Glutamic Acid-88 Is Close to SH-1 in the Tertiary Structure of Myosin Subfragment 1†

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ABSTRACT: The thiol-specific photoactivatable reagent benzophenone iodoacetamide (BPIA) can be selectively incorporated into the most reactive thiol, SH-1, of myosin S1, and upon photolysis, an intramolecular cross-link is formed between SH-1 and the N-terminal 25-kDa region of S1. If a Mg^{2+}-nucleotide is present during photolysis, cross-links can be formed either with the 25-kDa region or with the central 50-kDa region [Lu et al. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6392]. Comparison of the peptide maps of cross-linked and un-cross-linked S1 heavy chains indicates that the segment located about 12–16 kDa from the N-terminus of the heavy chain can be cross-linked to SH-1 via BPIA independently of the presence of a nucleotide whereas the segment located 57–60 kDa from the N-terminus can be cross-linked to SH-1 only in the presence of a Mg^{2+}-nucleotide [Sutoh & Lu (1987) *Biochemistry 26*, 4511]. In this report, S1 was labeled with radioactive BPIA, photolyzed in the absence of nucleotide, and then degraded with proteolytic enzymes. Peptides containing cross-links were isolated by liquid chromatography and subjected to amino acid sequence analyses. The results show that Glu-88 is the major site and Asp-89 and Met-92 are the minor sites involved in cross-linking with SH-1 (Cys-707) via BPIA. These residues are very near the reactive lysine residue (Lys-83) but relatively remote in the primary structure from the putative nucleotide binding region.

Myosin is one of the key proteins of muscle cells that converts the chemical energy of ATP to mechanical force. The globular portion of myosin, subfragment 1 (S1), containing the actin binding and ATP hydrolysis sites is made of the N-terminal portion of the heavy chain and two light chains. The heavy chains of S1 can be cleaved with proteolytic enzymes into three distinct fragments with apparent molecular masses of 50, 25, and 20 kDa on SDS–PAGE (Balint et al., 1975). The order of these fragments, 25–50–20, in the primary structure was at first established on the basis of partial amino acid sequences of the fragment (Lu et al., 1978). The nicked S1 retains the K^+- and Ca^{2+}-ATPase activities and the ability to bind actin.

There are two reactive thiols, SH-1 (Cys-707) and SH-2 (Cys-697), on the heavy chains of myosin S1. Modification of one of the reactive thiols results in the activation of Ca^{2+}-ATPase activity and the loss of K^+-ATPase activity; modification of both thiols leads to the abolition of Ca^{2+}-ATPase activity also (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966; Reisler et al., 1974). However, the direct involvement of reactive thiols in the binding of nucleotides has been ruled out (Burke & Reisler, 1977; Wells & Yount, 1979; Tao & Lamkin, 1981; Perkins et al., 1984).

Use of nicked S1 has allowed the localization of sites with functional interests; for example, reactive thiols are located in the 20-kDa fragment (Balint et al., 1978; Gallagher & Elzinga, 1980), analogues of nucleotides bind to the 25- or 50-kDa region (Szilagyi et al., 1979; Mahmood & Yount, 1984; Okamoto & Yount, 1985; Hiratsuka, 1985), and actin can be cross-linked to the 50- or 20-kDa region (Yamamoto & Sekine, 1979; Mornet et al., 1981; Sutoh et al., 1984; Chen et al., 1985). It has been proposed that three fragments fold independently into three domains and the functional sites probably involve one or two domains. This hypothesis appears attractive but awaits experimental support. Little is known about the folding of polypeptide chain within S1, and the knowledge on the proximity among residues in the tertiary structure is limited to thiols; namely, an intramolecular loop can be formed between SH-1 and SH-2 (Reisler et al., 1974; Wells & Yount, 1979; Graceffa & Seidel, 1980), SH-1 and Cys-522 (Mornet et al., 1985; Ue, 1987), or SH-2 and Cys-540 (Chaussepied et al., 1988).

We have shown recently that SH-1 can be selectively modified with the photoactivatable thiol reagent 4-(2-iodoacetamido)benzophenone (BPIA) and upon photolysis a cross-link is made to the 25-kDa region in the absence of nucleotides. Cross-linking can take place either with the 25-kDa or with the 50-kDa region if photolysis was carried out in the presence of a Mg^{2+}-nucleotide (Lu et al., 1986). Comparison of the peptide maps of cross-linked and un-cross-linked S1 heavy chains indicated that two regions, 12–16 and 57–60 kDa from the NH_2 terminus, respectively, are in proximity with SH-1 in the tertiary structure (Sutoh & Lu, 1987). In this work, we report the identification of amino acid residues in the 25-kDa region that can be cross-linked to SH-1. Our results show that Glu-88 is the major site to be cross-linked to SH-1 and this proximity relationship is nucleotide independent.

Materials and Methods

Proteins and Reagents. Myosin was prepared from rabbit back muscle, and S1 was made by chymotryptic digestion according to Weeds and Pope (1977). BPIA was purchased from Molecular Probes (Junction City, OR), and [^{3}H]BPIA was synthesized by Applied Protein Technologies (Cambridge, MA). Lysine endopeptidase was from Wako Chemical Co.

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(Tokyo, Japan), and trypsin was from Worthington (Freehold, NJ).

Modification and Photolysis of S1 with BPIA. S1 was modified with BPIA at a 1:1 molar ratio of S1 to BPIA in a buffer containing 40 mM KCl and 20 mM sodium phosphate, pH 7.0. The reaction was allowed to proceed for 30 min at 23 °C and then stopped by the addition of 2-mercaptoethanol (4 mol/mol of BPIA). Excess reagents were removed by extensive dialysis against 40 mM KCl/10 mM Hepes, pH 7.5 at 4 °C. Photolysis was carried out for 10 min at 4 °C in a Rayonet RPR-100 photochemical reactor.

Isolation of Peptides. Gel filtration through a G-50 column was carried out in 25% acetic acid by gravity. Chromatography on a Vydac(C_6H_5) column was performed on a Beckman 344 high-pressure liquid chromatography unit using a gradient made of acetonitrile in 0.1% TFA. Effluents of HPLC runs were monitored for absorbance at 220 and 300 nm. Radioactivity was determined on a Beckman 7500 scintillation counter using a xylene-based scintillation solution (Anderson & McClure, 1973).

Amino Acid Sequence Determination. At the early stage, automatic Edman degradations were carried out on Beckman sequenator 890, and PTHs were determined by HPLC using a Beckman ODS column and a solvent system developed by Kolbe et al. (1985). Some sequence analyses were carried out on a Applied Biosystem Sequencer 477 with a on-line PTH analyzer. All data reported here were obtained from a Beckman sequenator.

Results

Strategy. The iodoacetamide moiety of BPIA reacts with SH-1 selectively, whereas the benzophenone moiety can react with the C–H bond of any amino acid side chains or polypeptide backbones in the vicinity of SH-1. Consequently, it is possible that cross-linking may involve more than one site. To facilitate the isolation of cross-linked peptides and the identification of the cross-linked residues, radioactive labeled BPIA was used. For a given protein, the complexity of the peptide mixture is directly proportional to the number of peptides or the cleavage points, and, therefore, cross-linked S1 was first digested with lysine endopeptidase to produce a limited number of peptides. The radioactivity of the cross-linker would not be released until residues attached to both moieties of the cross-linker, in this case SH-1 and the unknown residue, are cleaved from the parent peptides by Edman degradation. Thus, in order to release the radioactivity and unknown residue simultaneously, it is necessary to have SH-1 cleaved by Edman degradation before the cross-linked residue(s). A second cleavage with trypsin was chosen since SH-1 is the second residue from the NH_2 terminus of the SH-1-containing tryptic peptide based on the known sequence.

Isolation of the Cross-Linked Peptides from the LEP Digest of S1. The LEP digest of S1 was at first gel filtered on a G-50 column, the radioactivity of effluent was measured, and fractions were pooled on the basis of the distribution of radioactivities (Figure 1). Each pooled fraction was further purified on HPLC chromatography, and peptide analyses were carried out on the major radioactive peak. LEP-4 contained mainly SH-1 peptide modified with BPIA but un-cross-linked to any other region. The chromatogram of LEP-3 on Vydac(C_6H_5) column showed fraction 25 was the major radioactive peak (Figure 2).

Amino Acid Sequence Analysis of LEP-3-25. Twenty-eight cycles of Edman degradation were carried out on LEP-3-25, and the PTH-amino acids released at the first 12 cycles were identified quantitatively. On comparison of the results with

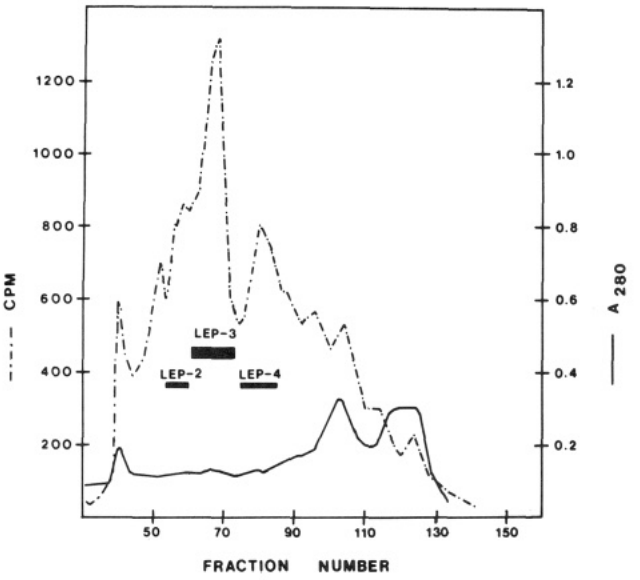

FIGURE 1: Gel filtration of peptides of cross-linked S1 cleaved with lysine endopeptidase on a G-50 column (1.9 × 200 cm) in 25% acetic acid; 4-mL fractions were collected, and 25-μL aliquots were taken from each fraction for determination of radioactivities.

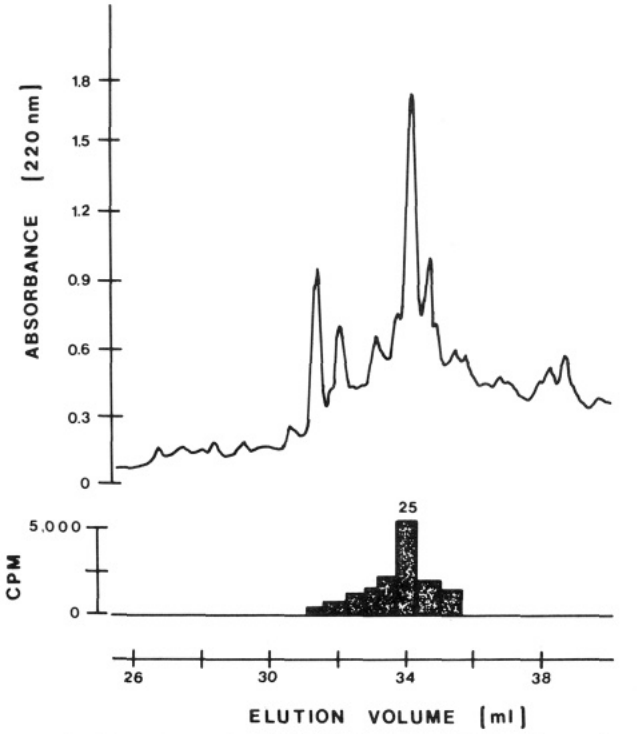

FIGURE 2: Chromatography of LEP-3 on a Vydac(C_6H_5) column using a gradient made of 0.1% TFA and 90% acetonitrile in 0.1% TFA, 0–20% acetonitrile in 5 min, and then 20–60% acetonitrile in 50 min at a flow rate of 1 mL/min. Peaks were collected manually on the basis of the absorbance at 220 nm, and 25 μL from each fraction was used for determination of radioactivities. Only the portion of the chromatogram containing the radioactive fractions was shown.

the published sequence data on S1 (Elzinga & Collins, 1977; Tong & Elzinga, 1983; numbering was based on the complete sequence of rabbit skeletal S1; Elzinga, personal communication), it became clear that two peptides correspond to the SH-1-containing peptide, Thr_{682}-Lys_{709} and another peptide, Ile_{87}-Lys_{106} (Figure 3). Aliquots of each cycle were monitored for the release of radioactivities, and it was found at cycle 26 when Cys-707 was cleaved. Although this data did not reveal which residue was involved in cross-linking, it confirmed that cross-linking occurred via BPIA attached on SH-1.

87 106
Ile-Glu-Asp-Met-Ala-Met-Met-Thr-His-Leu-His-Glu-Pro-Ala-Val-Leu-Tyr-Asn-Leu-Lys

682 709
Thr-Pro-Gly-Ala-Met-Glu-His-Glu-Leu-Val-Leu-His-Gln-Leu-Arg-Cys----Arg-Ile-Cys-Arg-Lys

FIGURE 3: Amino acid sequences of the cross-linked peptides correspond to Ile-87 to Lys-106 and Thr-682 to Lys-709 (Elzinga & Collins, 1977; Tong & Elzinga, 1983; Strehler et al., 1986). Radioactivities were detected at cycle 26 as shown by the asterisk.

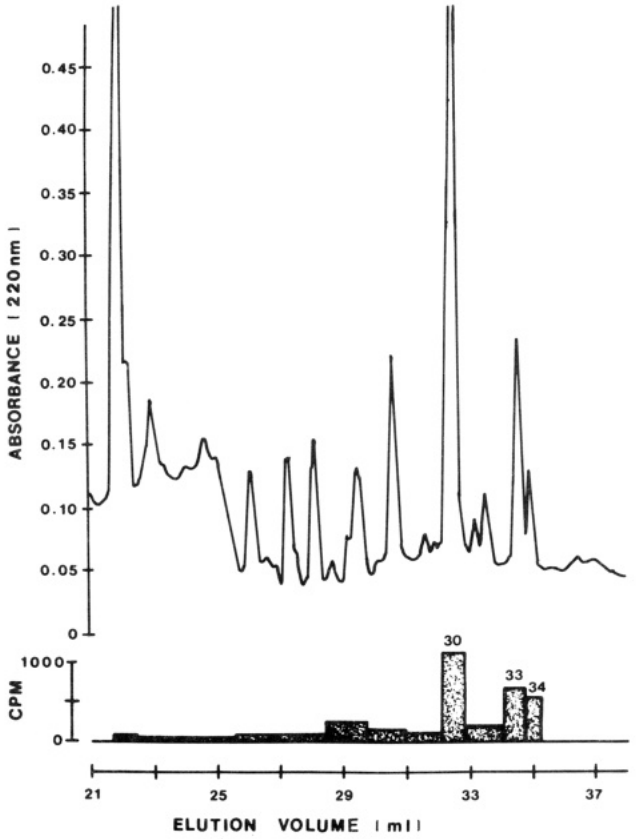

FIGURE 4: Chromatography of the tryptic digest of LEP-3 fraction 25 on a Vydac(C_6H_5) column. Conditions were the same as for Figure 2.

87 106
Ile-Glu-Asp-Met-Ala-Met-Met----------Leu-Lys

706 708
Ile-Cys-Arg

FIGURE 5: Amino acid sequences of LEP-3-25-T fractions 30 or 33 correspond to Ile-87 to Lys-106 and Ile-706 to Arg-708. Radioactivities were found at cycles 2, 3, and 6 as shown by asterisks.

Identification of Residues That Are Cross-Linked to SH-1. Peptide LEP-3-25 was cleaved with trypsin and then chromatographed on a Vydac(C_6H_5) column. Three radioactive fractions were found (Figure 4), and 20 cycles of Edman degradation were carried out on fractions 30 and 33. The results of sequence analyses showed that SH-1 containing tripeptide Ile-Cys-Arg was cross-linked to peptide Ile_{87}-Lys_{106} (Figure 5). At the first cycle, a high yield of Ile was obtained (derived from both peptides), and the yields of amino acids at later cycles decreased rapidly, probably due to the involvement in cross-linking. For fraction 30, radioactivities were detected at cycles 2, 3, and 6 (Table I), indicating that we were dealing with a mixture of peptides that were cross-linked at different sites, which was consistent with the fact that a substantial amount of PTH-Glu was found at cycle 2. These two peptides would not have been eluted together if they were not cross-linked. If all cross-linking occurred via Glu-88, no PTH-Glu would have been detected at cycle 2 (Table I).

Table I: Release of Radioactivity after Edman Degradation of LEP-3-25-T Fractions 30 and 33[a]

		fraction 30		fraction 33	
cycle	amino acid found	nmol	cpm	nmol	cpm
1	Ile	1.25	40	0.28	11
2	Glu	0.32	240	0.09	596
3	Asp	0.14	169	0.12	68
4	Met	0.02	64	0.02	33
5	Ala	0.07	44	0.02	21
6			130		72
7	Met	0.02	38		24

[a] Corrections have not been made for variations in recovery rate for each amino acid; 20% of the samples at each cycle was used for PTH analysis, and 20% was used to determine the radioactivities.

For fraction 33, radioactivity predominantly appeared at cycle 2 (Table I), but the presence of a residual amount of PTH-Glu at cycle 2 and some radioactivities at cycle 6 indicate that fraction 33 also contained a mixture of peptides that are cross-linked at different sites. The appearance of multiple peaks on HPLC is not an uncommon phenomenon, and the reasons were not fully understood. In our case, it might be related to the status of the cross-linker since the spectroscopic properties of fractions 30 and 33 were different. All the HPLC runs were monitored at 220 nm as well as 300 nm, and the absorbance ratio (220/300) of fraction 30 was about 5 times that of fraction 33 or 34.

Discussion

Using BPIA, we have shown that the cross-linking between SH-1 and the 25-kDa region is nucleotide independent whereas that between SH-1 and the 50-kDa region requires the presence of a Mg^{2+}-nucleotide (Lu et al., 1986). Later similar results were obtained by Rajasekharan et al. (1987) using the multifunctional cross-linker 4,4′-bis(*N*-maleimido)benzophenone. It has also been reported that cross-links can form between SH-1 and the 50-kDa region via 1,2,4-trinitrobenzene (Muno et al., 1987), and between SH-2 and the 25-kDa region via *p*-nitrophenyl iodoacetate in the absence of nucleotide (Hiratsuka, 1987).

Taking advantage of the fact that the efficiency of our cross-linking system is very high and the availability of radioactive reagent, we were able to isolate the cross-linked peptides and to identify that Glu-88 is the major site and Asp-89 and Met-92 are the minor sites involved in cross-linking. The chain weight from the NH_2 terminus to Glu-88 is about 10 kDa which is lower than our earlier estimation, 12–16 kDa, based on peptide mapping (Sutoh & Lu, 1987), indicating the mobility of NH_2-terminal peptide on SDS–PAGE was anomalous.

The results show that the sites on the 25-kDa region that are close to SH-1 are not in the putative nucleotide binding pocket, segment from Tyr-109 to Tyr-142 (Tong & Elzinga, 1983), but are only few residues away from the reactive lysine, Lys-83. The proximity of Lys-83 and SH-1 in tertiary structure is consistent with the observation that the modification of reactive lysine has similar effect as modification of SH-1, namely, the loss of ATPase activity (Kubo et al., 1965). The distance between SH-1 and Lys-83 measured by the fluorescence resonance energy transfer technique is 2.6 nm (Takashi et al., 1982), in good agreement with our finding that Glu-88 can be reached by SH-1 via cross-linker spans 1 nm.

Electron microscopic mappings of myosin head structure have shown that SH-1 is about 13 nm from the head/rod junction (Sutoh et al., 1984) and the N-terminus of the 25-kDa fragment and Trp-130 are 12 and 14 nm from the head/rod

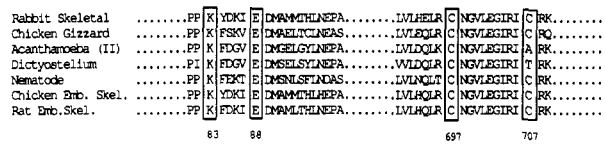

FIGURE 6: Comparison of amino acid sequences of regions containing the reactive thiols (Cys-697 and Cys-707), reactive lysine (Lys-83), and Glu-88. Rabbit skeletal myosin: Elzinga & Collins, 1977; Tong & Elzinga, 1983. Chicken gizzard myosin: Onishi et al., 1986; Maita et al., 1987. *Acanthamoeba* (II): Hammer et al., 1987. *Dictyostelium*: Warrick et al., 1986. Nematode: Karn et al., 1983. Chicken embryonic skeletal myosin: Molina et al., 1987. Rat embryonic skeletal myosin: Strehler et al., 1986.

junction, respectively (Sutoh et al., 1987; Sutoh, 1987; Tokunaga et al., 1987). Our result showed that Glu-88 to Met-92 are also in this part of the head structure.

Studies have shown that sequence conservation at certain segments of the primary structure of proteins can be an indication of functional importance. Comparison of amino acid sequences of S1 from a variety of origins has shown that both SH-1 and Glu-88 are in the very conserved region (Figure 6). Although they are not directly involved in nucleotide binding, the integrity of these regions may be important to maintain the conformation of the catalytic site.

Similar studies have been carried out on smooth muscle myosin from chicken gizzard, and we found that cross-links can be made only to the 50-kDa region but not to the 25-kDa region (Nag et al., 1988). Since the photoreaction is not limited to a certain type of side chain, replacement at Glu-88 by a different amino acid in S1 from gizzard would not account for the absence of 20–25-kDa-type cross-linking. In fact, Glu-88 and Asp-89 have been conserved in gizzard myosin (Onishi et al., 1986; Maita et al., 1987), suggesting that the folding of gizzard myosin S1 is different from that of the skeletal myosin.

Work is in progress to determine the residues that are close to SH-1 in the presence of Mg^{2+}-nucleotide. For the 20–25-kDa-type cross-linking, results were similar to that reported above, confirming our earlier studies that the proximity relationship between SH-1 and the 25-kDa region is nucleotide independent (Lu et al., 1986; Sutoh & Lu, 1987).

Registry No. BPIA, 76809-63-7; Glu, 56-86-0; Asp, 56-84-8; Met, 63-68-3; Cys, 52-90-4; ATPase, 9000-83-3.

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