Chemical Identification of Serine 181 at the ATP-binding Site of Myosin as a Residue Esterified Selectively by the Fluorescent Reagent 9-Anthroylnitrile*

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The esterification reagent 9-anthroylnitrile (ANN) reacts with a serine residue in the NH2-terminal 23-kDa peptide segment of myosin subfragment-1 heavy chain to yield a fluorescent S1 derivative labeled by the anthroyl group (Hiratsuka, T. (1989) J. Biol. Chem. 264, 18188–18194). The labeling was highly selective and accelerated by nucleotides. In the present study, to determine the exact location of the labeled serine residue, the labeled 23-kDa peptide fragment was isolated. The subsequent extensive proteolytic digestion of the peptide fragment yielded two labeled peptides, a pentapeptide and its precursor nonapeptide. Amino acid sequence and composition analyses of both labeled peptides revealed that the anthroyl group is attached to Ser-181 involved in the phosphate binding loop for ATP (Smith, C. A., and Rayment, I. (1996) Biochemistry 35, 5404–5417). We concluded that ANN can esterify Ser-181 selectively out of over 40 serine residues in the subfragment 1 heavy chain. Thus ANN is proved to be a valuable fluorescent tool to identify peptides containing the phosphate binding loop of S1 and to detect the conformational changes around this loop.

Myosin subfragment-1 (S1) is the globular head region of the myosin molecule that contains the sites for ATPase and actin binding (1, 2). During ATP hydrolysis in the actomyosin complex, transient conformational changes in the ATPase site take place and are thought to be transmitted to the remote actin-binding site, thereby controlling the interaction of S1 with actin (3). On the other hand, the binding of actin to S1 is supposed to affect the conformation of the ATPase site, resulting in the acceleration of product release. Thus, elucidation of the mechanism of the energy transduction process in muscle contraction requires detailed knowledge of conformational changes of S1 that are involved in such communication between the ATPase- and actin-binding sites.

One approach for monitoring the ligand-induced conformational changes around a specific residue in proteins is to attach a sensitive fluorescent probe covalently to the residue. Because of its inherent high sensitivity, such a method is a powerful technique to obtain information about the conformational changes in proteins. However, in the case of tyrosine, threonine, and serine residues it is difficult to label them by a fluorescent reagent because the chemical reactivity of their hydroxyl groups in aqueous solution is low. This is also the case for S1. Although a series of photoactive analogs of ATP can modify Ser-181, Ser-243, and Ser-324 of S1 heavy chain (4, 5) (residue numbers in chicken skeletal S1, Ref. 6), there had been no fluorescent reagent available for the labeling not only of serine but also threonine and tyrosine residues in S1.

ANN appeared as a unique example of the serine-directed fluorescent reagent (7). ANN can esterify a serine residue of S1 located within the NH2-terminal 23-kDa peptide segment of the heavy chain (residues 2–204). It is characteristic that the labeling is highly selective and accelerated by nucleotides. The extrinsic fluorescence from the AN group labeled to S1 is sensitive to the binding of nucleotides and ATP hydrolysis (8, 9). Thus, the AN group is useful not only as a fluorescent tag for the 23-kDa segment of S1 heavy chain (10, 11) but also as a fluorescent conformation probe for the S1 ATPase (8, 9). However, the chemical determination of the exact location of the labeled serine residue has yet not been done.

In the present study, we isolate the pentapeptide and its precursor nonapeptide containing the AN group-labeled Ser-181, which is involved in the phosphate binding loop at the ATPase site of S1 (residues 179–186 of the heavy chain) (1, 2, 6). The results suggest that Ser-181 in S1 exhibits unusual reactivity as well as the reactive serine at the active site in serine peptidases (12). Labeling with ANN promises to be a valuable method to identify the peptides containing the phosphate binding loop of S1 and to detect ligand-induced conformational changes around this loop.

**EXPERIMENTAL PROCEDURES**

**Materials**—The commercial compounds used and their sources were as follows: ANN, LEP, and V8 (Wako Pure Chemicals Co.); CT, diphenyldimethylaminochlorotriptysin, soybean trypsin inhibitor, and SerNAc (Sigma); ATP (Kohjin Co.); Ultrogel AcA44 (IBF Biotechnics). AN-SerNAc was prepared as described previously (8).

**Protein Preparation**—Rabbit skeletal myosin and cryotryptic S1 were prepared as described previously (8). AN-S1, S1 labeled with ANN in the presence of ATP, was prepared as described previously (7). The labeled S1 contained 0.9 mol of AN group/mol of S1.

**Isolation of the 23-kDa Tryptic Fragment from AN-S1**—Experiments were carried out using the following buffers: buffer A (40 mM KCl and 5 mM 3-N-morpholino)propanesulfonic acid, pH 7.0), buffer B (0.1% SDS, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, and 20 mM sodium phosphate, pH 7.0), and buffer C (6 mM urea, 10 mM 2-mercaptoethanol, and 50 mM Tris acetate, pH 7.8).

The tryorphilized AN-S1 (160 mg) was dissolved in 26 ml of buffer A and dialyzed against the same buffer at 4 °C overnight. The AN-S1 was digested with trypsin (1.6 mg) at 25 °C for 1 h. The reaction was terminated by the addition of soybean trypsin inhibitor (2.4 mg). Dithi-
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othioctic acid, Gdn-HCl, and EDTA were added to 2 mM, 6 mM, and 2 mM, respectively, and the reaction mixture was incubated for 30 min at 25 °C. Then 3 volumes of cold ethanol were added to remove the COOH-terminal 20-kDa fragment and most of the 50-kDa fragment of S1 heavy chain and light chains. After the sample was left for 9 h at −30 °C, the resulting precipitate containing the 23-kDa fragment and a small amount of the 50-kDa fragment was collected by centrifugation, dissolved in 5 ml of buffer B, and dialyzed against the same buffer overnight. The dialysate was then applied to an Ultrogel AcA44 gel filtration column (2.2 × 84 cm) pre-equilibrated with buffer B. Elution with buffer B was carried out at a flow rate of 17 ml/h, and fractions of 3.4 ml were collected. Fractions containing the fluorescent 23-kDa tryptic fragment detected by SDS-PAGE were pooled and dialyzed against buffer C. The dialysate was then passed through a Dowex 1×2 column pre-equilibrated with buffer C. Fractions showing fluorescence were collected and pooled, followed by dialysis against water for 2 days. The dialysate was lyophilized and subjected to isolation of the labeled peptide by HPLC.

Isolation of Labeled Peptides—The fluorescent 23-kDa fragment (4.5 mg/ml) was first digested by LEP (0.18 mg/ml) in 7.4 mM urea, 2 mM dithiothreitol, and 20 mM imidazole, pH 7.0. Digestion was performed at 25 °C for 24 h and terminated by the addition of trifluoroacetic acid to pH 2. After passage through a membrane filter (Millipore Ultrafree-MC, 0.45 μm), the digest was subjected to reverse-phase HPLC on a TSK-gel (ODS-80TS) packed column (4.6 × 250 mm) connected to a TOYOBO SC-8020 HPLC system. Peptides were eluted at a flow rate of 1 ml/min with a linear gradient of 0–60% acetonitrile containing 0.1% trifluoroacetic acid at 25 °C. The elution was monitored at 225 nm (for peptides) and 360 nm (for the labeled AN group). The labeled peptide was eluted from the column as a single peak. Aliquots of the peptide (48 μg with respect to the AN group) were next digested at 25 °C with CT (2.3 μg) in 20 mM Tris-HCl, pH 8.1, for 24 h. After another addition of 2.3 μg CT, digestion was further continued for 25 h, followed by the addition of 0.5 mM ammonium acetate (pH 4.0) and V8. Final concentrations of the peptides, the ammonium acetate buffer, and V8 were 36 μg, 0.1 mM, and 1.5 μg, respectively. Digestion with V8 was performed at 25 °C for 24 h and terminated by the addition of trifluoroacetic acid. After passage through the membrane filter, the digest was applied to HPLC as described above. The labeled peptides were eluted from the column as two peaks. Each peptide fraction was subjected to spectral measurements and amino acid sequence and composition analyses.

Amino Acid Sequence and Composition Analyses—The amino acid sequence of a peptide was determined from the amino terminus using a pulse-liquid protein sequencer, Procise 492 (Applied Biosystems). The amino acid composition of a peptide was determined using an AccQ-Tag amino acid analysis system (Waters) after vapor phase acid hydrolysis with 7 M HCl, 10% trifluoroacetic acid, 0.1% phenol at 160 °C for 30 min. Spectral Measurements—Absorption spectra were measured at room temperature with a Hitachi U-3210 spectrophotometer. Amounts of the AN group labeled to S1 and peptides were estimated using an absorption coefficient of 8.4 × 10³ M⁻¹ cm⁻¹ at 361 nm (7). Fluorescence emission spectra (uncorrected) were recorded at 25 °C in a JASCO FP-770 spectrophluorometer as described previously (8).

RESULTS

It has been well established that ANN reacts with S1, selectively labeling a serine residue in the NH₂-terminal 23-kDa peptide segment of the heavy chain, to yield fluorescent AN-S1 (7). In the present work, to identify an exact labeled residue in the 23-kDa segment, the labeled 23-kDa peptide fragment and its proteolytic products, labeled oligopeptides, were isolated. AN-S1 was digested with trypsin, fractionated with ethanol in the presence of 6 M Gdn-HCl, and applied to Ultrogel AcA44 gel filtration in the presence of SDS. Experimental details are given under “Experimental Procedures.” Elution of peptides was monitored by absorbance at 280 nm. Fractions containing the fluorescent 23-kDa fragment (indicated by a solid bar) were pooled.

The tryptic fragment of rabbit skeletal S1 contains 12 serine residues (6). Thus the fluorescent 23-kDa tryptic fragment was first digested with LEP, and the digest was subjected to reverse-phase HPLC. When the AN group attached to the peptides was monitored by the absorption at 360 nm, the AN group-labeled peptide denoted by L eluted as a single peak at 49 min (Fig. 2A). This labeled peptide was isolated and further digested with CT and subsequently with V8. The digest was subjected to HPLC to yield two labeled peptides denoted by the letters V and C (Fig. 2B). HPLC inspections of aliquots of the reaction mixture revealed that peptide C, which was produced by CT digestion of peptide L, was converted to peptide V upon digestion by V8 (not shown). Peptides V and C were characterized by sequencing (Fig. 3).

For both peptides, lysine was identified at the last cycle and no further PTH-amino acids were found. The peptide V was a pentapeptide with a sequence of SGAGK, identical to residues 181–185 in the chicken skeletal S1 heavy chain (6) (Fig. 3A). The peptide C was a nonapeptide with a sequence of ITGESGAGK, identical to residues 177–185, a precursor of peptide V (Fig. 3B). These results were consistent with the observation that peptide V was produced from peptide C by V8 digestion (Fig. 2B). However, it should be noted that yields (6–10%) for PTH-Ser-181 of both peptides were significantly lower than that (31%) for PTH-Glu-180 (peptide C) and those (26–31%) for PTH-Gly-182 (both peptides). The results are reminiscent of the previous reports that an abnormally low yield of PTH derivative for serine residue can be considered an indication of the presence of the chemically modified serine residue in peptides (13–15). Using procedures and instruments similar to those in the present analysis, 5–10-fold lower yields of PTH derivatives for the esterified serine residues but not for non-esterified ones have been reported (13–15). Thus, the sequence data for peptides V and C suggest that Ser-181 has been esterified by ANN.

To further confirm this point we next determined the amino acid compositions of both these peptides (Table I). The analysis revealed the existence of 0.8–0.9 mol of serine residue/mol of each peptide, which was not fully detected by the sequencing (Fig. 3). The results suggest that serine residues are regenerated from the labeled residues by acid hydrolysis treatment for the amino acid analysis. This was confirmed by a control experiment in which the attached AN group of AN-SerNAC was found to be released by such a treatment. Thus, we concluded from the sequence data (Fig. 3) and the amino acid compositions (Table I) that a single serine residue in peptides V and C, Ser-181, was labeled with ANN.

Although data are not shown, the fluorescence and absorption spectra of peptide V were measured and compared with those of a model compound, AN-SerNAC. There was no signif-

FIG. 1. Isolation of the AN group-labeled 23-kDa fragment by Ultrogel AcA44 gel filtration. AN-S1 was digested with trypsin, fractionated by ethanol in the presence of 6 M Gdn-HCl, and applied to Ultrogel AcA44 gel filtration in the presence of SDS. Experimental details are given under “Experimental Procedures.” Elution of peptides was monitored by absorbance at 280 nm. Fractions containing the fluorescent 23-kDa fragment (indicated by a solid bar) were pooled.
icant difference in the fluorescence spectrum in 80% acetonitrile between peptide \( V \) and AN-SerNAc with fluorescence emission peaks at 474 and 471 nm, respectively. This was also the case for the absorption spectra. The spectrum of AN-SerNAc exhibited four maxima at the longer wavelengths (332, 346, 363, and 382 nm) that had been established to be associated entirely with the AN group (7). The spectrum of peptide \( V \) also exhibited similar four maxima at 333, 349, 364, and 384 nm. This spectrum was identical to that of peptide \( C \). Because both peptides \( V \) and \( C \) contain no chromophoric amino acid residues (Table I), the amounts of the AN group labeled to peptides can be estimated from the absorption spectrum of the AN group. The two obtained values, 0.73 and 0.72 mol of AN group/mol of peptide for peptides \( V \) and \( C \), respectively, were essentially identical (Table I). The results indicate again that peptide \( C \) is a precursor of peptide \( V \), supporting our assignment of the peptide sequences (Fig. 3). These spectral data for the labeled peptides strengthen the conclusion that only Ser-181 is selectively labeled with ANN.

**DISCUSSION**

In the present study, we have shown that Ser-181 in S1 heavy chain is specifically esterified with ANN. A specific reaction of Ser-181 was already reported by Cremo et al. (4). The UV irradiation of the S1 complex with Mg/ADP/V\( _i \) was shown to be induced by the transition state for ATP hydrolysis (16), resulting in a specific V\( _i \)-induced photooxidation by which Ser-181 is converted to a "serine aldehyde" (17). Because V\( _i \) in this complex mimics the \( \gamma \)-phosphate in the S1 complex with ATP, such a V\( _i \)-induced reaction served to identify a critically important serine residue in the coordination of the \( \gamma \)-phosphate of ATP. This result was verified by the x-ray structure of the S1 complex with MgADP-V\( _i \) (18). The x-ray structure revealed that Ser-181 is

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**Fig. 2.** Isolation of the labeled peptides by reverse-phase HPLC. A, the fluorescent 23-kDa fragment was digested with LEP and subjected to reverse-phase HPLC on a TSK-gel (ODS-80TS) with a linear gradient of 0–60% acetonitrile containing 0.1% trifluoroacetic acid. The elution was monitored at 225 nm (for peptides) and 360 nm (for the labeled AN group). B, the labeled peptide obtained by LEP digestion (indicated by a solid bar in panel A) was further digested with CT and subsequently with V8 and subjected to HPLC under the same conditions as described above. Experimental details for enzymatic digests of samples are given under "Experimental Procedures." Labeled peptides produced by digestions with LEP, CT, and V8 are expressed as \( L \), \( C \), and \( V \), respectively.

**Fig. 3.** Sequence analyses of peptides \( V \) and \( C \). The yields of PTH amino acids were indicated as percentages of the amount of each sample loaded to the sequencer. Samples of 36 and 35 pmol were employed for the sequence analyses of peptides \( V \) and \( C \), respectively, as described under "Experimental Procedures." A, peptide \( V \); B, peptide \( C \).

**Table I**

Amino acid compositions and amounts of the labeled AN group of peptides \( V \) and \( C \).

| Amino acid | Peptide \( V \) | Peptide \( C \) |
|------------|----------------|----------------|
| residues/mol | Actual theory | Actual theory |
| D/N | 0.1 | 0.1 |
| S | 0.9 | 1.0 |
| E/Q | 0.2 | 1.0 |
| G | 2.2 | 3.2 |
| H | 0.0 | 0.0 |
| R | 0.0 | 1.0 |
| T | 0.1 | 0.7 |
| A | 1.0 | 1.1 |
| P | 0.1 | 0.1 |
| C | 0.0 | 0.0 |
| Y | 0.2 | 0.3 |
| V | 0.1 | 0.1 |
| M | 0.0 | 0.0 |
| K | 1.0 | 1.0 |
| I | 0.1 | 0.9 |
| L | 0.1 | 0.2 |
| F | 0.1 | 0.1 |
| Total Position | 5 | 9 |
| AN group | 0.73 mol/mol | 0.72 mol/mol |

\( ^a \) The labeled peptide, which was obtained by HPLC as specified in Fig. 2B, was subjected to amino acid analysis as described under "Experimental Procedures."

\( ^b \) The positions of the amino acid sequences are expressed as corresponding numbers of chicken skeletal S1 heavy chain (6).

\( ^c \) The concentrations of AN group were determined spectrophotometrically. Peptide concentrations were determined by amino acid analysis.
in the phosphate binding loop for nucleotides and its side chain is involved in coordination of $V_i$ that mimics the $\gamma$-phosphate of ATP.

It is surprising that the labeling reaction of S1 with ANN is highly selective. Even when a 50-fold molar excess of ANN over S1 was used, only one serine residue, Ser-181, out of the 42 serine residues in the S1 heavy chain was labeled (Ref. 7 and present results). In the case of the photo-modification reaction (4), it is reasonable that Ser-181 is specifically oxidized by the $V_i$-induced reaction, because Ser-181 is adjacent to $V_i$ coordinated with its oxygen atom in the S1 complex with MgADP-$V_i$ (18). However, it is unusual that ANN with structure dissimilar to a nucleotide can approach Ser-181 and result in a selective esterification of the residue. One possible explanation for such an unusual specificity for the ANN labeling is that ANN forms a non-covalent complex with S1 prior to the reaction in which the functional group of ANN is in a favorable position for a subsequent esterification of Ser-181. In fact, the selective labeling of Ser-181 was not observed upon the labeling of S1 with a structural isomer of ANN, 1-anthroylnitrile, in which the functional group acylnitrile is at C1 of the anthracene ring instead of its C9 in ANN, suggesting that its functional group was not situated near Ser-181. It is also likely that a hydrophobic pocket near Ser-181 composed of Ser-243, Ser-244, Phe-246, Ala-465, Gly-466, and Phe-467 (1, 2), interacts with the anthrachene moiety of ANN and helps the acylnitrile moiety to approach Ser-181, resulting in the specific labeling of the residue.

The first pentapeptide sequence in the phosphate binding loop of the S1 ATPase site (GESGA, residues 179–183 of S1 heavy chain) is equivalent to a highly conserved pentapeptide sequence GXSXG/A found around active serine residues in serine peptidases (12). Serine peptidases occur in three distinct families, represented by CT, subtilisin, and wheat serine carboxypeptidase II. The CT-like enzymes have glycine in the fifth position of the pentapeptide sequence. On the other hand, the latter two enzymes have another small amino acid, alanine, in this position. This is also the case for the peptide sequence of S1. It should be emphasized that among all the 42 serine residues in the S1 heavy chain only Ser-181 is in the sequence GXSXG/A (6). Such pentapeptide segments form a tight turn in motifs of strand-turn-helix, which confers a reactive nature of the serine residue in the third position (12). This may be why Ser-181 of S1 has unusual reactivity against ANN.

It is interesting to compare the enzymatic properties of AN-S1 (7) with those of the photo-modified S1 (19). In both S1 derivatives, Ser-181 is chemically modified. AN-S1 had Mg$^{2+}$-ATPase activity 2.2-fold higher than that of the control S1, whereas the K$^+$- and Ca$^{2+}$-ATPase activities were below 30% of the control. The photo-modified S1 had Ca$^{2+}$-ATPase activity 4–5-fold higher than that of the control S1, whereas the K$^+$- ATPase activity was below 10% of the control. It is clear that a chemical modification of Ser-181 itself does not result in abolishment of the S1 ATPase activity. Thus, the Mg$^{2+}$-ATPase reaction of AN-S1 can be monitored continuously by changes in fluorescence emitted from the AN group attached to S1 (8, 9).

In the course of the present study, we became aware of a study carried out by Szarka et al. (20). To obtain information about the labeling sites with ANN in S1, they measured the distances between the AN group labeled to S1 and known positions of S1 (Lys-553 and Cys-707) using the technique of fluorescence resonance energy transfer. They suggested that the most probable labeled residue is Ser-181, consistent with our present result.

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$^2$ T. Hiratsuka, unpublished result.
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