Serine-324 of Myosin's Heavy Chain Is Photoaffinity-Labeled by 3′(2′)-*O*-(4-Benzoylbenzoyl)adenosine Triphosphate†

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ABSTRACT: A portion of the active site of rabbit skeletal myosin near the ribose ring of ATP can be labeled by the photoaffinity analogue 3′(2′)-*O*-(4-benzoylbenzoyl)adenosine triphosphate (Bz_2ATP). The specificity of the photolabeling was assured by first trapping [^{14}C]Bz_2ATP at the active site by use of thiol cross-linking agents [Mahmood, R., Cremo, C., Nakamaye, K., & Yount, R. (1987) *J. Biol. Chem. 262*, 14479–14486]. Five radioactive peptides were isolated by high-performance liquid chromatography after extensive trypsin and subtilisin digestion of photolabeled myosin subfragment 1. Four of these peptides were sequenced by Edman techniques, and all originated from a region with the sequence Gly-Glu-Ile-Thr-Val-Pro-Ser-Ile-Asp-Asp-Gln, which corresponds to rabbit myosin heavy chain residues 318–328. The fifth labeled peptide had an amino acid composition appropriate for residues 312–328. Amino acid composition, radiochemical analysis, and sequence data indicate that Ser-324 is the major amino acid residue photolabeled by Bz_2ATP. Spectrophotometric evidence indicates that the benzophenone carbonyl group has inserted into a C–H bond from either the α- or β-carbon of serine. These results place Ser-324 at a distance of 6–7 Å from the 3′(2′) ribose oxygens of ATP bound at the active site of myosin.

The location of the force-generating ATPase site in myosin is of fundamental importance to understand how the chemical energy released from ATP hydrolysis is converted into mechanical force in muscle contraction. One of our goals has been to define the nucleotide binding region of S1[1] with various photoreactive ATP analogues. The general approach involves the trapping of nucleotide analogues at the active site of S1 by cross-linking to two reactive thiols, termed SH_1 and SH_2. The bridging of these thiols by bifunctional thiol reagent leads to the loss of ATPase activity and stable trapping of nucleotides at the active site (Wells & Yount, 1979, 1982). The trapped S1–nucleotide complexes typically have half-lives of days and, thus, can be purified free of nontrapped nucleotide and excess modifying reagents by ammonium sulfate precipitation and gel filtration. Subsequent irradiation of the purified S1–analogue complex then assures specific photolabeling of residues at or near the active site. Such an approach has been successfully used with 2-azido-ADP (Grammer et al., 1985; H. Kuwayama and R. G. Yount, unpublished results) and NANDP, a photoaffinity analogue of ADP (Okamoto & Yount, 1985; Nakamaye et al., 1985). Both of these analogues label the adenine binding site and were found to label Trp-130 in the NH_2-terminal 23-kDa tryptic peptide of the S1 heavy chain fragment. In the case of smooth muscle myosin, NANDP labels both the heavy chain and the essential light chains (Okamoto et al., 1986), demonstrating that light chains provide part of the adenine binding site for ATP. Thus, the use of ATP analogues has provided valuable information about both the subunit composition and the amino acid residues at the active site of myosin.

We have previously shown that Bz_2ATP, an analogue of ATP containing a photoreactive benzophenone moiety (Williams & Coleman, 1982) esterified at the 2′- or 3′-hydroxyl group of ribose (Mahmood et al., 1987), can be trapped[2] at the active site of S1 by the cobalt(III) phenanthroline cross-linking system (Mahmood & Yount, 1984). Irradiation of the purified S1–Bz_2ATP complex with UV light gave 50–60% covalent incorporation of trapped analogue into the enzyme. Gel electrophoretic analysis of S1 after limited trypsin digestion showed that only the central 50-kDa tryptic peptide of the heavy chain was labeled.

In this paper we report the purification and identification of peptides photolabeled by [^{14}C]Bz_2ATP and show that Ser-324 is the major amino acid residue modified. Since the photoreactive carbonyl group of Bz_2ATP is 6–7 Å from the 2′- or 3′-OH of ribose, these results place Ser-324 at this distance from the ribose ring of ATP bound at the active site of myosin. These results identify for the first time the part of the 50-kDa tryptic peptide that is close to the active site and should prove valuable in defining the nucleotide binding site when the crystal structure of S1 (Rayment & Winkelmann, 1984) is solved.

Materials and Methods

The sources of commercial compounds were as follows: subtilisin BPN′, TPCK–trypsin, Sephadex G-50 (fine), dansyl chloride, and dansyl amino acid standards (Sigma); ultrapure

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[1] Abbreviations: S1, skeletal myosin subfragment 1; Bz_2ATP, 3′(2′)-*O*-(4-benzoylbenzoyl)adenosine triphosphate; Bz_2acid, 4-benzoylbenzoic acid; CBH, 4-carboxybenzhydrol; CBH-ADP, 3′(2′)-*O*-(4-carboxybenzhydrol) ester of ADP; Co(phen), Co(III)–1,10-phenanthroline complex that cross-links SH_1 and SH_2; Bicine, *N*,*N*-bis-(2-hydroxyethyl)glycine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; RP-HPLC, reversed-phase HPLC; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; TEA, triethylamine; TEAP, triethylammonium phosphate; PTH, phenylthiohydantoin; NANDP, 2-[(4-azido-2-nitrophenyl)amino]ethyl diphosphate.

[2] For convenience, we use Bz_2ATP throughout this paper although it is known (Mahmood et al., 1987) that it is Bz_2ADP which is stably trapped and photoincorporated into S1.

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urea and ammonium sulfate (Schwarz/Mann); Bicine (Research Organics); TFA and polyamide TLC Plates (Pierce); 1,10-phenanthroline (Aldrich); H_3PO_4, TEA, HPLC-grade H_2O, and CH_3CN (Baker). TEA was redistilled before use.

[1-^{14}C-*carboxy*]-4-Benzoylbenzoic acid ([^{14}C]Bz_2acid) was prepared according to the procedure of Nakamaye and Yount (1985). [^{14}C]Bz_2ATP (5400 cpm/nmol) was synthesized from ATP and [^{14}C]Bz_2acid as described previously (Mahmood et al., 1987). Preparation of rabbit skeletal myosin S1, protein determination, and synthesis of $[Co^{III}(phen)_2CO_3]^+$ were as described (Mahmood et al., 1984). 4-Carboxybenzhydrol was prepared by the Zn/NaOH reduction of the keto group of Bz_2acid (Cremo & Yount, 1987). Mass spectrometric analysis of Bz_2acid and 4-carboxybenzhydrol were done on a VG7070E high-resolution mass spectrometer (electron voltage 70 eV, source temperature 150 °C).

Trapping of [^{14}C]Bz_2ATP on S1 by use of the cobalt(II)/cobalt(III) phenanthroline system was essentially as described earlier (Mahmood & Yount, 1984). A 30 μM solution of S1 (110 mg of protein) in 0.1 M KCl and 50 mM Bicine, pH 8.0, was incubated on ice with 20 mM $MgCl_2$, 60 μM [^{14}C]Bz_2ATP, 0.3 mM $CoCl_2$, 0.3 mM 1,10-phenanthroline, and 3 mM $[Co^{III}(phen)_2CO_3]^+$. After 70 min, the Co(phen)-modified enzyme was purified by precipitating it twice with 2.5 volumes of saturated ammonium sulfate, pH 8.0, containing 20 mM EDTA. The protein precipitate was dissolved in minimal KCl–Bicine buffer and was purified by gel filtration on a Sephadex G-50 (fine) column (2.6 × 21 cm). This procedure gave >90% recovery of enzyme with 0.82 ± 0.02 mol of [^{14}C]Bz_2ATP trapped per mole of S1. Solutions (12–15 mL) of Co(phen)-modified S1 (24–34 μM) were placed in 9-cm covered glass Petri dishes kept on ice and irradiated with UV light (>300 nm) for 30 min as described previously (Mahmood & Yount, 1984). This procedure gave 50–60% photoincorporation of the trapped [^{14}C]Bz_2ATP into the enzyme.

Proteolytic Digestion of [^{14}C]Bz_2ATP-Labeled S1. The [^{14}C]Bz_2ATP-labeled S1 was precipitated with 2.5 volumes of saturated ammonium sulfate, pH 8.0, containing 20 mM EDTA and was collected after centrifugation at 14 000 rpm for 30 min. The precipitate was dissolved in 2 M urea, 50 mM NH_4HCO_3, and 0.1 mM $CaCl_2$ buffer to give a protein concentration of 4 mg/mL and dialyzed overnight against the same solution to remove residual ammonium sulfate. S1 was digested at 37 °C by addition of 1/100 (w/w) TPCK–trypsin at 0, 4, and 8 h followed by overnight incubation. A second digestion was done at 37 °C by adding 1/50 (w/w) subtilisin BPN′ at 0, 3, and 6 h followed by lyophilization after overnight incubation.

High-Performance Liquid Chromatography. HPLC was carried out at room temperature (22 °C) using a microprocessor-controlled Altex/Beckman dual pump set up connected to a Beckman Model 165 dual-wavelength detector. RP-HPLC utilized a semipreparative (6 mm × 25 cm) or analytical (4.6 mm × 25 cm) 300-Å pore C_8 column (RP-300, Brownlee Labs, Santa Clara, CA), and ion-exchange HPLC utilized a 6 mm × 15 cm IEX 545 DEAE-TSK column (Beckman). The column eluents were monitored for absorbance at 214 and 260 nm. A flow rate of 1 mL/min was used in all separations, and 1-mL fractions were collected. Aliquots of each fraction were mixed with 5 mL of ACS scintillant (Amersham) and assayed for radioactivity by using a Beckman LS 7500 counter.

Sodium phosphate and TEAP buffers were prepared by titrating H_3PO_4 solutions to the desired pH with NaOH and TEA, respectively. TEAP (0.1%) in CH_3CN/2-propanol (60:40) was prepared by adding 1.38 mL of TEA to 700 mL of 0.1% H_3PO_4 in CH_3CN/1-propanol (60:40). All solutions were prepared from HPLC-grade solvents (Baker) and filtered through 0.45-μm nylon-66 membrane filters (Rainin) before use. All peptide solutions were also filtered through 0.45-μm nitrocellulose filters before injecting on the column.

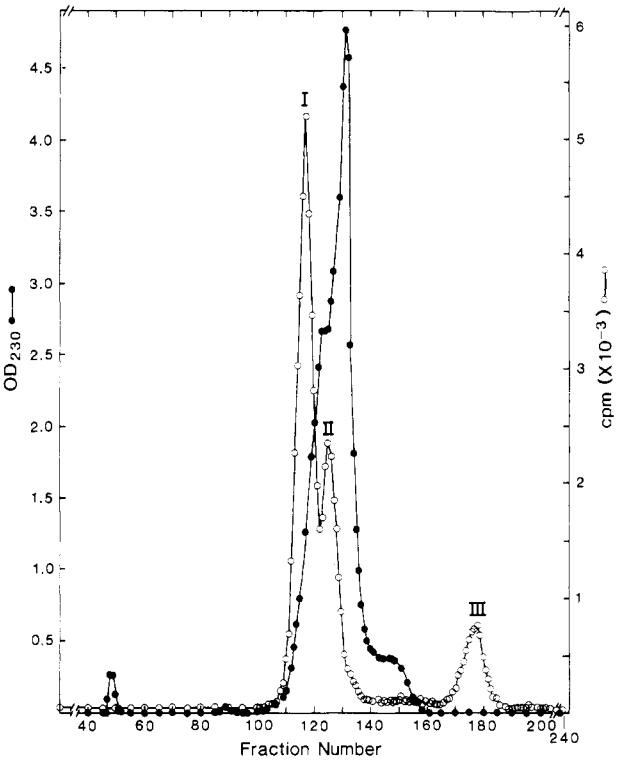

FIGURE 1: Gel filtration of a tryptic and subtilisin digest of [^{14}C]-Bz_2ATP-labeled S1. [^{14}C]Bz_2ATP–S1 was digested with trypsin and subtilisin as described under Materials and Methods and lyophilized. The powder was redissolved in 0.1% TFA (total volume 10 mL) and chromatographed on a 2.5 × 150 cm column of Sephadex G-50 (fine). The column was eluted with 0.1% TFA containing 0.01% NaN_3 at room temperature at a flow rate of 27 mL/h. Fractions of 5 mL were collected and assayed for absorbance at 230 nm (●). Aliquots from each fraction (0.1 mL) were also assayed for radioactivity (○).

Sequence Determination of Peptides. NH_2-terminal amino acids were determined by the dansylation procedure of Gray (1972) using polyamide TLC plates. Amino acid composition analyses were done with a Beckman MB121 analyzer single-column system using norleucine as the internal standard. Peptides were sequenced with an Applied Biosystems gas-phase sequencer and a Beckman Model 890C sequencer. The PTH-amino acids were identified by HPLC (Johnson et al., 1979) using a 250 × 4.6 mm (cyanopropyl)silane column (Du Pont Zorbax) or by use of an ABI 120-A PTH analyzer and assayed for radioactivity to identify the residue modified by [^{14}C]Bz_2ATP.

Results

Gel Filtration of Trypsin and Subtilisin Digest of [^{14}C]-Bz_2ATP–S1. The digestion of [^{14}C]Bz_2ATP-photolabeled S1 with trypsin left a trypsin-resistant core that contained the majority of the radioactivity. A further digestion with subtilisin yielded small radioactive peptides which were chromatographed on a column of Sephadex G-50 (Figure 1). Elution with 0.1% TFA showed an asymmetric broad peak of absorbance at 230 nm but three major peaks of radioactivity. Fractions 109–122 (peak I), 123–131 (peak II), and 170–185 (peak III) were pooled separately, lyophilized, and

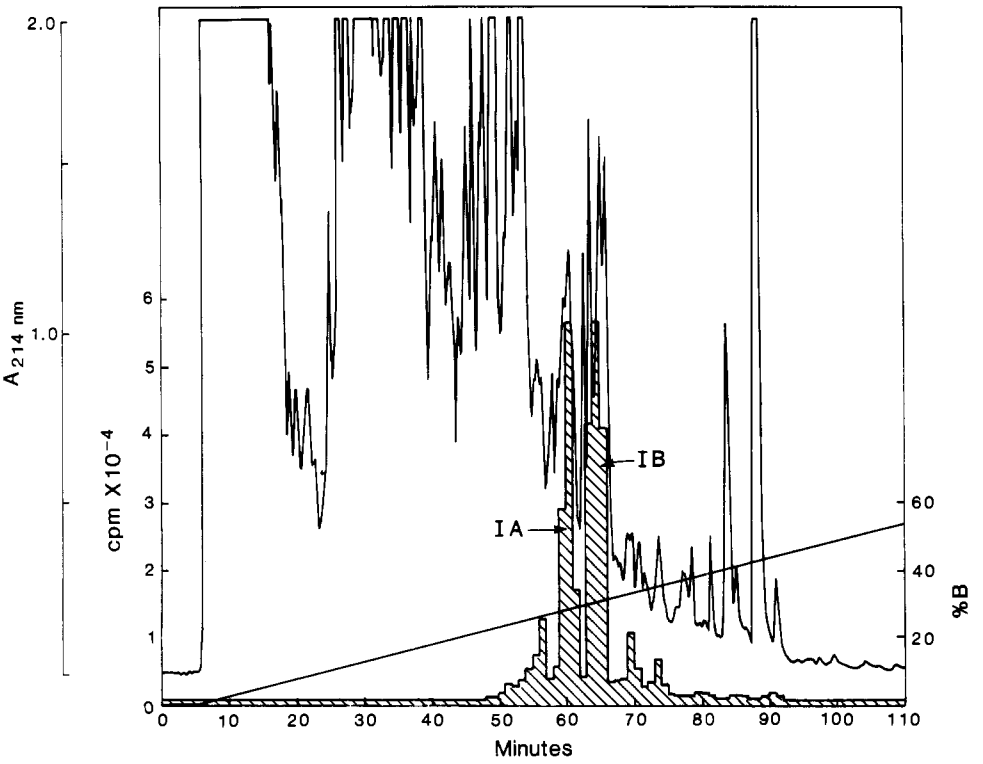

FIGURE 2: HPLC of peptides from peak I (Figure 1). The lyophilized fractions (109–122) were dissolved in 10 mM sodium phosphate, pH 6.9 (solvent A), and applied on a semipreparative RP-300 column. The peptides were eluted with a 0.5%/min gradient of solvent B (60% CH_3CN). The labeled peptides eluting at 60–62 min (IA) and 64–66 min (IB) were pooled separately.

stored at −80 °C. Peaks I, II, and III contained 50–55%, 20–27%, and 12–15% of the radioactivity recovered from the column, respectively. About 85–90% of the radioactivity applied on the column was recovered.

Purification of [^{14}C]Bz_2ATP*-Labeled Peptides from Peak I of Sephadex G-50 Column.* The lyophilized residue from peak I was dissolved in a small volume of 10 mM sodium phosphate, pH 6.9, and chromatographed on a semipreparative RP-300 column equilibrated in the same buffer. The histogram showed two major radioactive peaks, which were labeled IA and IB (Figure 2) and contained 25 and 40% of the counts recovered from the column, respectively. Both peaks were pooled separately and rechromatographed on the same column with a shallow gradient of 60% acetonitrile (0.25% B/min) to remove several unlabeled peptides (Figure 3).

Peak IA was pooled from the NaP_i, pH 6.9/60% CH_3CN system (Figure 3A), concentrated to half the volume on a Speed-Vac, and separated on an analytical C_8 RP-300 column by using the TEAP/CH_3CN solvent system. This fractionated IA into two well-resolved radioactive peaks, IA_1 and IA_2, which contained 60 and 40% of the radioactivity (Figure 3A, inset). Dansylation followed by TLC showed IA_1 was pure with isoleucine as the single NH_2-terminal amino acid. Similar analysis of IA_2 gave several dansyl-amino acids. This peptide mixture was then further purified by use of a DEAE-TSK column with a gradient of 0.5 M KCl (data not shown). The major peak was pooled, was desalted by use of the 0.1% TFA/CH_3CN solvent system, and gave glycine as the single NH_2-terminal amino acid when analyzed by dansyl TLC.

Peak IB was also purified further by HPLC on the analytical C_8 column (Figure 3B, inset). Although several unlabeled peptides were removed, the major radioactive peak required further purification on a DEAE-TSK column with a 0.5 M KCl gradient (data not shown). The single radioactive peak when analyzed as above gave isoleucine as the single NH_2-terminal amino acid.

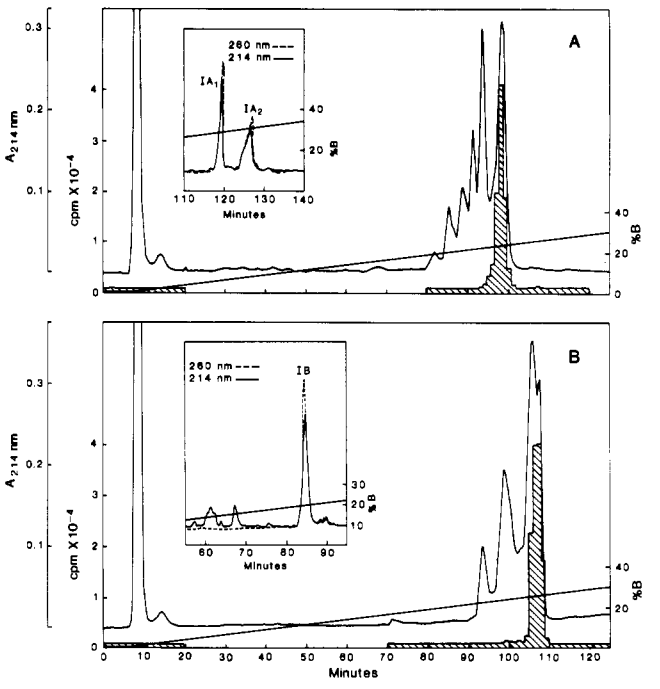

FIGURE 3: Purification of peptides from IA and IB. (A) and (B) show chromatography of IA and IB, respectively, on the semipreparative RP-300 column using the 10 mM sodium phosphate, pH 6.9 (solvent A), and 60% CH_3CN (solvent B) system as in Figure 2 but with a shallow gradient of CH_3CN (0.25% B/min). The peak fractions containing radioactivity (shaded area) were pooled, concentrated to half the volume, and rechromatographed on an analytical RP-300 C_8 column. Inset (A) shows the rechromatography of IA. Solvent A = 0.1% TEAP, pH 3.5; solvent B = 0.2% TEAP in 60% CH_3CN, pH 3.5. Inset (B) shows rechromatography of IB. Solvent A = 0.1% TEAP, pH 3.5; solvent B = 0.1% TEAP in CH_3CN/2-propanol (60:40).

Purification of [^{14}C]Bz_2ATP*-Labeled Peptides from Peak II of Sephadex G-50 Column.* The lyophilized powder con-

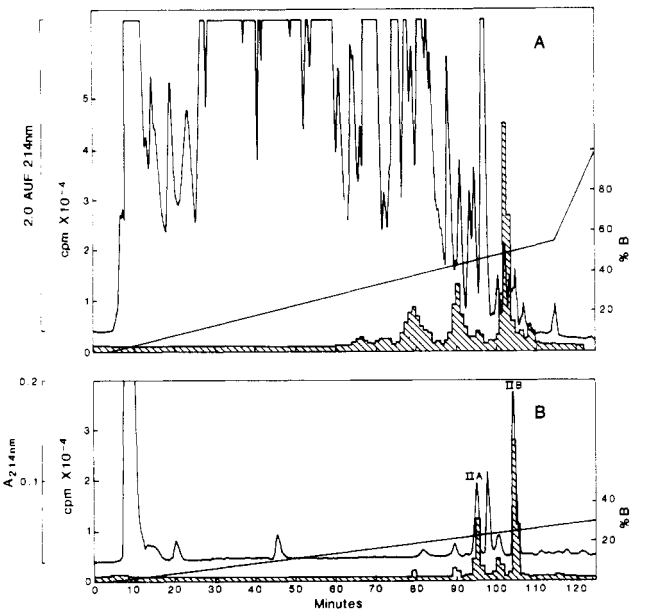

FIGURE 4: HPLC of peptides from peak II (Figure 1). (A) The lyophilized fractions (123–131) corresponding to peak II were dissolved in 2 mL of 0.1% TFA (solvent A) and separated on a semipreparative Brownlee RP-300 column using 0.1% TFA in 60% CH_3CN as solvent B. (B) Fractions 103–105 were pooled and rechromatographed on the same column with 10 mM sodium phosphate (pH 6.9) and 60% CH_3CN as the starting and limiting solvents, respectively.

taining peak II peptides was dissolved in 0.1% TFA and separated by RP-HPLC (Figure 4). The radioactivity in fractions 90–95 was due to contaminating peptides from peak I and was not analyzed further. Fractions 103–105, which contained 40% of the total radioactivity, were pooled and rechromatographed on the same column at pH 6.9 with a shallower gradient of acetonitrile (Figure 4B). Four peaks of 214-nm absorbance were obtained, but only the first (IIA) and last (IIB) peaks contained significant radioactivity. About 25 and 60% of the radioactivity were present in IIA and IIB, respectively. NH_2-terminal amino acid analysis of IIB gave dansylisoleucine after further purification by RP-HPLC using 0.1% H_3PO_4 (starting solvent) and 60% CH_3CN (limiting solvent) to remove a contaminating unlabeled peptide (data not shown). There was insufficient IIA to determine its NH_2-terminal amino acid.

Peak III from Figure 1 was shown to be free [^{14}C]Bz_2acid by several criteria. Rechromatography of peak III on the same RP-HPLC column and with the same conditions used in Figure 4A gave a single peak with a UV spectrum and elution time identical with those of Bz_2acid standards. TLC analysis on silica gel 60 plates (F_{254}, EM Reagents) with 0.5 M LiCl or 1-butanol/acetic acid/H_2O (5:1:3) gave a single spot with R_f values the same as those for standard Bz_2acid. Determination of the concentration of [^{14}C]Bz_2acid samples from peak III from the known specific activity (5400 cpm/nmol) and from the ϵ^{M}_{264nm} for Bz_2COO^- (20 000 M^{-1} cm^{-1}; Mahmood et al., 1987) gave identical values. Finally, mass spectra analyses gave a parent peak of mass 226, the molecular weight of Bz_2acid. The origin of the [^{14}C]Bz_2acid likely results from the hydrolysis of the unincorporated Bz_2ATP by the slightly basic conditions used for the trypsin and subtilisin digestions. The ribose ester linkage in CBH-ADP (see below) attached to peptides apparently is stable to the acidic conditions used in the Sephadex G-50 and some RP-HPLC separations as several of the labeled peptides (IA, IA_2, IB) contained both ADP and CBH (see Figure 5B).

Absorption Spectra of [^{14}C]Bz_2ATP-Labeled Peptides.

Table I: Amino Acid Composition of [^{14}C]Bz_2ATP-Labeled Peptides[a]

	IA_1	IA_2	IB	IIA	IIB
aspartic acid	2.0 (2)	1.4 (1)	2.0 (2)	2.3 (2)	1.7 (2)
threonine	0.87 (1)	0.72 (1)	0.97 (1)	1.2 (1)	0.80 (1)
serine	0.29 (1)	0.63 (1)	0.18 (1)	0.63 (2)	0.30 (1)
glutamic acid	1.1 (0)	1.4 (1)	1.25 (1)	2.4 (3)	1.1 (1)
proline	0.84 (1)	0.6 (1)	1.1 (1)	1.3 (1)	0.87 (1)
glycine	1.0 (0)	1.9 (1)	0.83 (0)	1.8 (1)	0.54 (0)
alanine	– (0)	– (0)	– (0)	0.21 (1)	– (0)
valine	1.3 (1)	0.75 (1)	1.04 (1)	1.3 (2)	0.99 (1)
isoleucine	2.0 (2)	1.2 (2)	1.66 (2)	2.1 (2)	2.6 (2)
leucine	0.84 (0)	– (0)	– (0)	0.53 (0)	0.33 (0)
tyrosine	– (0)	– (0)	– (0)	0.68 (1)	– (0)
phenylalanine	– (0)	– (0)	– (0)	0.98 (1)	– (0)
NH_2 terminus	Ile	Gly	Ile	ND	Ile

[a] Aliquots containing 1–4 nmol of purified [^{14}C]Bz_2ATP–peptides were freeze-dried and hydrolyzed with constant-boiling HCl at 110 °C for 24 h. NH_2-terminal analyses were done by using the dansyl–TLC method of Gray (1972). The numbers in parentheses are expected values determined from the known heavy chain sequence (see text). ND = not determined. A dash (–) indicates not detected. Note that the compositions in some cases do not agree exactly with the expected values; this likely reflects the presence of impurities in the preparation analyzed. These apparent impurities did not affect the identification of Ser-324 as the labeled residue.

Table II: Amino Acid Sequences of [^{14}C]Bz_2ATP-Labeled Peptides

peptide	sequence
IA_1	Ile-Thr-Val-Pro-X[a]-Ile-Asp-Asp
IA_2	Gly-Glu-Ile-Thr-Val-Pro-X-Ile-Asp
IB	Ile-Thr-Val-Pro-X-Ile-Asp-Asp-Gln
IIB	Ile-Thr-Val-Pro-X-Ile-Asp-Asp-Gln

[a] In this sequence "X" is Ser-324 in rabbit skeletal myosin heavy chain.

Figure 5 (panel A) shows the absorption spectrum of peptide IIB and of the anionic forms of Bz_2acid and 4-carboxybenzhydrol (Cremo & Yount, 1987). As expected, the peptide spectrum closely resembles that of the anion of 4-carboxybenzhydrol rather than that of Bz_2acid because photoinsertion of the carbonyl group into a C–H bond gives a tertiary alcohol product. The spectrum of IIB further indicates the absence of ATP/ADP and of any aromatic amino acids. In addition, the concentration of peptide calculated from the ^{14}C content and the absorbance of 4-carboxybenzhydrol (ϵ^{M}_{236nm} = 15 100 M^{-1} cm^{-1}) agreed within ±5%, indicating essentially all the peptide absorbance was due to the reduced benzophenone moiety.

Panel B (Figure 5) shows similar UV spectra for labeled peptides IA, IA_2, and IB and for CBH-ADP. The absorption maxima (~251 nm) and the shape of all four curves are essentially identical, indicating these ^{14}C-labeled peptides contain both ADP and carboxybenzhydrol moieties. Surprisingly, the ester bond between CBH and ADP remained intact through lengthy enzymatic digestions and multiple chromatographic separations. In addition, these peptides also appear to contain no aromatic amino acids, a result consistent with the amino acid compositon of all three peptides (see below).

Amino Acid and Sequence Analysis of [^{14}C]Bz_2ATP-Labeled Peptides and Determination of Modified Residue(s). The TLC analysis of IA_1, IA_2, IB, and IIB after reaction with dansyl chloride gave Ile, Gly, Ile, and Ile as the NH_2-terminal residues, respectively. The amino acid compositions of these peptides and of IIA are summarized in Table I. Peptides IA_1, IA_2, IB, and IIB have very similar amino acid compositions, indicating they come from the same region of the heavy chain. The amino acid sequences of these peptides determined by

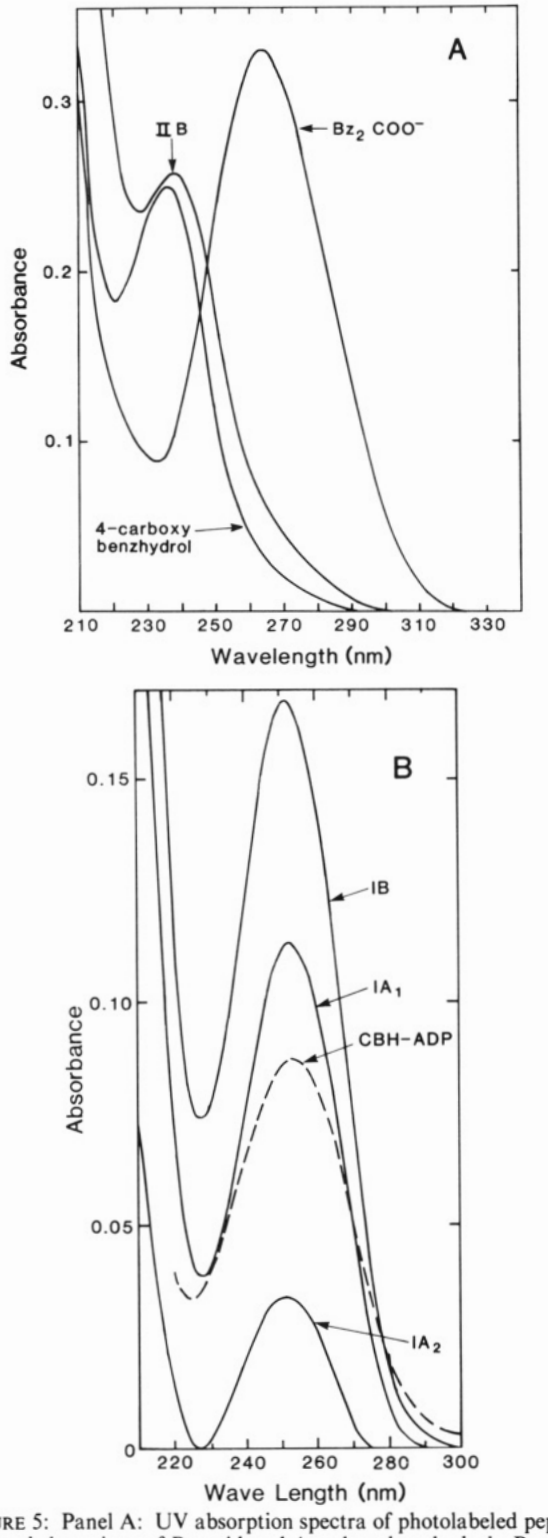

FIGURE 5: Panel A: UV absorption spectra of photolabeled peptide IIB and the anions of Bz_2acid and 4-carboxybenzhydrol. Panel B: UV absorption spectra of 4-carboxybenzhydrol–ADP and photolabeled peptides, IA_1, IA_2, and IB. All spectra were recorded at pH 6.9 on a Varian-220 spectrophotometer.

automated Edman degradation are shown in Table II. The sequence of these peptides is contained in the sequence of rabbit skeletal myosin spanning heavy chain residues 318–328 (S.-L. Davidoff and M. Elzinga, unpublished results). In all cases no PTH-amino acid corresponding to Ser-324 was found, suggesting this amino acid has been modified by [^{14}C]Bz_2ATP. In addition, the amino acid compositions (Table I) show the general absence of one serine. Although peptide IIA was not sequenced, its amino acid composition matches that of a 17

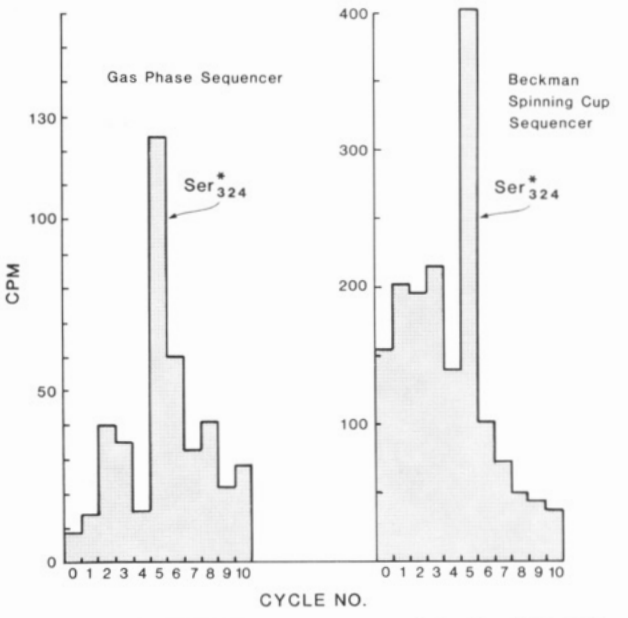

FIGURE 6: Automated Edman sequence analysis of peptide IIB on an Applied Biosystems gas-phase sequencer (left) or a Beckman spinning cup sequencer (right). The ^{14}C radioactivity in PTH-amino acids released versus cycle number is shown. In both cases radioactivity was released at cycle number 5 which corresponds to Ser-324 in the myosin heavy chain sequence.

amino acid peptide (residues 312–328) in which the sequence Tyr-Ala-Phe-Val-Ser-Glu is present at the NH_2-terminal end. Peptides IB and IIB had identical amino acid compositions (Table I) and sequences (Table II). The reason for their different behavior on RP-HPLC was the loss of ADP from the [^{14}C]CBH-ADP adduct on peptide IIB (Figure 5A). These results indicate it would have simplified peptide analyses if ADP was removed from peptides labeled with Bz_2ADP before HPLC separations were initiated.

To obtain additional evidence for the site of modification by [^{14}C]Bz_2ATP, the PTH-amino acids from the sequence analysis of peptide IIB were assayed for radioactivity. Figure 6 shows the majority of radioactivity was released at cycle 5 where in both gas-phase and spinning cup analyses no PTH-amino acid was detected. Since Ser-324 is present at this position, this is further evidence that this residue is the site of modification.

Discussion

The aim of the present study was to identify amino acid residue(s) photolabeled by Bz_2ATP and to place these in the S1 heavy chain sequence. Bz_2ATP has previously been shown to be stably trapped on S1 by cross-linking SH_1 and SH_2 by cobalt(III) phenanthroline (Mahmood & Yount, 1984) or by *p*-phenylenedimaleimide (Mahmood et al., 1987). Irradiation of the purified S1–Bz_2ATP complex gave 50–60% incorporation of trapped analogue into the S1 heavy chain. This high level of incorporation made it possible to isolate several Bz_2ATP-labeled peptide(s). Since ester linkages are both acid and base labile, it was deemed important initially to prepare Bz_2ATP in which the Bz_2acid was radioactive. Accordingly, a radiochemical synthesis of [1-^{14}C-*carboxy*]-4-benzoylbenzoic acid was devised (Nakamaye & Yount, 1985) and the product used to prepare [^{14}C]Bz_2ATP. Five major Bz_2ATP-labeled peptides were purified from the first two radioactive peaks of the Sephadex G-50 column by using RP-HPLC and ion-exchange HPLC. Four of these peptides were sequenced, and all come from the sequence Gly-Glu-Ile-Thr-Val-Pro-Ser-

Table III: Comparative Sequences from Myosin Heavy Chains[a]

Species	Sequence	Reference
Rabbit skeletal	QGEITVPSI•DDQEE	Davidoff and Elzinga[b]
Rat skeletal	QGEILVASI•DDREE	Strehler *et al.* (1986)
Chicken skeletal	QGEITVPSIN•DQEE	Molina *et al.* (1987)
Nematode (*C. elegans*)	AQAELIIDGIDDVEE	Karn *et al.* (1983)
Slime mold (*D. discoideum*)	QSGYVDIKGVSDSEE	Warrick *et al.* (1986)
Acanthamoeba (Myosin II)	QNACYTVDDMDDAKE	Hammer *et al.* (1987)

[a] Amino acid sequences for the region near Ser-324 (boldface) in rabbit skeletal myosin and comparable regions from five other heavy chains. Hydrophilic residues are underlined. [b] Unpublished results (1988).

Ile-Asp-Asp-Gln, which corresponds to the sequence of residues 318–328 present in the 50-kDa tryptic peptide of the S1 heavy chain. The amino acid analysis of the fifth peptide indicated that it was peptide 312–328 with six extra amino acids at the NH_2-terminal end. No other significantly labeled peptides were detected.

The amino acid composition of the region surrounding Ser-324 is predominately hydrophilic. Table III shows this sequence for rabbit skeletal myosin and for comparable sequences from five other myosin heavy chains. All contain larger numbers of charged residues, particularly glutamic and aspartic acids, indicating this region (and Ser-324) is likely at the surface of myosin heads. Ser-324 is flanked by two hydrophobic residues, Val-322 and Ile-325, which may provide binding sites for the aromatic rings of the benzophenone moiety. This factor may account for the high degree of specificity of photolabeling observed. The fact that Ser-324 (and the region immediately around it) are not conserved in all myosins means it is likely this region is not essential for ATP binding. Rather, because the reactive carbonyl of Bz_2ATP is some 6–7 Å from the ribose hydroxyls, this region is likely simply near the active site, possibly in a cleft between the 23- and 50-kDa peptides.

Three observations indicate that Ser-324 is the primary amino acid photolabeled by [^{14}C]Bz_2ATP. The serine content was low or absent in the amino acid analyses of all five labeled peptides (Table I). In addition, no Ser-PTH amino acid was identified at position 324 as would be predicted from the amino acid sequence of this part of the heavy chain. .Finally, the major release of radioactivity at cycle 5 of the Edman degradation of the nine amino acid peptide IIB agreed with the absence of an identifiable PTH-amino acid at this position.

The absence of complete photoincorporation of trapped Bz_2ATP into S1 suggested that side reactions could be occurring. For example, the benzophenone moiety could have reacted with the Bicine buffer upon irradiation since it is likely this part of the trapped Bz_2ATP sticks out into the solution. In addition, the benzophenone group possibly could have been reduced to carboxybenzhydrol by abstraction of two hydrogens from the same amino acid side chain. Such a reaction occurs upon irradiating sterol esters of carboxybenzophenones (Baldwin et al., 1970; Breslow et al., 1973). However, the observation that peak III (Figure 1) was [^{14}C]Bz_2acid (presumably from hydrolysis of trapped but unincorporated Bz_2ATP) and not [^{14}C]-4-carboxybenzhydrol rules out both of these possibilities. A more likely explanation is that only one isomer, i.e., either the 3′ or 2′ ester of Bz_2ATP, photoincorporates efficiently (see below).

A low level of labeling of other residues around Ser-324 cannot be ruled out conclusively by our data. It is unlikely, however, that another peptide region was labeled as was observed in the photolabeling of chloroplast coupling factor 1 (CF_1) by Bz_2ATP (Admon & Hammes, 1987). These workers observed covalent labeling of two β chain peptides, residues 360–378 and 393–397. In peptide I, both Tyr-362 and Asp-369 were labeled while all five amino acids in the second peptide appeared to have a low level of labeling. Multiple labeling of several adjacent amino acid residues was also observed in the photo-cross-linking studies of Leszyk et al. (1987). Here four different amino acids in the same peptide (Arg-103, Lys-107, Arg-108, Pro-110) from troponin I were photolabeled by 4-maleimidobenzophenone attached to Cys-98 of troponin C. This heterogeneity of labeling may reflect the greater flexibility of the cross-linker compared to the substrate analogue bound tightly at the active site. It should be pointed out, however, that Bz_2acid is esterified to both the 2′- and 3′-hydroxyls (Mahmood et al., 1987) not just to the 3′-hydroxyl as was previously believed (Williams & Coleman, 1982; Kambouris & Hammes, 1985; Williams et al., 1986). It is possible that the presence of these two isomers (at pH 7, 40% 2′ and 60% 3′ isomers; Mahmood et al., 1987) bound on CF_1 could have been responsible for the heterogeneity of labeling observed since some 70% photoincorporation of tightly bound Bz_2ATP was observed (Admon & Hammes, 1987). With myosin, such multiple labeling was not observed. This might occur if only one isomer was in the correct stereochemical position for efficient photoinsertion into the heavy chain. Such a possibility could also explain why only 50–60% photolabeling is observed of the active sites containing trapped Bz_2ADP. Thus, if both the 2′ and 3′ isomers are trapped equally, it is possible only one isomer is effective at photolabeling.

The photoreactive carbonyl group of benzophenone inserts into C–H bonds, and therefore, it can potentially react with either the α- or the β-carbons of serine. Sperling and Elad (1971a,b) have shown that the carbonyl group of acetone preferentially abstracts the hydrogen from the α-carbon of glycine residues in small peptides upon irradiation with UV light. Thus, it appears reasonable that the benzophenone carbonyl group may also abstract a hydrogen from the α-carbon of serine and then form a carbon–carbon bond by radical coupling. However, at this time we cannot rule out a similar reaction with the β-carbon C–H bond of serine.

The spectral characteristics of all four of the photolabeled peptides sequenced (Figure 5) confirm the formation of a tertiary alcohol from the carbonyl group of the benzophenone moiety. Surprisingly, in three of the four labeled peptides (Figure 6B) the ester linkage between ADP and the carboxybenzhydrol group remained intact through all the varied purification steps. It had been assumed that all ester linkages would be cleaved early in the purification scheme (Williams et al., 1986). Since this does not always occur, it may be useful in future photolabeling studies with Bz_2ATP to treat labeled peptides with mildly alkaline conditions to cleave the 3′(2′) ester bond before HPLC separations are attempted. Such a step would decrease the potential number of radioactive peptides by a factor of 2.

In summary, a specific region near Ser-324 in the 50-kDa heavy chain fragment has been placed near the ribose portion of the ATP binding site in myosin. Such structural information should prove useful in future studies aimed at characterizing the active site in crystals of S1 (Rayment & Winkelmann, 1984).

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Registry No. ATPase, 9000-83-3; Bz_2ATP, 105638-39-9; ATP, 56-65-5; L-Ser, 56-45-1.

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High Levels of Sodium–Calcium Exchange in Vascular Smooth Muscle Sarcolemmal Membrane Vesicles

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ABSTRACT: Membrane vesicles which exhibit high levels of Na_i-dependent Ca^{2+} uptake have been prepared from either porcine or bovine aortic smooth muscle. These membranes are identified as being of sarcolemmal origin by enrichment of marker activities associated with the sarcolemma (e.g., binding of the ligands PN 200–110, iodocyanopindolol, and ouabain). The V_{max} of Na–Ca exchange in the two aortic sarcolemmal preparations [0.5–3.5 nmol s^{-1} (mg of protein)$^{-1}$] is significantly higher than that previously reported with membrane preparations derived from visceral and vascular smooth muscle and compares favorably with maximal values recorded in cardiac sarcolemmal membrane vesicles [5–20 $nmol^{-1}$ s^{-1} (mg of protein)$^{-1}$] under identical experimental conditions. The K_m of Ca^{2+} (15 ± 5 μM) and the K_m of Na^+ (15 ± 7 mM) are similar values as determined in heart. Aortic and cardiac Na–Ca exchange activities are equivalent in their sensitivity to inhibition by La^{3+} and two known classes of mechanism-based organic blockers of transport activity (i.e., amiloride analogues and bepridil-like agents). Both also display electrogenic behavior. However, Li^+, K^+, and choline all inhibit the smooth muscle transporter with markedly greater potency than found in heart, and intravesicular Ca^{2+} does not affect transport activity in smooth muscle membranes as it does in the cardiac system. When maximal transport velocities are compared, aortic membrane vesicles have 3–6-fold higher Na–Ca exchange than sarcolemmal Ca^{2+}-ATPase Ca^{2+} transporting capacities. The ratio of these activities and the specific activity of Na–Ca exchange in this tissue suggest that Na–Ca exchange is a major pathway for mediating sarcolemmal Ca^{2+} flux in vascular smooth muscle.

Tension development in vascular smooth muscle under conditions which implicate a Na–Ca exchange mechanism was originally reported by Bohr et al. (1958) and Reuter et al. (1973). Subsequent tissue-based contracture experiments proved equivocal because, while evidence continued to build in support of the presence of Na–Ca exchange (Ozaki & Urakawa, 1979, 1981), many experiments produced data inconsistent with a significant role for this process in controlling smooth muscle contractility (Mulvany et al., 1984; Mulvany, 1984; Aalkjaer & Mulvany, 1985). In addition, purified

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