yielding the elution profile shown in Figure 5. The major radioactive peak was pooled, and, after lyophilization, it was subjected to a shallower gradient on the C-4 column (insert of Figure 5) resulting in the radioactivity being present in two poorly resolved peaks which could not be adequately separated. Since our previous experience with N-substituted maleimidyl derivatives has shown that the maleimidyl ring can be opened by hydrolysis, it was assumed that these two peptides represented closed- and opened-ring forms of the pPDM-labeled tryptic peptide and were analyzed together in the automated sequencer through 10 cycles of Edman degradation without release of radioactivity and yielded the following sequence data: Glu(Ile)-Gly-Ile(Arg)-Glu-Trp-Glu-Phe-Ile-Asp-Phe. This sequence is compatible with cross-linking of the tryptic peptide Ile-Cys-Arg, containing SH1 to the tryptic peptide starting at Glu-506 which would contain cys-522.[3] Therefore, it appears that the cross-link formed when MgADP binds to S1 involves Cys-707 (SH1) to Cys-522 of the 50-kDa middle segment of S1.

Discussion

The present work has established that pPDM reacts readily with SH2–NEM–S1 irrespective of whether MgADP is bound to the protein and that, in the presence of this ligand, it forms a cross-link between the 21-kDa and the 50-kDa segments.

[3] Sequence kindly provided by Dr. Marshall Elzinga.

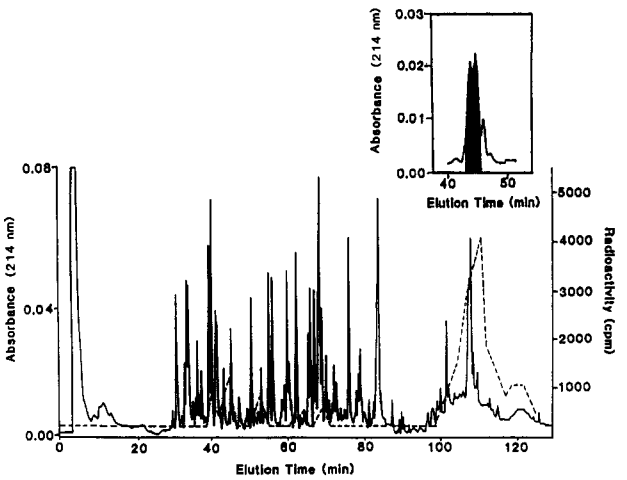

FIGURE 5: HPLC of tryptic digest on a "new" C-4 column (Synchropac) of the [^{14}C]pPDM-labeled (21 + 50)-kDa adduct isolated as shown in Figure 4. The solid and dashed plots represent the absorbance at 214 nm and the radioactive profile, respectively. The insert shows the further purification of the radioactively labeled tryptic peptide on C-4 column using shallower gradients. The blocked fractions were used for sequence analyses.

Thus, in a general manner, these results are in agreement with other recent cross-linking studies which have shown that SH1 is close to the 50-kDa segment in the absence (Muno & Sekine, 1988; Mornet et al., 1985) and presence of Mg-nucleotide (Lu et al., 1986). Furthermore, partial sequence analysis of the major labeled tryptic peptide, isolated from tryptic digestion of the [^{14}C]pPDM-labeled, 21–50-kDa cross-linked adduct, indicates that the cross-link was formed between SH1 and Cys-522 of the 50-kDa segment. The failure of pPDM to readily cross-link from SH1 to the 50-kDa segment in the absence of MgADP cannot be attributed to the inability of pPDM to react with SH1 in the absence of nucleotide, since the changes in the Ca^{2+}-ATPase activity indicate that SH1 is modified in this situation. Moreover, it is possible to rule out cross-linking within the 21-kDa segment for being responsible for this failure, since subsequent addition of MgADP to the protein, modified in the absence of nucleotide and on removal of residual pPDM, resulted in the formation of the cross-link to the 50-kDa segment (Figure 3, lanes e and f).

The data, therefore, indicate that binding of MgADP to S1 is required to position Cys-522 in the appropriate spatial geometry to enable pPDM to cross-link SH1 to it. This conclusion is also supported by results of the distribution of radioactivity among the three heavy-chain fragments and light chain when SH2–NEM–S1 was modified in the presence and absence of MgADP with [^{14}C]pPDM. In the absence of MgADP, labeling was confined solely to the 21-kDa and light chain (data not shown), indicating that Cys-522 in the 50-kDa segment was not reacting under these conditions. On the other hand, as shown in Figure 4, in the presence of MgADP, the radioactivity was also found in the cross-linked 21–50-kDa adduct as well as in the 50-kDa fragment. Thus, these observations indicate that the binding of MgADP perturbs the structure in the 50-kDa segment in such a way that Cys-522 (and perhaps Cys-540; Chaussepied et al., 1986) becomes reactive and that it can now be cross-linked to SH1 by pPDM.

This dependency on nucleotide binding is opposite to what was previously observed by modification of S1 with dibromobimane, where it was shown that cross-linking between SH1 and Cys-522 occurred independently of the presence or absence of Mg-nucleotide (Mornet et al., 1985; Ue, 1987). Thus, while the data with pPDM would suggest that the loop between SH1 and Cys-522 forms as a consequence of the

binding of MgADP, the data previously obtained with dibromobimane modification (Mornet et al., 1985; Ue, 1987) indicated that the loop preexisted prior to nucleotide binding. The two apparently contradicting observations can be reconciled if it is assumed that, in the absence of MgADP, the distance between SH1 and Cys-522 is restricted to the cross-linking range of dibromobimane (0.3–0.6 nm; Mornet et al., 1985) and cannot extend to 1.2 nm, which is the lower extreme of the cross-linking range of pPDM (Burke & Reisler, 1977; Wells et al., 1980). The ability of both cross-linking reagents to cross-link SH1 to Cys-522, when MgADP is bound to the protein, could then reflect an increase in the flexibility within the loop between these two thiols, allowing them to now move in a range where both dibromobimane and pPDM can link them. Consistent with this view are preliminary results with 4,4′-bis(*N*-maleimido)benzophenone (cross-linking span of 1.3–1.76 nm) indicating that cross-linking from SH1 in SH2–NEM–S1 to the 50-kDa segment occurs only in the presence of MgADP (unpublished data). This interpretation would be consistent with the loop preexisting prior to the addition of the Mg-nucleotide (Mornet et al., 1985; Ue, 1987) and with MgADP affecting the flexibility (and movement) within the loop. An increase in the flexibility in regions within this loop on binding MgADP has also been implicated from the ability of a variety of cross-linking agents of different spans to cross-link SH1 to SH2 (Wells et al., 1980; Huston et al., 1988) or for pPDM to link SH2 to Cys-540 (Chaussepied et al., 1986, 1988) only when nucleotide is bound.

The observations that regions within the three tryptic heavy-chain segments are in close proximity and that, in some cases, this proximity is dependent on the binding of nucleotide (Lu et al., 1986; Hiratsuka, 1987; Sutoh & Hiratsuka, 1988; Muno & Sekine, 1988; Rajasekharan et al., 1987) raise the question of how these regions are related to and affected by the binding of nucleotide. Significant progress in this regard has been recently made by Botts et al. (1989), who have defined a three-dimensional structure for the S1 heavy chain, based primarily on distance estimates from previous fluorescence energy-transfer data. This has enabled them to draw inferences on how bound Mg-nucleotide may be situated in this structure and how its binding may affect the structure in the presence and absence of actin. Of particular interest is that their lattice constrains particular sections of the three heavy-chain segments to be proximal to each other: (i) the "glycine-rich" loop from Gly-178 to Ile-192 (27 kDa); (ii) Cys-522 to Cys-540 (50 kDa); and (iii) SH2 (Cys-697) to SH1 (Cys-707) (21 kDa). Moreover, their model indicates that binding of nucleotide would result in a relative movement of the latter two segments [Figure 2B of Botts et al. (1989)], and the present results would not be inconsistent with this interpretation.

Of these three segments, only the "glycine-rich" loop [sequence A of Walker et al. (1982)] has been definitively shown to be at the ATP site (Grammer et al., 1988). In this connection, we have recently indicated that a region, in the 21-kDa segment near SH2 and SH1, bears some similarities with a second consensus sequence [sequence B of Walker et al. (1982)] of some ATP-requiring proteins (Burke et al., 1990). This sequence can be represented by the general pattern $EX_2RX_mGX_n$, followed by a five-residue segment ending with an aspartate, which has been proposed to interact directly or indirectly with the Mg^{2+} of the bound substrate (Walker et al., 1982; Fry et al., 1985, 1986). The five-terminal residue segment possesses a rather well-conserved motif in the F1 ATP synthases and in mammalian cytosolic adenylate kinase in which (i) the first and second residues are generally aliphatic, hydrophobic residues such as Ile or Leu, (ii) the third residue is aromatic and generally Tyr or Phe, (iii) the fourth residue is generally uncharged or, in some cases, is Asp, and (iv) the fifth residue is Asp or Glu. For the F1 ATP synthases and adenylate kinase, *m* is 2 or 3 and *n* is 3 or 4. This pattern, where *m* is 4 in myosin, begins five residues downstream from SH2 (Cys-697) at Glu-702 and terminates at Asp-719. It appears in all of the vertebrate myosins (allowing for conservative substitutions), but the terminal five residues are not fully conserved in some of the invertebrate myosins. A search of the SWISS PROT protein database of this general pattern, EX(2)RX(2,3,4)GX(3,4)(ILVM)X(FY)X(DE) (where the numbers in parentheses indicate the number of variable residues X between the invariant ones, and in which the second and fourth residues of the terminal five were allowed to be completely variable), scored only F1 ATP synthases, mammalian cytosolic adenylate kinases, and myosins, indicating that this sequence is fairly specific for ATP-requiring proteins (Burke et al., 1990).

If this assignment is valid, it would suggest that Cys-522 and Cys-540 in the 50 kDa are also close to this consensus sequence in the 21 kDa when MgADP is bound to the ATP site, in agreement with the structure proposed by Botts et al. (1989). Moreover, there is direct evidence that regions near the SH2 thiol are close to Lys-184 or Lys-189 when the ATP site is unoccupied (Hiratsuka, 1987; Sutoh & Hiratsuka, 1988) and that residues extending from SH2 on its C-terminal side must also be close to Ser-180 [identified as part of the ATP site by Grammer et al. (1988)]. In this regard, it has recently been reported by Maruta et al. (1989) that the ATP analogue 3′-*O*-(*N*-methylanthraniloyl)-8-azidoadenosine 5′-triphosphate photolabels avian skeletal S1 in the 21-kDa segment in a 50-residue peptide containing SH1 and SH2. Moreover, it appears that the bifunctional photoreactive ATP analogue 3′-*O*-(azidonitrobenzoyl)-8-azidoadenosine 5′-triphosphate can covalently link the 27- and 21-kDa segments of rabbit skeletal myosin (Maruta et al., 1990), consistent with regions of these two domains residing next to the bound Mg-nucleotide. Secondary structure predictions (unpublished data) indicate that SH1 is adjacent to a turn and is followed by an extended structure containing the common five-residue terminal segment. This could allow for the chain, proximal and C-terminal to SH1, to bend back, placing the five terminal residues close to the glycine-rich loop in the 27-kDa segment [Figure 2B of Botts et al. (1989)].

This proposal, although admittedly speculative, is consistent with numerous modification studies showing an intimate relationship between these thiols and the ATPase function of the protein. SH1 is assumed not to be part of the ATP site, but to be near a segment (the terminal five-residue segment) which may contribute directly to the binding of the Mg polyphosphate moiety. Thus, modifications at SH1 would not be expected to abolish the Mg^{2+}- or Ca^{2+}-ATPases of S1, but would be expected to alter the activity due to its proximity to the terminal five residues. This is in accord with reports for modifications at SH1, where both activation (Sekine & Kielley, 1965; Duke et al., 1973; Bailin & Barany, 1972) and inactivation (Mornet et al., 1985) have been observed depending on the modifying reagent used. On the other hand, modifications at SH2 result in smaller changes in the Ca^{2+}-ATPase (Seidel, 1969; Reisler et al., 1974; Miyanishi & Borejdo, 1989; Ajtai & Burghardt, 1989).

It is tempting to suggest that formation of cross-links from SH1 or SH2 to one another or to thiols in the 50-kDa segment

in the presence of MgADP "locks" the ATP site by preventing or restricting movement of segments in these consensus sequences associated with nucleotide binding. The likelihood that SH1 is also at an actin binding contact (Suzuki et al., 1987) and the observations that actin binding causes release of trapped nucleotide (Perkins et al., 1981; Goodno, 1982; Greene et al., 1986; Chaussepied et al., 1988) could be explained by the ability of actin to "unlock" the trap by binding near SH1 and perturbing the nearby "five-residue" segment, which we propose is part of the ATP binding site. This would imply that elements associated with nucleotide and actin binding are close to one another in the folded structure, for at least part of the ATPase cycle.

A significant difference between the reaction with pPDM and dibromobimane is that cross-linking of Cys-522 to SH1 by the former in the presence of MgADP leads to the trapping of the nucleotide. The reason for this difference is unclear at present, since it is difficult to understand why cross-linking between the same two sites (albeit at different separations) would lead to trapping in one case and not in the other. However, the data with pPDM extend the number of cross-linking sites which are capable of trapping MgADP in S1, e.g., SH1 to SH2 (Wells & Yount, 1979, 1980) and SH2 to Cys-540 (Chaussepied et al., 1986). It is apparent that these cross-links must result in some form of stabilization of the ATP binding site which markedly lowers the off rate for MgADP.

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Registry No. ATPase, 9000-83-3; MgADP, 7384-99-8; pPDM, 3278-31-7.

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The Rigor Configuration of Smooth Muscle Heavy Meromyosin Trapped by a Zero-Length Cross-Linker†

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ABSTRACT: When chicken gizzard heavy meromyosin (HMM) in its rigor complex with actin was reacted with the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), HMM cross-linked with actin but also the two heads of the HMM molecule cross-linked to each other [Onishi, H., Maita, T., Matsuda, G., & Fujiwara, K. (1989) *Biochemistry 28*, 1898-1904, 1905-1912]. By ultracentrifugal fractionation of the EDC-treated acto–HMM in the presence of Mg-ATP, we obtained a preparation enriched for gizzard HMM with cross-linked heads. When HMM molecules in this preparation were rotary-shadowed and observed in an electron microscope, many head pairs were in contact with each other. The amount of HMM with cross-linked heads determined by electron microscopy was equal to that of the cross-linked NH_2-terminal 24K tryptic fragments of HMM heavy chains determined by NaDodSO$_4$ gel electrophoresis, indicating that this cross-linking is primarily responsible for the contact observed between two HMM heads. Most pairs of the contacted heads originated in the same HMM molecule, although a few pairs belonged to different HMM molecules. Cross-linking between the two heads of the same HMM molecule appeared to occur within the distal, more globular half of each head. However, the cross-linking sites were located at different positions within the globular portion. The actin-activated Mg-ATPase activity of the HMM sample treated with EDC in the presence of actin increased in a biphasic manner, depending on the concentration of F-actin, with two apparent association constants: 2.9×10^4 M^{-1} and one much less than 1×10^4 M^{-1}. Since the apparent association constant obtained with the HMM control was similar to the latter value, the association constant for HMM molecules with cross-linked heads was identified to be the former value. The binding of HMM to actin was thus strengthened at least by a factor of 3 by the cross-linking between two HMM heads. These results suggest that HMM heads are trapped by treatment with EDC in the rigor complex configuration and that this configuration is retained even after the HMM has been released from actin. The EDC reactivity of rabbit skeletal muscle HMM, however, was different from that of chicken gizzard HMM. The treatment of acto–HMM complexes with EDC did not generate cross-linking between two skeletal muscle HMM heads.

Actin and myosin are two essential proteins for muscular contraction. The myosin molecule contains two pear-shaped heads at one end of a long tail (Slayter & Lowey, 1967). The heads are important, because they bear the actin binding capability, the ATPase activity (Mueller & Perry, 1962), and the site for binding of regulatory light chains (Szent-Györgyi et al., 1973). Moreover, because of their cyclic interaction with actin, they are believed to be involved in the generation of the contractile force (Huxley, 1969). Our recent carbodiimide cross-linking study with the rigor acto–smooth muscle

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