Nucleotide-Induced Change of the Interaction between the 20- and 26-Kilodalton Heavy-Chain Segments of Myosin Adenosinetriphosphatase Revealed by Chemical Cross-Linking via the Reactive Thiol SH_2

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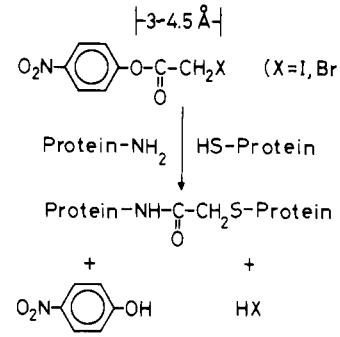
Received October 22, 1986; Revised Manuscript Received December 26, 1986

ABSTRACT: When myosin subfragment 1 (S-1) reacts with the bifunctional reagents with cross-linking spans of 3–4.5 Å, *p*-nitrophenyl iodoacetate and *p*-nitrophenyl bromoacetate, the 20-kilodalton (20-kDa) segment of the heavy chain is cross-linked to the 26-kDa segment via the reactive thiol SH_2. The well-defined reactive lysyl residue Lys-83 of the 26-kDa segment was not involved in the cross-linking. The cross-linking was completely abolished by nucleotides. Taking into account the recent report that SH_2 is cross-linked to a thiol of the 50-kDa segment of S-1 using a reagent with a cross-linking span of 2 Å [Chaussepied, P., Mornet, D., & Kassab, R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2037–2041], present results suggest that SH_2 of S-1 lies close to both the 26- and 50-kDa segments of the heavy chain. The data also encourage us to confirm our previous suggestion that the ATPase site of S-1 resides at or near the region where all three segments of 26, 50, and 20 kDa are contiguous [Hiratsuka, T. (1984) *J. Biochem.* (*Tokyo*) *96*, 269–272; Hiratsuka, T. (1985) *J. Biochem.* (*Tokyo*) *97*, 71–78].

Myosin subfragment 1 (S-1)[1] is the globular head region of the myosin molecule containing the sites responsible for ATPase activity and binding of actin (Mueller & Perry, 1962). Limited tryptic proteolysis of the heavy chain of S-1 produces mainly three peptide fragments of 26, 50, and 20 kDa which are aligned in this order within the heavy chain (Balint et al., 1978; Lu et al., 1978). Analysis by electrophoresis under nondenaturing conditions has revealed that even after limited proteolysis of S-1 the resultant three fragments remain associated, suggesting that the three segments of S-1 are held together by strong noncovalent forces (Hiratsuka, 1985). Indeed, it has previously been suggested that all three segments of S-1 are contiguous at least in some regions (Hiratsuka, 1984). A number of workers have referred to these segments of S-1 as tertiary structural domains (Mornet et al., 1984; Muhlrad & Morales, 1984; Botts et al., 1984). Recent measurements of transient electric birefringence of S-1 have also suggested that S-1 is composed of two tertiary structural domains, being connected by a flexible linkage with a substantial restoring force (Highsmith & Eden, 1986). Therefore, if it is accepted that 26-, 50-, and 20-kDa segments are actually domains of the S-1 heavy chain, it is time to consider the relation of interdomain interactions to function of the myosin head. Such specific interactions would be expected to affect conformations of both the ATPase and the actin binding sites (Botts et al., 1984; Hiratsuka, 1984, 1986).

Among three segments of S-1, the largest central 50-kDa segment is of particular interest as it seems to undergo some kind of temperature-dependent melting, which is prevented upon the addition of nucleotides or actin to S-1 (Setton & Muhlrad, 1984; Mocz et al., 1984). Moreover, the intrinsic conformational instability of the segment has recently been reported (Mornet et al., 1985). These properties of the 50-kDa segment can have functional implications with regard to the idea that the elastic and contractile properties of the cross-bridges arise primarily in the S-1 portion of myosin (Cross et al., 1984). As a part of our strategy for studying the specific interactions between three segments of S-1, we have recently used glutaraldehyde as an amino-specific cross-linker for the investigation of role of the 50-kDa segment (Hiratsuka, 1986). Limited glutaraldehyde modification of tryptic S-1 resulted in the selective cross-linking of the 50- and 20-kDa fragments, affecting the ATPase and actin binding properties. The results have suggested that the 50-kDa segment acts as a communicating apparatus between the ATPase and actin binding sites of myosin, interacting with the 20-kDa segment in a cooperative manner. The selective cross-linking of the two segments has also been achieved by the inter-thiol cross-linkers dibromobimane (Mornet et al., 1985) and *N,N'-p*-phenylenedimaleimide (Chaussepied et al., 1986). They involve SH_1 and SH_2 thiols of the 20-kDa segment, respectively. In particular, the experiments with the latter reagent have suggested the prominent cooperative role of the 20-kDa segment and the proximal segment of 50 kDa in modulating the conformation

Scheme I: Reaction Scheme for Cross-Linking of Amino and Thiol Groups in Protein with NPIA and NPBA (Haugland, 1985)



[1] Abbreviations: S-1, myosin subfragment 1; NPIA, *p*-nitrophenyl iodoacetate; NPBA, *p*-nitrophenyl bromoacetate; SDS, sodium dodecyl sulfate; PP_i, pyrophosphate; P_i, orthophosphate; V_i, orthovanadate; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; TNBS, 2,4,6-trinitrobenzenesulfonate; NEM, *N*-ethylmaleimide; kDa, kilodalton(s); Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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of the ATPase site, being consistent with our results (Hiratsuka, 1986).

In contrast to this, little is known about the specific interactions between the 20- and 26-kDa segments of S-1. In the present study, we have utilized *p*-nitrophenyl iodoacetate (NPIA) and *p*-nitrophenyl bromoacetate (NPBA) for investigation of such interactions. While the use of NPIA and NPBA in protein cross-linking has not been reported, they are capable of converting protein lysyl residues to iodoacetamides and bromoacetamides, respectively, which can be cross-linked to protein thiols with cross-linking spans of 3–4.5 Å (Haugland, 1985) (Scheme I). We have found that the 20- and 26-kDa segments of S-1 are preferentially cross-linked by the nitrophenyl esters via the reactive thiol SH_2 and that the cross-linking is completely abolished by the addition of nucleotides. The data encourage us to propose that the ATPase site of S-1 resides at or near the region where the 26-, 50-, and 20-kDa segments are contiguous.

Materials and Methods

Reagents. NPIA and NPBA were purchased from Molecular Probes Inc. TNBS and NEM were from Nakarai Chemical Co. IAEDANS was from Aldrich Chemical Co. α-Chymotrypsin, diphenylcarbamyl chloride treated trypsin, and soybean trypsin inhibitor were from Sigma Chemical Co. ATP, ADP, and AMP were from Kohjin Co. All other reagents were of reagent or biochemical research grade.

Preparations of Proteins. Rabbit skeletal myosin was prepared by the method of Perry (1955) with slight modification. S-1 was prepared by chymotryptic digestion of myosin as described by Weeds and Taylor (1975). Tryptic S-1 was prepared as described previously (Hiratsuka, 1986).

Denaturation of tryptic S-1 was carried out by incubating the protein (8 mg/mL) in 50 mM KCl and 5 mM Mops (pH 7.0) with 2% SDS for 24 h at 36 °C.

Specific Modifications of S-1. The specific fluorescence labeling of SH_1 of tryptic and undigested S-1's with IAEDANS was done according to the procedure of Takashi (1979). Thiol titrations indicated the loss of 0.7–0.8 mol of SH/mol the protein after labeling. The S-1 derivative exhibited about 6–7% of the K^+-EDTA–ATPase activity of unmodified S-1, whereas the Ca^{2+}-ATPase was enhanced to 320–420% of the control value.

SH_1 and SH_2 of S-1 were sequentially blocked with NEM in the presence of 1 mM MgADP as described by Reisler (1982). Thiol titrations indicated the loss of 2.3 mol of SH/mol of S-1. The S-1 derivative exhibited 1–2% of the K^+-EDTA–ATPase activity of unmodified S-1, and Ca^{2+}-ATPase was 1–7% of the control value.

Trinitrophenylation of the reactive lysyl residue of S-1 was carried out with TNBS by the method of Muhlrad et al. (1975). Under our conditions, 0.96–1.0 mol of the dye was incorporated per mole of S-1. The K^+-EDTA–ATPase and Ca^{2+}-ATPase activities of the S-1 derivative were 25–35% of the control value.

All modification reactions were stopped by the addition of 2-mercaptoethanol or 1 M glycine/KOH (pH 8.0) at a final concentration of 35 or 15 mM, respectively. The reaction mixture was then dialyzed overnight against 5 mM Mops (pH 7.0) and 40 mM KCl.

Cross-Linking Experiments. Tryptic and undigested S-1's (1–1.5 mg/mL) were incubated with NPIA or NPBA in 40 mM triethanolamine/NaOH (pH 8.4), 50 mM NaCl, and 0.5 mM $MgCl_2$ in the presence and absence of 0.2 mM ligand at 25 °C. NPIA and NPBA were dissolved in dimethylformamide immediately before use. Final concentrations of the reagent and dimethylformamide were 0.05–0.5 mM and 2–4%, respectively. The cross-linking reaction was stopped by the addition of 1 M glycine/NaOH (pH 8.4) and 2-mercaptoethanol when necessary at final concentrations of 20 and 70 mM, respectively.

ATPase Measurements. The Ca^{2+}-ATPase and K^+-EDTA–ATPase activities were measured at 25 °C in 1 mM ATP, 0.5 M KCl, and 50 mM Tris-HCl (pH 8.0) in the presence of 5 mM $CaCl_2$ and EDTA, respectively. P_i liberated was determined by the method of Fiske and SubbaRow (1925).

Determination of Thiols. The thiol content of S-1 was measured by Ellman's titration using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959) in the presence of urea, as described previously (Uchida & Hiratsuka, 1971).

Tryptic Digestion of S-1. Limited cleavage by trypsin was performed at 25 °C in 40 mM triethanolamine/NaOH (pH 8.4), 50 mM NaCl, and 0.5 mM $MgCl_2$ using a molar ratio of 1:100 for trypsin/S-1.

SDS–Polyacrylamide Gel Electrophoresis. Cross-linked proteins were analyzed by SDS–polyacrylamide gel electrophoresis by the system of Weber and Osborn (1969) using 7.5% acrylamide gels; it is well-known that cross-linked proteins migrate anomalously on Laemmli gels although the gels can give better resolution. To follow processes of cross-linking and tryptic cleavage of the protein, 50–200-μL aliquots of sample were withdrawn periodically and pipetted into equal volumes of a solution containing 0.1 M phosphate (pH 7.0), 2% SDS, 10% 2-mercaptoethanol, and 34% sucrose. Each mixture was then heated in boiling water for 1–3 min. Gels were stained with Coomassie Brilliant Blue.

For quantitative analysis of the formation of cross-linked products, the gels were scanned with a Yamato Ozumor 82 densitometer at 570 nm. Areas under peaks of the protein bands were measured.

Protein Determinations. The concentration of S-1 was determined from the extinction coefficient ($A^{1\%}_{1cm}$) at 280 nm of 7.5 (Wagner & Weeds, 1977). Protein concentrations of modified S-1 were determined by the biuret method (Gornall et al., 1949), standardized by using the $A^{1\%}_{1cm}$ of unmodified S-1. S-1 was assumed to have a molecular weight of 120 000 (Weeds & Taylor, 1975).

Results

Cross-Linking of Tryptic S-1. It is well-known that although the limited tryptic digestion of S-1 lowers the protein's affinity for actin, it does not have a significant effect on the ATPase properties (Mornet et al., 1979; Botts et al., 1982; Hiratsuka, 1986). These properties of tryptic S-1 allow ready use of the protein in various experiments as a useful model for myosin ATPase. Thus, we first studied the reaction of NPIA and NPBA with tryptic S-1, which mainly consists of 26-, 50-, and 20-kDa peptide fragments.

As shown in Scheme I, NPIA and NPBA are capable of converting protein lysyl residues to iodoacetamides and bromoacetamides, respectively, which can be cross-linked to protein thiols (Haugland, 1985). Their reaction with lysyl residues can be followed by measuring the release of chromophoric *p*-nitrophenol on the basis of the absorbance change at 400 nm. When tryptic S-1 was incubated with NPIA or NPBA, the increase of absorbance at 400 nm was observed (data not shown).

Effects of the concentration of NPIA on protein cross-linking were tested (Figure 1). Tryptic S-1 was exposed to various concentrations of the reagent for 1 h at 25 °C. The cross-linked products were analyzed by SDS–polyacrylamide gel electrophoresis. As shown in Figure 1A, the electrophoretic

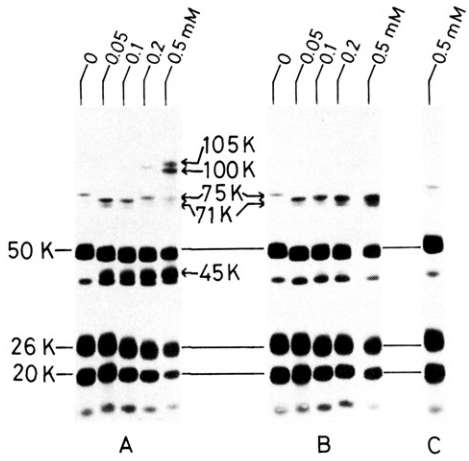

FIGURE 1: Effects of NPIA concentration on cross-linking of tryptic S-1: (A) −ATP; (B) +ATP (0.2 mM); (C) denatured tryptic S-1, ±ATP. The protein (1 mg/mL) was incubated with NPIA at various concentrations (0–0.5 mM) in 40 mM triethanolamine/NaOH (pH 8.4), 50 mM NaCl, and 0.5 mM $MgCl_2$ for 1 h at 25 °C. The reaction mixture was then subjected to SDS gel (7.5%) electrophoresis.

pattern showed clearly that in the absence of ATP the amount of 20- and 26-kDa fragments decreased with the NPIA concentration. At the same time, a new prominent protein band of the cross-linked product with an apparent molecular mass of 45 kDa appeared. At lower concentrations of the reagent, minor bands of 71- and 75-kDa cross-linked products were also seen. These products were converted to 100- and 105-kDa products at higher concentrations of the reagent.

Next, we examined whether ATP changes the formation of the cross-linked products. As shown in Figure 1B, the addition of 0.2 mM ATP to the incubation mixture completely abolished the formation of the 45-kDa cross-linked product even at 0.5 mM reagent. On the other hand, the 71- and 75-kDa cross-linked products were formed only slightly, but they were no longer converted to the cross-linked products with higher molecular weights. Similar results were obtained with NPBA, except that the reactivity of this reagent was lower than NPIA (data not shown). When the protein had been denatured prior to incubation with NPIA or NPBA, no cross-linked product was detected regardless of the presence or absence of ATP (Figure 1C). Thus, these results suggest that we are looking only at interfragment cross-linking of tryptic S-1 and NPIA and NPBA would therefore be potentially useful as tools for "freezing" the existing interactions predominantly between the 20- and 26-kDa fragments.

To confirm that the major 45-kDa cross-linked product, which is generated in the absence of ATP, indeed contains the 20-kDa fragment, we attempted to fluorescently label the 20-kDa fragment of tryptic S-1 (Figure 2). When the labeling was carried out with IAEDANS (Takashi, 1979), the 20-kDa fragment was predominantly labeled. The fluorescently labeled protein was incubated with NPIA or NPBA in the presence and absence of ATP and analyzed by SDS–polyacrylamide gel electrophoresis. When the protein was incubated with the reagent in the absence of ATP, fluorescence of the 20-kDa fragment decreased significantly. At the same time, major fluorescence appeared at the position of the 45-kDa product while minor fluorescence at the positions of the 100- and 105-kDa products. On the other hand, when the cross-linking was carried out in the presence of ATP, fluorescence originally seen on the band of 20-kDa fragment was scarcely decreased. New minor fluorescent bands appeared at the positions of the 71- and 75-kDa products. Therefore, we concluded that not only the major 45-kDa product but also the minor 71-, 100-,

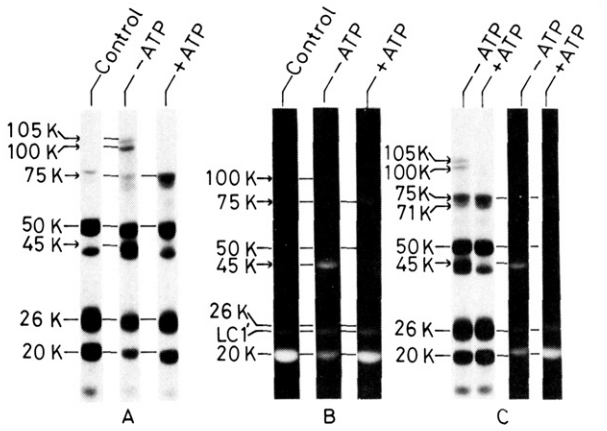

FIGURE 2: Cross-linking of fluorescently labeled tryptic S-1 with NPIA (A, B) and NPBA (C). Prior to the cross-linking reaction, tryptic S-1 was fluorescently labeled with IAEDANS (Takashi, 1979). The labeled protein was incubated with the reagent (0.5 mM) for 1 h in the presence and absence of 0.2 mM ATP. Fluorescence photographs of the gels (B, C) were taken under UV illumination in the dark. LC1′, degradation product of light chain 1.

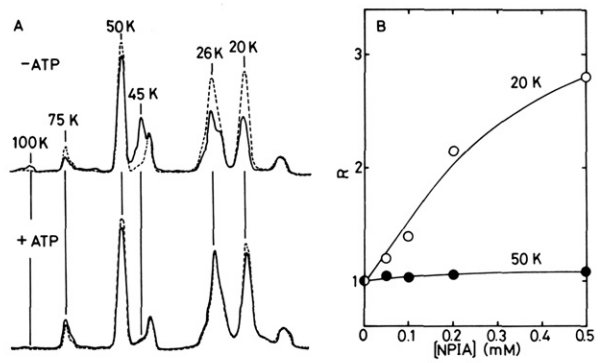

FIGURE 3: (A) Cross-linking of tryptic S-1 with NPIA as assessed by densitometric scanning of the gel. Tryptic S-1 was incubated with 0.2 mM NPIA for 1 h in the presence and absence of 0.2 mM ATP; (---) control tryptic S-1. (B) Effects of ATP on cross-linking of the 20- and 50-kDa fragments at varying NPIA concentrations. The *R* value was obtained by dividing the remaining amount of 20- or 50-kDa fragment after cross-linking in the presence of ATP by the amount after cross-linking in the absence of ATP at given NPIA concentration using the gels shown in Figure 1.

and 105-kDa products indeed contain the 20-kDa fragment.

Quantitative analysis of the formation of cross-linked products was carried out by densitometric scanning of the gels. Figure 3A shows the resulting densitometric traces of the samples, which were incubated with 0.2 mM NPIA for 1 h in the presence and absence of 0.2 mM ATP. In the presence of ATP, about 50% and 55% of the 26- and 20-kDa fragments, respectively, were cross-linked, generating the 45-kDa product. On the other hand, the 50-kDa fragment was converted to the cross-linked products of 71–105 kDa only slightly (15%). In the presence of ATP, however, most of the 26-kDa fragment was left un-cross-linked. The 20- and 50-kDa fragments were converted to the cross-linked products of 71 and 75 kDa only slightly (10–15%). However, the cross-linked products of 100 and 105 kDa were not detectable in this case. The data confirmed the results of photography shown in Figures 1 and 2; the major 45-kDa product is the 1:1 complex of the 20- and 26-kDa fragments, and the minor products of 71–75 and 100–105 kDa are complexes of 20- and 50-kDa fragments and of 20-, 50-, and 26-kDa fragments, respectively.

Figure 3B shows the effects of ATP on the cross-linking of the 20- and 50-kDa fragments at varying NPIA concentrations. The effects were studied by determining the ratio, *R*. *R* was obtained by dividing the remaining amount of 20- or

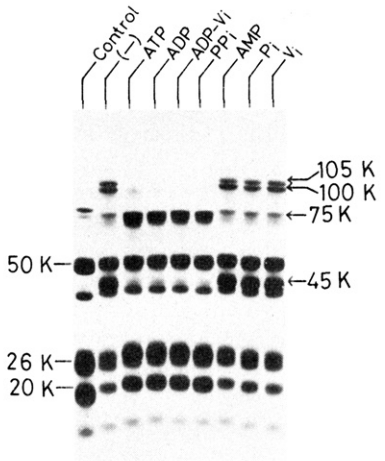

FIGURE 4: Effects of adenine nucleotides and ligands on the formation of cross-linked products of tryptic S-1. Tryptic S-1 was incubated with 0.5 mM NPIA for 1 h in the presence and absence of 0.2 mM ligands; (–) –ligand.

50-kDa fragment after cross-linking in the presence of ATP by the amount after cross-linking in the absence of the nucleotide at a given concentration of NPIA. When the *R* value was plotted against the NPIA concentration, the *R* value of the 20-kDa fragment increased gradually from 1 to 2.8 up to 0.5 mM NPIA. However, the *R* value of the 50-kDa fragment was nearly equal to 1 over the NPIA concentration range 0.05–0.5 mM. These results suggest that ATP exerts effects on the formation of the major 45-kDa cross-linked product but not on the formation of the minor cross-linked products of the 20- and 50-kDa segments.

Effects of nucleotides other than ATP and various ligands on the formation of the cross-linked products were also examined (Figure 4). Like ATP, ADP, ADP–V_i, and PP_i completely abolished the formation of the major 45-kDa cross-linked product but not the formation of the minor 71- and 75-kDa products. However, no effect was observed with AMP, P_i, and V_i, which are neither substrate nor competitive inhibitor for the myosin S-1 ATPase. These results clearly indicate that the conformational changes induced in the ATPase site are entirely transmitted to the cross-linking sites of the 20- and 26-kDa segments.

Cross-Linking of Intact S-1. In a second phase of this work, we investigated the cross-linking of intact S-1 with NPIA. Since the fluorescence labeling of tryptic S-1 with IAEDANS had no effect on the cross-linking reaction (Figure 2), we first attempted to fluorescently label S-1 with the reagent prior to the cross-linking reaction. The fluorescently labeled protein was incubated with NPIA in the presence and absence of ATP and subjected to limited tryptic digestion (Figure 5A,B).

The control S-1, which had been fluorescently labeled with IAEDANS but not incubated with NPIA, was found to be digested by trypsin in a manner similar to unlabeled S-1, as indicated by finally generating main peptide fragments of 50, 26, and 20 kDa (a). The fluorescence of the bound fluorophore, originally seen predominantly in the S-1 heavy chain, finally appeared in the 20-kDa peptide band. In contrast to this, when fluorescently labeled S-1 had been incubated with NPIA in the absence of ATP, a new 45-kDa peptide fragment was generated while the formation of the 20- and 26-kDa fragments was significantly suppressed (b). Densitometric traces of gels showed that only less than 10% of the 20-kDa fragment was found in the tryptic digest, indicating the cross-linking of almost stoichiometric amounts of the S-1 heavy chain (data not shown). The intense fluorescence of the 45-

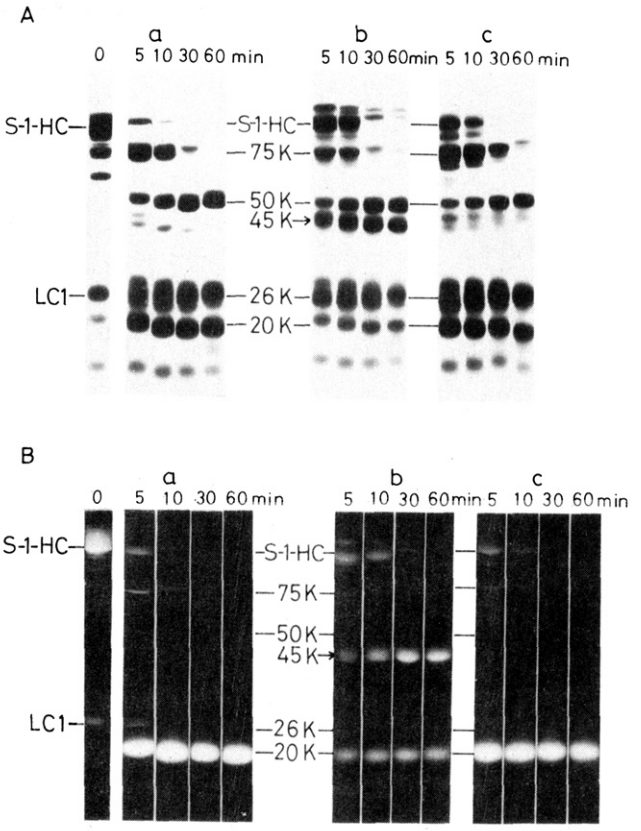

FIGURE 5: Time courses of limited tryptic cleavage of fluorescently labeled intact S-1 after cross-linking with NPIA: (a) S-1 before cross-linking; (b) S-1 cross-linked in the absence of ATP; (c) S-1 cross-linked in the presence of ATP. Intact S-1 (1.5 mg/mL), which had been fluorescently labeled with IAEDANS, was cross-linked with 0.5 mM NPIA for 1 h in the presence and absence of 0.2 mM ATP. Limited cleavage of the S-1 derivatives was performed at 25 °C in 40 mM triethanolamine/NaOH (pH 8.4), 50 mM NaCl, and 0.5 mM $MgCl_2$ using a molar ratio of 1:100 for trypsin/S-1. (A) Gels stained with Coomassie Brillian Blue; (B) gels viewed under UV light. HC, heavy chain; LC1, light chain 1.

kDa fragment indicates that this fragment contains the 20-kDa peptide. When the protein had been incubated with NPIA in the presence of ATP, the sample was digested by trypsin in a manner similar to the control S-1, generating the highly fluorescent peptide of 20 kDa (c).

In conclusion, the 20- and 26-kDa segments of the intact S-1 are also cross-linked with NPIA in a manner similar to in the case of tryptic S-1, and the cross-linking is completely abolished by the addition of ATP. Therefore, it seems that there is no significant difference between the intact and tryptic S-1's in the interactions between these two segments.

Determination of the Cross-Linking Sites. To obtain information about the cross-linking sites between the 20- and 26-kDa segments of S-1, we first measured the ATPase activities of S-1 after cross-linking. Upon a 1-h incubation with 0.5 mM NPIA, the Ca^{2+}-ATPase and K^+-EDTA–ATPase of S-1 were significantly decreased to less than 5% of the original activity. However, this was also the case for S-1, which had been incubated with the reagent in the presence of ATP. Thus, loss of ATPase activity of S-1 is not directly related to the cross-linking of the 20- and 26-kDa segments. Measuring the ATPase activity did not tell us the information about the cross-linking sites between the two segments.

It is well-known that S-1 contains one reactive lysyl residue (Lys-83) in the 26-kDa segment (Mornet et al., 1980; Miyanishi & Tonomura, 1981) and two reactive thiols, SH_1 (Cys-707) and SH_2 (Cys-697), in the 20-kDa segment (Ya-

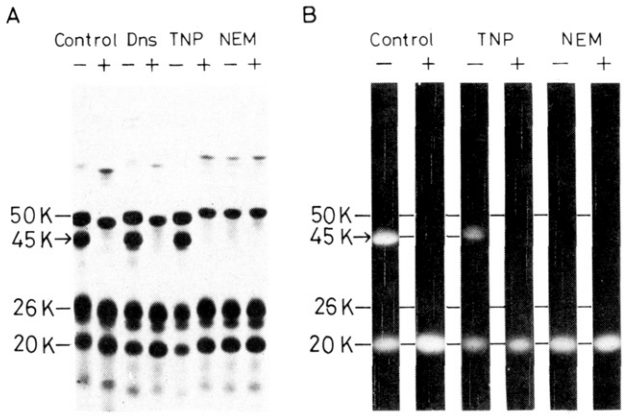

FIGURE 6: Effects of blockage of SH_1, SH_2, and Lys-83 of S-1 on the cross-linking reaction with NPIA. (A) Dns, SH_1 was blocked with IAEDANS (Takashi et al., 1979); TNP, Lys-83 was blocked with TNBS (Muhlrad et al., 1975); NEM, SH_1 and SH_2 were blocked with NEM (Reisler, 1982). (B) S-1, in which SH_1 had been blocked with IAEDANS, was modified with TNBS or NEM for the subsequent blockage of Lys-83 or SH_2, respectively. The S-1 derivatives were incubated with 0.5 mM NPIA for 1 h in the presence (+) and absence (−) of 0.2 mM ATP and then subjected to limited tryptic cleavage for 45 min as descirbed in Figure 5. (A) Gels stained with Coomassie Brilliant Blue; (B) gels viewed under UV light.

mashita et al., 1974; Elzinga & Collins, 1977). Because NPIA and NPBA are amino- and thiol-specific reagents (Haugland, 1985), we next checked the possibility that these reactive residues of S-1 are involved in the cross-linking reaction. We thus prepared the S-1 derivatives in which these residues were blocked by three reagents that react quantitatively and specifically with the residues. These were the fluorescent reagent IAEDANS for SH_1 (Takashi, 1979), TNBS for Lys-83 (Kubo et al., 1960), and NEM for SH_1 and SH_2 (Sekine & Yamaguchi, 1963). The resultant S-1 derivatives were incubated with NPIA in the presence and absence of ATP and subjected to limited tryptic digestion (Figure 6).

As shown in Figure 6A, blocking of SH_1 of S-1 with IAEDANS had no effect on the formation of the 45-kDa cross-linked product, as described above. However, unexpectedly, this was also the case for the blocking of Lys-83. The S-1 derivative, in which Lys-83 had been quantitatively blocked with TNBS, formed the 45-kDa cross-linked product to a similar extent to in the case of control S-1. In contrast to this, when both SH_1 and SH_2 of S-1 had been blocked with NEM, the S-1 derivative no longer formed the 45-kDa cross-linked product. However, it should be noted that the minor products of 71 and 75 kDa were formed in the presence of ATP, suggesting that neither SH_1 nor SH_2 was the cross-linking site between the 20- and 50-kDa segments. Similar results were obtained with doubly blocked S-1 derivatives, in which SH_1 had been preblocked with IAEDANS and then blocked with TNBS or NEM (Figure 6B). Again, blocking of SH_1 and SH_2 with IAEDANS and NEM, respectively, resulted in abolition of the formation of the major 45-kDa cross-linked product.

In conclusion, NPIA and NPBA react with SH_2 (Cys-697) in the 20-kDa segment and an amino acid residue other than Lys-83 in the 26-kDa segment, generating a cross-link between the two segments.

Discussion

Until now, the use of NPIA and NPBA in protein cross-linking has not been reported (Haugland, 1985). When amino groups in proteins react with NPIA and NPBA, the groups are converted to iodoacetamides and bromoacetamides, respectively (Scheme I). These acetamide derivatives would be expected to be able to react with thiols like iodoacetamide and IAEDANS. S-1 contains two highly reactive thiols, SH_1 and SH_2 (Sekine & Kielley, 1964). However, the involvement of SH_1 in the present cross-linking reaction is ruled out, since blockage of this thiol has no effect on the cross-linking of the 20- and 26- kDa segments. Therefore, it is reasonable to conclude that one of two sites of the cross-linking is SH_2 in the 20-kDa segment. On the other hand, it is well-known that *p*-nitrophenyl esters have a high reactivity toward amino groups in protein. Indeed, Levy (1973) has demonstrated that only α- and ϵ-amino groups of insulin are modified with *p*-nitrophenyl-*p*-(chloromercuri)benzoate with slightly alkaline conditions. Because the N-terminal residue in the 26-kDa segment is acetylated (Tong & Elzinga, 1983), it is most likely that the cross-linking site in the 26-kDa segment is a lysyl residue other than Lys-83. However, the involvement of other amino acid residues cannot be ruled out at present, since amino acid residues in proteins have an unexpected chemical reactivity.

It is interesting that NPIA and NPBA cross-link the 20- and 26-kDa segments via SH_2 instead of SH_1, since iodoacetamide and IAEDANS are known to react readily with SH_1 (Kunz et al., 1977; Takashi, 1979). The reason appears to be that there is no suitable pair of thiol and lysyl residue for SH_1. Therefore, it cannot be ruled out at present that the reagents modify monofunctionally not only SH_1 but also other amino acid residues even when no cross-linked product is formed. This is also the case for SH_2, since the reactivity of SH_2 is significantly increased by PP_i and ADP (Reisler, 1982); SH_2 may be monofunctionally modified with reagents in the presence of nucleotides, generating no cross-linked product of the 20- and 26-kDa segments.

Mornet et al. (1985) have recently reported that cross-linking between SH_1 and a thiol in the 50-kDa segment is achieved by dibromobimane. More recently, Chaussepied et al. (1986) have also demonstrated that *N,N'-p*-phenylenedimaleimide and 5,5'-dithiobis(2-nitrobenzoic acid) cross-link SH_2 to a thiol in the 50-kDa segment. However, their reagents did not form any cross-link between the 20- and 26-kDa segments. These results seem to suggest that one thiol (Cys-122) in the 26-kDa segment (Tong & Elzinga, 1983) is close to neither SH_1 nor SH_2 in the 20-kDa segment. On the other hand, our previous study using the photosensitized direct cross-linking method suggested that certain residues including amino groups are involved in the interaction between the 26- and 20-kDa segments (Hiratsuka, 1984), consistent with the present results.

Our present results clearly show that SH_2 in the 20-kDa segment is cross-linked to the 26-kDa segment with reagents with cross-linking spans of 3–4.5 Å. Taking into account the fact that SH_2 is also cross-linked to a thiol in the 50-kDa segment with a thiol-specific reagent with a cross-linking span of 2 Å (Chaussepied et al., 1986), our results suggest that SH_2 is close to both the 26- and 50-kDa segments of S-1. Moreover, the formation of the 26- and 20-kDa cross-linked product was completely abolished by nucleotides. Since the reactivity of SH_2 is significantly increased by ADP and PP_i (Reisler, 1982), it is unlikely that this abolition is due to reduction of the reactivity of the thiol induced by nucleotide binding. Our results favor the interpretation that binding of nucleotides induces the conformational changes which cause the 26-kDa segment to become close to the 50-kDa segment [for a discussion, see Hiratsuka (1986)], resulting in increase of the distance between SH_2 in the 20-kDa segment and the cross-linking site in the 26-kDa segment.

The above conclusion also means that the ATPase site is close to the interacting site of SH_2 and the 26-kDa segment, i.e., the site where all three segments of S-1 are contiguous (Hiratsuka, 1984). The proximity of the ATPase site to such a specific site would allow nucleotides to abolish completely the cross-linking between the 20- and 26-kDa segments and to be trapped in the ATPase site when the 20- and 50-kDa segments are cross-linked via SH_2 (Chaussepied et al., 1986). Recent measurements using fluorescence energy transfer techniques has shown that the actual minimal separation between the ATPase site and SH_1, which is known to be close to SH_2, is probably less than 15 Å, suggesting the possibility of direct interaction between the two sites (Cheung et al., 1985).

If on the basis of the present results it is accepted that all three segments of S-1 are contiguous at or near the ATPase site, it will be time to reconsider the intersite communication between the ATPase and actin binding sites in understanding energy transduction in muscle contraction (Morales et al., 1982; Hiratsuka, 1984, 1986).

After completion of the manuscript, we became aware of a study carried out by Lu et al. (1986). These authors used the thiol-specific photoactivatable reagent 4-iodoacetamidobenzophenone and found that the 20- and 26-kDa segments were cross-linked via SH_1 too, confirming our present results. Thus, taking into account the fact that SH_1 is cross-linked to a thiol in the 50-kDa segment with dibromobimane with cross-linking spans of 3–6 Å (Mornet et al., 1985), we may conclude that not only SH_2 but also SH_1 is close to both the 26- and 50-kDa segments of S-1.

ACKNOWLEDGMENTS

I am indebted to Professor Koki Uchida for his encouragement and support. I am also grateful to Kyoko Nakamura for her skillful assistance and typing the manuscript.

Registry No. NPIA, 31252-85-4; NPBA, 19199-82-7; 5′-ATP, 56-65-5; ATPase, 9000-83-3.

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