A Short Hydrophobic Segment Next to Tryptophan-130 in Myosin Heavy Chain Is Close to the Ribose Ring of ADP Bound in the Adenosinetriphosphatase Site†

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ABSTRACT: The ATPase site of rabbit skeletal myosin was covalently labeled by an ADP analogue that carried a biotin moiety on its adenine ring and a photoreactive phenyl azide group on its ribose ring [Sutoh, K., Yamamoto, K., & Wakabayashi, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 212–216]. The ADP analogue was tightly trapped into the ATPase site in the presence of vanadate ions and then covalently cross-linked to the site by UV irradiation. The N-terminal 23 000-dalton tryptic fragment of the heavy chain was selectively labeled with the analogue. Further mapping of the labeled segment along the 23-kDa fragment was carried out by "end-label fingerprinting" which employed site-directed antibodies against both ends of the N-terminal heavy chain fragment. The mapping revealed that a hydrophobic segment of ~10 residues next to Trp-130, which was reported to be in proximity to the adenine ring of ADP bound to the ATPase site [Okamoto, Y., & Yount, R. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1575–1579], was the site of covalent labeling with the ADP analogue. The result indicates that the hydrophobic segment is close to the ribose ring of ADP bound to the ATPase site.

Cyclic association–dissociation of actin and myosin coupled with ATP hydrolysis by myosin ATPase[1] is the most essential process of muscle contraction. In order to understand the process at the molecular level, it is important to elucidate the molecular architecture of myosin, especially its structure around the ATPase site.

Location of the ATPase site along polypeptide chains of myosin has been investigated by using the affinity labeling technique. The N-terminal 23 000-dalton (23-kDa) tryptic fragment of the heavy chain was selectively labeled by a photoreactive ATP analogue, arylazido-β-alanyl-ATP (Szilagyi et al., 1979). Trp-130 in the 23-kDa tryptic fragment was a residue covalently labeled with another photoreactive ADP analogue, *N*-(4-azido-2-nitrophenyl)-2-aminoethyl diphosphate (Okamoto & Yount, 1985). Furthermore, Ser-324 in the 50 000-dalton (50-kDa) tryptic fragment of the heavy chain was identified as a residue labeled with 3′-*O*-(4-benzoylbenzoyl)-ADP (Mahmood & Yount, 1984; Yount et al., 1985). Another way to obtain information about residues or segments participating in the ATPase site is to compare sequences of ATP-binding proteins (Walker et al., 1982; Fry et al., 1986). Heavy chain sequence in the glycine-rich region (residues 178–185) is highly homologous to those of ATP-binding sites of other ATP-binding proteins, implying that the glycine-rich segment is in the ATPase site.

The ATPase site of myosin has been located on its three-dimensional structure by using a photoreactive biotinylated ADP analogue as an electron microscopic probe (Sutoh et al., 1986; Tokunaga et al., 1987). The site is on the middle part of the head where it shows a sharp bend. Distance from the tip of the head to the site is about 5 nm while total length of the head along its long axis is 20 nm. The actin-binding site is on the other side of the head. Therefore, the ATPase site is located outside when myosin head is on F-actin to form the rigor complex.

In this paper, location of the cross-linked biotinyl ADP analogue that was used as an electron microscopic probe of the ATPase site was mapped along the myosin heavy chain by the "end-label fingerprinting" technique which employed chemical labeling of the N-terminus and/or C-terminus of polypeptide chains (Sutoh, 1982, 1983; Jay, 1984; Jue & Doolittle, 1985) or antibody staining of the termini (Matsudaira et al., 1985; Sutoh & Mabuchi, 1986; Sutoh & Lu, 1987). The mapping showed that a hydrophobic segment of ~10 residues next to Trp-130 contained a residue (or residues) labeled by the biotinyl ADP analogue. Thus location of the ATPase site of myosin has been identified both in its three-dimensional structure and in its primary structure by using the same ADP analogue.

Materials and Methods

Proteins and Reagents. Myosin subfragment 1 (S1) was prepared from rabbit skeletal muscle myosin according to the method of Weeds and Taylor (1975). Avidin D was purchased from Vector Laboratories (Burlingame, CA). DCCP-treated trypsin and TLCK-treated chymotrypsin were purchased from Sigma (St. Louis, MO). Alkaline phosphatase labeled anti-rabbit IgG (affinity purified) was from Kirkegaard & Perry Laboratories, Inc. (KPL) (Gaithersburg, MD). The photoreactive biotinylated ADP analogue was synthesized as previously described (Sutoh et al., 1986). 2-Nitro-5-thiocyanobenzoic acid (NTCB) was synthesized according to methods of Vanaman and Stark (1970) and Degani and Patchornik (1974). Biotinylated agarose was purchased from Vector Laboratories. Durapore membrane (type GV, 0.22 μm) was from Millipore (Bedford, MA).

Antibodies. A site-directed antibody against the heavy chain N-terminus of rabbit skeletal muscle myosin was produced by using a synthetic peptide with the sequence Ac-Ser-Ser-Asp-Ala-Asp-Met-Ala-Val-Lys as described (Sutoh et al., 1987). Another antibody was directed to the junction between the 23-

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[1] Abbreviations: ATPase, adenosinetriphosphatase; S1, myosin subfragment 1; NTCB, 2-nitro-5-thiocyanobenzoic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CNBr, cyanogen bromide; BSA, bovine serum albumin; L1, tryptic fragment of alkaline light chain 1; L3, alkaline light chain 2; kDa, kilodalton(s); TLCK, N^{α}-tosyllysine chloromethyl ketone; IgG, immunoglobulin G; DCCP, diphenylcarbamyl chloride.

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and 50-kDa segments of the heavy chain. A peptide with the sequence Cys-Thr-Gly-Asp-Lys-Lys-Lys-Glu-Glu-Pro-Thr-Pro-Gly-Lys-Met was synthesized by the solid-phase method. The peptide sequence corresponds to heavy chain residues 201–214 (Tong & Elzinga, 1983) except for the first Cys residue introduced as an anchoring site for BSA. The peptide was coupled with BSA by using sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate (sulfo-SMPB) (Pierce, Rockford, IL) as a cross-linker. To 10 mg of BSA in 500 μL of 0.1 M phosphate buffer (pH 7.0) was added 3 mg of sulfo-SMPB freshly dissolved in 50 μL of dimethyl formamide. The modification reaction was allowed to proceed for 30 min at room temperature. The solution was then passed through a Sephadex G-25 column to remove free sulfo-SMPB from the modified BSA. Elution was carried out by 0.15 M NaCl and 50 mM phosphate buffer (pH 7.0). The flow-through fraction was mixed with 10 mg of the peptide. The coupling reaction was allowed to proceed for 3 h at room temperature. The resulting solution was dialyzed against the same solvent as above. Epitope density of the resulting peptide–BSA complex was about 20, as revealed by quantitative amino acid analysis. The peptide–BSA complex was injected to a New Zealand White rabbit to raise the antibody against the peptide as described (Bulinski et al., 1983).

These antibodies were characterized by the Western blotting (Towbin et al., 1978) and by enzyme-linked immunosorbent assay (ELISA) as described (Sutoh & Mabuchi, 1986; Sutoh & Lu, 1987).

Covalent Incorporation of the ADP Analogue into the ATPase Site. The ATPase site of S1 was covalently labeled with the biotinylated ADP analogue as described (Sutoh et al., 1986). Extent of the modification of the ATPase site was about 10%.

Digestion of S1 Heavy Chain and Isolation of Biotinylated Peptides. S1 labeled with the biotinylated photoreactive ADP analogue was digested with trypsin in 50 mM NaCl and 20 mM MOPS (pH 7.0) at a weight ratio of 1/50 (trypsin/S1) at 25 °C for 20 min. The digest was mixed with 0.1 volume of 10% $NaDodSO_4$ and 0.01 volume of 2-mercaptoethanol and immediately boiled for 5 min. After dialysis against 20 mM Tris-HCl (pH 8.0) containing 0.1% $NaDodSO_4$ and 0.1% 2-mercaptoethanol, a portion of the digest (2 mg/mL) was further digested either with trypsin or with chymotrypsin at 25 °C for 20 min at weight ratios of 1/10 and 1/100, respectively. Cleavage reactions were quenched by boiling digests for 5 min.

Biotinylated peptides were isolated from the trypsin-treated S1 and also from the tryptic and the chymotryptic digests of the trypsin-treated S1 as follows. An aliquot in 20 mM Tris-HCl (pH 8.0) containing 0.1% $NaDodSO_4$ and 0.1% 2-mercaptoethanol (about 200 μL) was mixed with avidin D to make its final concentration 0.2 mg/mL. Large excess of avidin over an expected amount of biotinylated fragments was added to ensure complete recovery of these fragments. The solution was gently shaken for 30 min at room temperature. To the solution was added biotin–agarose beads prewashed with 20 mM Tris-HCl (pH 8.0) containing 0.1% $NaDodSO_4$ (20 μL). The resulting solution was gently shaken for 1 h at room temperature. The beads were collected by centrifugation in an Eppendorf centrifuge. The precipitated beads were washed with 1 mL of 20 mM Tris-HCl (pH 8.0) containing 0.1% $NaDodSO_4$ by gently shaking for 10 min. The beads were collected by centrifugation. The wash was repeated 3 times. The beads were then washed with 1 mL of 0.5 M NaCl and 20 mM Tris-HCl (pH 8.0) containing 0.05% Tween 20 (ELISA grade, Bio-Rad). The beads were finally washed with 1 mL of 20 mM Tris-HCl (pH 8.0) containing 0.1% $NaDodSO_4$. After centrifugation, 20 μL of Laemmli's sample buffer (Laemmli, 1970) was added to the washed beads. The resulting slurry was boiled for 5 min and centrifuged. The supernatant was used for the Western blotting analysis.

Chemical Cleavages. S1 in 50 mM NaCl and 20 mM MOPS (pH 7.0) was digested with trypsin as above. It was dialyzed against water and lyophilized. To 100 μg of protein, 100 μL of 50 mM CNBr in 70% formic acid was added. CNBr cleavage was allowed to proceed for 1 h at 25 °C. Then 2 μL of 2-mercaptoethanol was added and solvent was evaporated.

To 100 μg of the lyophilized trypsin-treated S1 was added 100 μL of 6 M urea–50 mM Tris-HCl (pH 8.0). To the resulting solution was added 2 μL of 0.1 M NTCB. After the solution was incubated for 30 min at 25 °C, the pH of the reaction mixture was raised to 9.0 by Tris base to initiate the cleavage reaction. The reaction was allowed to proceed for 6 h at 37 °C. It was quenched just by adding excess amount of 2-mercaptoethanol.

Western Blotting. Western blotting (Towbin et al., 1978) was carried out as previously described (Sutoh & Mabuchi, 1986; Sutoh & Lu, 1987) with slight modifications. Antisera against synthetic peptides were diluted 1000-fold with 0.5 M NaCl and 20 mM Tris-HCl containing 0.05% Tween 20. Blots were washed with the same solvent. Secondary antibody was affinity-purified anti-rabbit IgG coupled with alkaline phosphatase (KPL). It was diluted 1000-fold with the above solvent. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (KPL) were used as chromogen.

Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was carried out according to Laemmli (1970), using acrylamide gels [15% acrylamide–0.43% bis(acrylamide)]. Apparent molecular weights were estimated by using standard proteins and peptides: aldolase (M_r 40 000), carbonic anhydrase (M_r 29 000), and myoglobin and its CNBr fragments (M_r 17 000, 14 400, 8200, 6200, and 2500).

RESULTS

Site-Directed Antibodies as Probes of N- and C-Termini of the N-Terminal Heavy Chain Fragment. S1 was digested with trypsin to cleave the 95 000-dalton (95-kDa) heavy chain into three fragments (the 23-, 50-, and 20-kDa fragments) (Mornet et al., 1979). The trypsin-treated S1 was electrophoresed in the presence of $NaDodSO_4$, blotted onto membrane, and then stained with the antibody against the N-terminal eight residues of the rabbit skeletal myosin heavy chain (Sutoh & Lu, 1987). On the blot, the N-terminal 23-kDa fragment of the heavy chain spanning residues 1–204 (Tong & Elzinga, 1983; Maita et al., 1987) was visible (lane B in Figure 1), consistent with location of the sequence used for antibody production. Besides the major 23-kDa fragment, a minor band with its apparent molecular weight of 24 000 was also visible just above the 23-kDa band (lane B). The same 24-kDa band was detected on the blot stained by gold colloid to visualize all peptides (Moeremans et al., 1985) (arrowhead in lane A in Figure 1). Intensity ratio of the major band to the minor band was about 10. Judging from its molecular weight, it seemed that the minor band was generated by trypsin cut at Lys-213 (Tong & Elzinga, 1983; Maita et al., 1987). Thus the 24-kDa fragment was identified as a peptide consisting of the 23-kDa fragment and an extra segment of residues 205–213. This identification of the 24-kDa fragment was further supported by the Western blotting with the antibody against the 23–50-kDa junction peptide whose

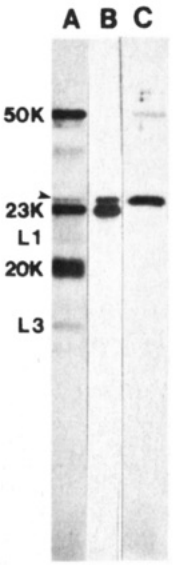

FIGURE 1: Specificity of antibodies against heavy chain N-terminus and against the 23–50-kDa junction peptide. Trypsin-treated S1 was electrophoresed in the presence of $NaDodSO_4$ and then blotted onto Durapore membrane. Lane A: Blot was stained with colloidal gold to visualize all peptides. The 24-kDa fragment is indicated by an arrowhead. Lane B: Blot was stained with antibody against heavy chain N-terminus. Lane C: Blot was stained with antibody against the 23–50-kDa junction peptide. Note that the 23- and 24-kDa fragments are visible on lane B while only the 24-kDa fragment is visible on lane C. L1: Alkaline light chain 1 degraded to a shorter fragment by tryptic digestion. L3: Alkaline light chain 2.

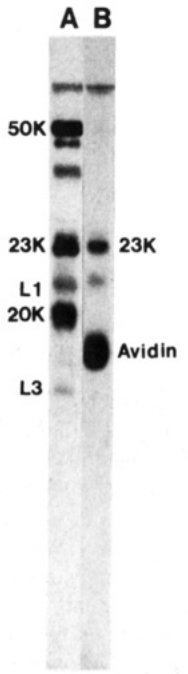

FIGURE 2: Identification of biotinylated fragments by avidin–biotin retrieving system. Trypsin-treated S1 and retrieved biotinyl peptides were electrophoresed in the presence of $NaDodSO_4$, blotted on membrane, and then stained with colloidal gold to visualize all peptides. Lane A: Blot of trypsin-treated S1. Lane B: Blot of biotinylated peptides retrieved from the trypsin-treated S1. L1: Alkaline light chain 1 degraded to a shorter fragment by tryptic digestion. L3: Alkaline light chain 2.

sequence was Thr-Gly-Asp-Lys-Lys-Lys-Glu-Glu-Pro-Thr-Pro-Gly-Lys-Met (residues 201–214 in the heavy chain sequence) (Tong & Elzinga, 1983). When a blot of the trypsin-treated S1 was stained with the antibody, the 24-kDa fragment was visible while the 23-kDa fragment was missing (lane C in Figure 1). The 50-kDa tryptic fragment was only slightly stained with the antibody, indicating that its major epitope spanned the C-terminal region of the 23-kDa fragment and the N-terminal region of the 50-kDa fragment. Since the trypsin-treated S1 always contained the 24-kDa fragment, though in a small amount, the antibody against the 23–50-kDa junction was used to map the biotinylated site along the 24-kDa fragment (the 23-kDa fragment plus an extra C-terminal segment of nine residues) from its C-terminus while the antibody against the heavy chain N-terminus was used to map the site from the N-terminus.

Biotinylated ADP Analogue Was Incorporated into the 23-kDa Fragment. S1 was covalently labeled with the biotinylated ADP analogue as described (Sutoh et al., 1986). Under the present conditions, about 10% of the ATPase site was labeled with the analogue. The biotinylated S1 was then treated by trypsin to cleave the 95-kDa heavy chain into the 23-, 50-, and 20-kDa fragments. Biotinylated peptides in the trypsin-treated S1 were retrieved by avidin and biotin–agarose under denaturing conditions (see Materials and Methods). Examination of the retrieved fraction by the $NaDodSO_4$ gel electrophoresis revealed that the 23-kDa fragment was the main heavy chain fragment retrieved from the trypsin digest (lane B in Figure 2). Besides the 23-kDa fragment, a peptide with a molecular weight of 21 000 was detected as a minor component (lane B). Since the amount of this fragment increased on longer trypsin treatment with concomitant decrease of the 23-kDa fragment, the 21-kDa fragment seems to be a breakdown product of the 23-kDa fragment. This 21-kDa fragment was not stained with the antibody against the N-terminus of the 23-kDa fragment (lane B in Figure 1). Therefore, the N-terminal region of the parent 23-kDa fragment is missing in the 21-kDa peptide. Besides the 23- and 21-kDa fragments, the 24-kDa fragment was also present in the retrieved fraction as revealed by the fact that the 24-kDa band was clearly visible on the blot of the retrieved fraction stained by the antibody against the 23–50-kDa junction peptide (data not shown). Neither the 50-kDa fragment nor the 20-kDa fragment was retrieved from the digest by the avidin–biotin system. Two light chains (alkaline light chains 1 and 2) were also missing in the retrieved fraction. These results led us to conclude that the biotinylated ADP analogue was selectively incorporated into the 23-kDa heavy chain fragment.

Strategy for Mapping the Biotinylated Site along the 23-kDa Fragment. Location of the covalently cross-linked ADP analogue along the 23-kDa fragment was determined by using end-label fingerprinting, schematically illustrated in Figure 3. First, the biotinylated 23-kDa fragment was further subjected to partial cleavage by an enzyme. For simplicity of our argument, we assume that there are two cleavage sites along the 23-kDa fragment (Figure 3). Cleavage sites are indicated as closed triangles 1 and 2. Partial cleavages of the 23-kDa fragment at sites 1 and 2 result in generation of six fragments, among which four peptides shown in the figure carry the biotinylated ADP. Those biotinylated fragments are retrieved by avidin and immobilized biotin, electrophoresed in the presence of $NaDodSO_4$, and then blotted on membrane. On the blot, peptides containing the N-terminus of the 23-kDa fragment are visualized with the antibody against the heavy chain N-terminus. We observe two bands corresponding to a peptide spanning the N-terminus and the cleavage site 2 (N-2) and the parent 23-kDa fragment (N-C) (Figure 3). When the total digest is blotted as a control, however, three bands (N-1, N-2, and N-C) are visible. Thus comparison of these two blots indicates that the fragment spanning the N-terminus and the cleavage site 1 (N-1) does not carry the

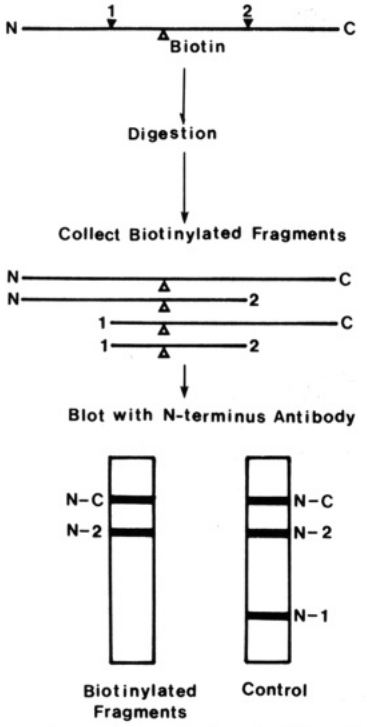

FIGURE 3: Scheme of experiments for locating biotinylated fragments along the tryptic 23-kDa fragment. The 23-kDa fragment is shown as a bar at the top of the figure. The cross-linked biotin moiety is represented by an open triangle on the bar. Cleavage sites 1 and 2 are also indicated. Four types of biotinylated fragments generated by partial cleavages at sites 1 and 2 are shown. Band patterns of blots stained with the heavy chain N-terminus are shown for the total digest (control) and for retrieved biotinyl fragments (biotinylated fragments).

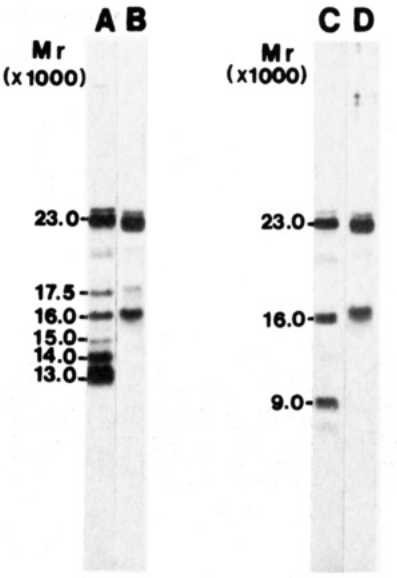

FIGURE 4: Mapping of biotinylated segment by end-label fingerprinting. Trypsin-treated biotinyl S1 was further digested either by chymotrypsin or by trypsin under denaturing conditions. From the digest, biotinylated fragments were retrieved by the avidin–biotin system. Peptides were blotted onto membranes after the $NaDodSO_4$ gel electrophoresis and visualized by the antibody against the heavy chain N-terminus. Lane A: Total chymotryptic digest. Lane B: Biotinylated fragments retrieved from the chymotryptic digest. Lane C: Total tryptic digest. Lane D: Biotinylated fragments retrieved from the tryptic digest.

biotin moiety while the longer N-2 fragment does. In other words, the biotinylated site is located between cleavage sites 1 and 2. The antibody against the heavy chain N-terminus thus detects the biotinylated site from the N-terminus of the 23-kDa fragment. Similar experiments using the antibody against the 23-50-kDa junctional peptide will reveal the location of the biotinylated site from the C-terminus of the 24-kDa fragment. Results obtained by mappings with the two types of antibodies are complementary.

In actual experiments, trypsin-treated S1 was used in place of the isolated 23-kDa fragment. Since other heavy chain fragments and light chains are not visible on blots stained with the antibodies, the same argument as above holds in experiments described below.

Mapping the Biotinylated Segment by Chymotrypsin Digestion. The biotinylated and trypsin-treated S1 was further digested with chymotrypsin in the presence of $NaDodSO_4$. The Western blot of the total digest (the control blot) was stained with antibody against the heavy chain N-terminus. A ladderlike band pattern was observed on the blot (lane A in Figure 4). When biotinylated fragments retrieved from the chymotryptic digest were blotted and stained with the N-terminus antibody, however, the 23-, 17.5-, and 16-kDa fragments were visible while the 13-, 14-, and 15-kDa fragments observed on the control blot were missing (lane B in Figure 4). All the fragments visible on the control blot were generated when the biotinylated 23-kDa fragment was first isolated by avidin and immobilized biotin (see Materials and Methods) and then subjected to chymotryptic digestion (data not shown). Therefore, the 13-, 14-, and 15-Da fragments were missing in the retrieved fraction because they did not contain the cross-linked biotin moiety, not because the digestion pattern was disturbed due to the biotinylation. All fragments visible on these blots carried the heavy chain N-terminus since they were stained with the N-terminus antibody. Thus the above results indicated that the N-terminal 16-kDa fragment of the heavy chain carried the biotin moiety while the N-terminal 15-kDa fragment did not. Furthermore, the intensity ratio of the visible bands in lane B was very similar to that of corresponding bands in the control blot (lane A). These results led us to conclude that the cross-linked biotin moiety was mainly within a heavy chain segment between two cleavage points generating the N-terminal 15-kDa and 16-kDa chymotryptic fragments (Figure 6).

The total chymotryptic digest and the biotinylated fragments retrieved from the total digest were blotted as above. These two blots were stained by the antibody against the 23–50-kDa junction peptide (lanes A and B in Figure 5). These two blots showed very similar band patterns. The shortest fragment visible on both of these blots had an apparent molecular weight of 12 000. Therefore, the main biotinylated site was located within the C-terminal 12-kDa segment of the 24-kDa fragment (Figure 6).

Mapping the Biotinylated Site with Trypsin Digestion. The biotinylated and trypsin-treated S1 was further cleaved by trypsin in the presence of $NaDodSO_4$. Blots of the total digest and the retrieved biotinyl fragments were stained with the antiobody against the heavy chain N-terminus (lanes C and D in Figure 4). On the blot of the total digest (lane C), fragments with apparent molecular weights of 9000 and 16 000 were visible besides the parent 23-kDa fragment. On the blot of the retrieved biotinyl fragments, however, the 9-kDa fragment was missing. The shortest fragment containing both the N-terminus and the biotin moiety was the 16-kDa fragment. The intensity ratio of the visible bands in lane D was very similar to that of the corresponding bands in lane C. These results led us to conclude that the main biotinylated site was within a segment spanning two trypsin cleavage sites generating the 9- and 16-kDa N-terminal fragments (Figure 6).

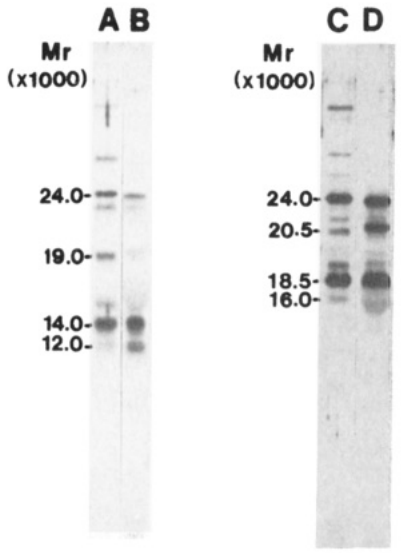

FIGURE 5: Mapping of biotinylated segments by end-label fingerprinting. Trypsin-treated biotinyl S1 was further digested either by chymotrypsin or by trypsin under denaturing conditions. Total digests and biotinylated fragments retrieved from the digests were blotted and stained by the antibody against the 23–50-kDa junction peptide. Lane A: Total chymotryptic digest. Lane B: Biotinylated fragments retrieved from the chymotryptic digest. Lane C: Total tryptic digest. Lane D: Biotinylated fragments retrieved from the tryptic digest. Note that apparent molecular weight of the parent peptide is 24 000.

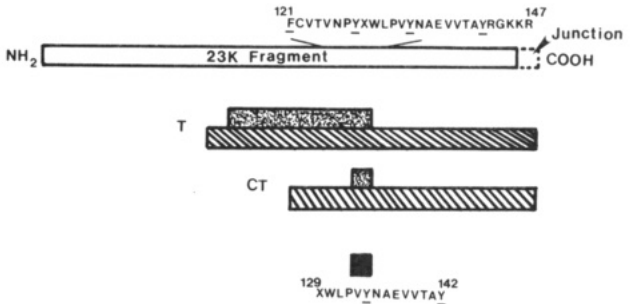

FIGURE 6: Maps of biotinylated segment in 23-kDa fragment. The top open bar represents the 23-kDa fragment. The 23–50-kDa junction peptide is also indicated by the open bar outlined with dots. The amino acid sequence from Phe-121 to Arg-147 is shown (X is trimethyllysine). Bars shaded with dots represent differential segments containing the cross-linked biotin moiety as identified by the antibody against heavy chain N-terminus. Cross-hatched bars represent segments containing the biotin moiety as identified by the antibody against the 23–50-kDa junction peptide. T and CT represent mappings by trypsin and chymotrypsin, respectively. Some residues in the segment shown by the closed bar (residues 129–142) are labeled with the biotinylated ADP analogue.

The total tryptic digest and the biotinylated fragments retrieved from the total digest were blotted and stained by the antibody against the 23–50-kDa junction peptide (lanes C and D in Figure 5). The band pattern generated from the biotinylated fragments was very similar to that from the total tryptic digest (lane C vs lane D in Figure 5). The shortest fragment visible on both of these blots was one with an apparent molecular weight of 16 000. Thus the main biotinylated site was located within the C-terminal 16-kDa fragment of the 24-kDa fragment (Figure 6).

Identification of the Biotinylated Segment. Maps of the biotinylated segment obtained by end-label fingerprinting with chymotryptic and tryptic cleavages are summarized in Figure 6. From these maps, it is concluded that the biotinylated site is within the segment between two cleavage sites generating the N-terminal 15- and 16-kDa fragments (closed box in Figure 6).

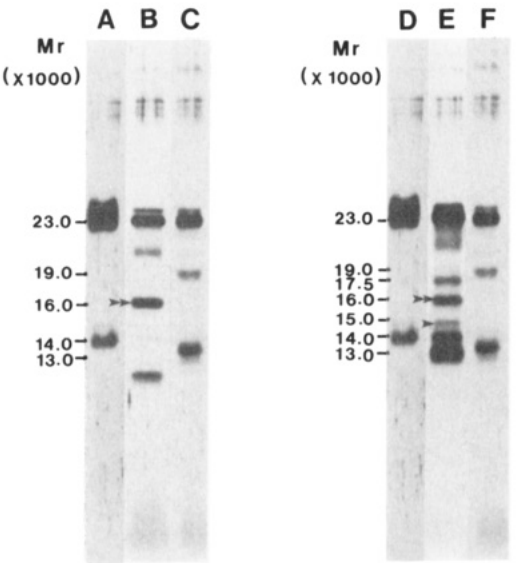

FIGURE 7: Western blots of tryptic and chymotryptic digests of the 23-kDa fragment are compared with those of NTCB and CNBr digests. Blots were stained by the antibody against heavy chain N-terminus. Lanes A and D: NTCB digest of the 23-kDa fragment. Lanes C and F: CNBr digest. Lane B: Tryptic digest. Lane E: Chymotryptic digest. The 16-kDa fragments in the tryptic and chymotryptic digests are indicated by double arrowheads in lanes B and E. The 15-kDa fragment in the chymotryptic digest is indicated by an arrowhead in lane E.

In order to identify the actual sequence corresponding to the segment, cleavage patterns of the 23-kDa fragment by chymotrypsin and trypsin were compared with those generated by CNBr and NTCB, which cleave peptide bonds at Met and Cys residues, respectively. The trypsin-treated S1 was further digested with NTCB to cleave the 23-kDa fragment at Cys-122, a single Cys residue in the 23-kDa fragment (Tong & Elzinga, 1983). When the digest was blotted after the Na-DodSO_4 gel electrophoresis and stained with the antibody against the heavy chain N-terminus, a fragment with an apparent molecular weight of 14 000 was visible (lanes A and D in Figure 7). Considering the specificity of the NTCB cleavage (Vanaman & Stark, 1970; Degani & Patchornik, 1974), it was most likely that the 14-kDa fragment spanned residues 1–121. The true molecular weight of the fragment was calculated as 13 800 from its amino acid sequence (Tong & Elzinga, 1983), consistent with its apparent molecular weight. The trypsin-treated S1 was also digested with CNBr. The digest was blotted and stained with the N-terminus antibody as above. Two fragments with apparent molecular weights of 19 000 and 13 000 were visible on the blot. The 23-kDa tryptic fragment of the heavy chain contains six Met residues: Met-165, Met-113, Met-93, Met-92, Met-90, and Met-79 (Tong & Elzinga, 1983). From its molecular weight, it was likely that the 19-kDa fragment spanned residues 1–165. Its molecular weight was calculated as 18 800 from its amino acid sequence (Tong & Elzinga, 1983). When the blot of the trypsin digest was stained with the antibody against the heavy chain N-terminus and compared with those of the NTCB and CNBr digests (lane B vs lanes A and C in Figure 7), it was apparent that the cleavage site generating the 16-kDa fragment was located between Cys-122 and Met-165. Examination of the sequence between Cys-122 and Met-165 (Tong & Elzinga, 1983) revealed that possible trypsin cleavage sites generating the 16-kDa fragment were within a cluster of basic residues, i.e., Arg-Gly-Lys-Lys-Arg (residues 143–147).

The N-terminal 16-kDa fragment in the chymotryptic digest of the trypsin-treated S1 (lane E in Figure 7) comigrated with

the N-terminal 16-kDa fragment in the tryptic digest. Considering its molecular weight and the amino acid sequence around the cluster of basic residues mentioned above, the most likely candidate of the chymotryptic cleavage site for the 16-kDa fragment is Tyr-142. Cleavages at other Tyr or Phe residues [for example, Phe-155 or Tyr-134 (Tong & Elzinga, 1983)] will generate fragments distinguishable from the tryptic 16-kDa fragment. Thus the chymotryptic cleavage site generating the 15-kDa fragment (lane E in Figure 7) must be within a segment spanning Cys-122 and Tyr-142. Candidates for the site are Tyr-128 and Tyr-134. Cleavages at Tyr-128 and Tyr-134 will generate fragments with molecular weights of 14 500 and 15 300, respectively. Considering errors in molecular weight estimation, it is difficult to decide which residue is the cleavage site generating the 15-kDa fragment. However, we can conclude that the biotinylated ADP is within the segment spanning residues, at most, 129–142. The sequence of residues 129–142 is Me_3-Lys-Trp-Leu-Pro-Val-Tyr-Asn-Ala-Glu-Val-Val-Thr-Ala-Tyr (Tong & Elzinga, 1983).

Discussion

Biotinylated photoreactive ADP analogue was previously used to introduce a biotin moiety into the ATPase site (Sutoh et al., 1986). The biotin moiety of the ADP analogue covalently cross-linked into the ATPase site was then visualized by attaching avidin. Electron microscopic examinations revealed that avidin bound at the middle part of the myosin head which was labeled with the ADP analogue (Sutoh et al., 1986), indicating that the ATPase site was located there. The avidin–biotin probe was further exploited to determine three-dimensional location of the ATPase site of myosin by using the three-dimensional reconstruction of electron microscopic images of the avidin–S1–tropomyosin–actin complex in which S1 was labeled with the biotinylated ADP analogue (Tokunaga et al., 1987). The ATPase site was located at the middle of the head and on the side opposite to the actin-binding site.

In this paper, location of the covalently bound biotinylated ADP analogue was identified along the myosin heavy chain by using end-label fingerprinting. The biotinylated site was mapped within a segment spanning residues 129–142. Since the photoreactive group is on the 3′-OH group of the ribose ring of the ADP analogue, the result indicates that some residues in the segment are close to the ribose ring of ADP bound in the ATPase site. It must be noted here that *N*-(4-azido-2-nitrophenyl)-2-aminoethyl diphosphate, a photoreactive ADP analogue, covalently labeled Trp-130 (Okamoto & Yount, 1985). Since its phenyl azide group seems to be an analogue of the adenine ring of ADP, it is likely that Trp-130 is very close to the adenine ring of ADP bound in the ATPase site. Taking these findings into account, it is concluded that the segment of ~10 residues following Trp-130 is in proximity to the ribose ring while several residues around Trp-130 are close to the adenine ring. It is noteworthy that this segment is rather hydrophobic except Glu-137. It has been previously shown that in adenylate kinase the ribose ring of bound ATP is surrounded by hydrophobic residues (Fry et al., 1986).

Examination of the heavy chain sequence around the hydrophobic segment reveals that a cluster of basic residues, i.e., Arg-Gly-Lys-Lys-Arg (residues 143–147), follows the hydrophobic segment (Tong & Elzinga, 1983). In nematode myosin, the sequence is Met-Gly-Lys-Arg-Lys (Karn et al., 1983) while it is Lys-Gly-Arg-Arg-Arg in *Dictyostelium* myosin (Warrick et al., 1986). Although the exact sequence of the basic cluster has not been conserved during evolution, its basic nature has been maintained. The fact implies some important functions for those basic residues. Thus it is tempting to speculate that the basic cluster might participate in binding the phosphate groups of ADP (Fry et al., 1986).

In the S1 heavy chain, a segment spanning residues 178–185 (Gly-Glu-Ser-Gly-Ala-Gly-Lys-Thr), the so-called "glycine-rich loop", has a sequence homologous to other sequences of ATP-binding sites of various ATPases and ATP-binding proteins (Walker et al., 1982; Fry et al., 1986), suggesting that the segment forms a part of the ATPase site in myosin too. However, a recent cross-linking experiment has shown that the segment is close to the SH_2 thiol (Sutoh and Hiratsuka, unpublished results). Since the SH_2 thiol is quite close to the SH_1 thiol in the primary sequence (Gallagher & Elzinga, 1980) and in the three-dimensional structure (Burke & Reisler, 1977), the result indicates a possibility that the glycine-rich loop is very close to the SH_1 thiol too. Three-dimensional reconstruction of electron microscopic images of the avidin–S1–tropomyosin–actin complex in which the SH_1 thiol of S1 was labeled with iodoacetamide derivative of biotin (IAA-biotin) (Sutoh et al., 1984) has shown that the SH_1 thiol is on the side opposite to the ATPase site, though both the ATPase site and the SH_1 thiol are at the middle region of the head (Sutoh et al., 1984, 1986; Tokunaga et al., 1987; Toyoshima et al., unpublished results). Therefore, some distance is expected between the ATPase site and the SH_1 thiol, consistent with previous fluorescence energy transfer experiments (Tao & Lamkin, 1981). Taking these results into account, it is possible that the glycine-rich loop does not directly participate in the ATPase site. It must be noted that the heavy chain segment around Trp-130 which has been identified to be in or, at least, close to the ATPase site has no homology to the glycine-rich loop or any other consensus sequences for the ATP-binding site (Walker et al., 1982; Fry et al., 1986).

Registry No. ATPase, 9000-83-3; Trp, 73-22-3.

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Reactions of the Sarcoplasmic Reticulum Calcium Adenosinetriphosphatase with Adenosine 5′-Triphosphate and Ca^{2+} That Are Not Satisfactorily Described by an E_1–E_2 Model†

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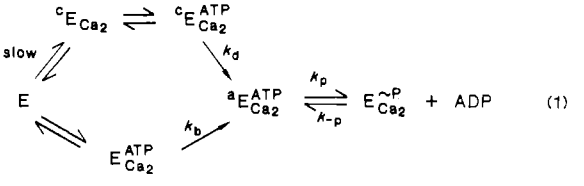
ABSTRACT: Phosphorylation of the sarcoplasmic reticulum calcium ATPase, E, is first order with k_b = 70 ± 7 s^{-1} after free enzyme was mixed with saturating ATP and 50 μM Ca^{2+}; this is one-third the rate constant of 220 s^{-1} for phosphorylation of enzyme preincubated with calcium, $^cE{\cdot}Ca_2$, after being mixed with ATP under the same conditions (pH 7.0, Ca^{2+}-loaded vesicles, 100 mM KCl, 5 mM Mg^{2+}, 25 °C). Phosphorylation of E with ATP and Ca^{2+} in the presence of 0.25 mM ADP gives ~50% $E{\sim}P{\cdot}Ca_2$ with k_{obsd} = 77 s^{-1}, not the sum of the forward and reverse rate constants, $k_{obsd} = k_f + k_r$ = 140 s^{-1}, that is expected for approach to equilibrium if phosphorylation were rate limiting. These results show that (1) k_b represents a slow conformational change, rather than phosphoryl transfer, and (2) different pathways are followed for the phosphorylation of E and of $^cE{\cdot}Ca_2$. The absence of a lag for phosphorylation of E with saturating ATP and Ca^{2+} indicates that all other steps, including the binding of Ca^{2+} ions and phosphoryl transfer, have rate constants of >500 s^{-1}. Chase experiments with unlabeled ATP or with ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) show that the rate constants for dissociation of [γ-^{32}P]ATP and Ca^{2+} are comparable to k_b. Dissociation of ATP occurs at 47 s^{-1} from $E{\cdot}ATP{\cdot}Ca^{2+}$ and at 24 s^{-1} from E·ATP. Approximately 20% phosphorylation occurs following an EGTA chase 4.5 ms after the addition of 300 μM ATP and 50 μM Ca^{2+} to enzyme. This shows that Ca^{2+} binds rapidly to the free enzyme, from outside the vesicle, before the conformational change (k_b). The fraction of Ca^{2+}-free E·[γ-^{32}P]ATP that is trapped to give labeled phosphoenzyme after the addition of Ca^{2+} and a chase of unlabeled ATP is half-maximal at 6.8 μM Ca^{2+}, with a Hill slope of n = 1.8. The calculated dissociation constant for Ca^{2+} from $E{\cdot}ATP{\cdot}Ca_2$ is ~2.2 × 10^{-10} M^2 ($K_{0.5}$ = 15 μM). The rate constant for the slow phase of the biphasic reaction of $E{\sim}P{\cdot}Ca_2$ with 1.1 mM ADP increases 2.5-fold when [Ca^{2+}] is decreased from 50 μM to 10 nM, with half-maximal increase at 1.7 μM Ca^{2+}. This shows that Ca^{2+} is dissociating from a different species, $^aE{\cdot}ATP{\cdot}Ca_2$, that is active for catalysis of phosphoryl transfer, has a high affinity for Ca^{2+}, and dissociates Ca^{2+} with $k \leq$ 45 s^{-1}. It is concluded that steady-state turnover of the ATPase under most conditions occurs through the $E{\cdot}ATP{\cdot}Ca_2$ pathway, which has a relatively low affinity for Ca^{2+}, not the pathway through $^cE{\cdot}Ca_2$ (or "$E_1{\cdot}Ca_2$"). This results in 11–17% unphosphorylated enzyme in the steady state at saturating [ATP] and [Ca^{2+}] because the k_b step is partly rate limiting. The two pathways for phosphorylation can result in nonlinear Lineweaver–Burk plots for ATP and initial overshoots of phosphoenzyme levels.

Reactions of the calcium ATPase (E)[1] of sarcoplasmic reticulum with its substrates ATP and Ca^{2+} can occur by two pathways, depending on the concentrations of ATP and Ca^{2+}. The upper pathway in eq 1 is the well-known pathway in which calcium binds first and causes a conformational change before



(1)

phosphorylation by ATP; the initial product of this reaction is designated $^cE{\cdot}Ca_2$ simply to indicate that it is the stable form

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