Direct Photoaffinity Labeling of Gizzard Myosin with Vanadate-Trapped Adenosine Diphosphate†

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ABSTRACT: The active-site topology of smooth muscle myosin has been investigated by direct photoaffinity-labeling studies with [^{3}H]ADP. Addition of vanadate (V_i) and Co^{2+} enabled [^{3}H]ADP to be stably trapped at the active site ($t_{1/2} > 5$ days at 0 °C). The extraordinary stability of the myosin·Co^{2+}·[^{3}H]ADP·V_i complex allowed it to be purified free of excess [^{3}H]ADP before irradiation began and ensured that only active-site residues became labeled. Following UV irradiation, approximately 10% of the trapped [^{3}H]ADP became covalently attached at the active site. All of the [^{3}H]ADP incorporated into the 200-kDa heavy chain, confirming earlier results using untrapped [α-^{32}P]ATP [Maruta, H., & Korn, E. (1981) *J. Biol. Chem. 256*, 499–502]. After extensive trypsin digestion of labeled subfragment 1, HPLC separation methods combined with alkaline phosphatase treatment allowed two labeled peptides to be isolated. Sequence analysis of both labeled peptides indicated that Glu-185 was the labeled residue. Since Glu-185 has been previously identified as a residue at the active site of smooth myosin using [^{3}H]UDP as a photolabel [Garabedian, T. E., & Yount, R. G. (1990) *J. Biol. Chem. 265*, 22547–22553], these results provide further evidence that Glu-185, located immediately adjacent to the glycine-rich loop, is located in the purine binding pocket of the active site of smooth muscle myosin.

A clear understanding of the structure of the active site of myosin is an essential first step toward elucidating the mechanism of force generation in muscle. In the absence of X-ray crystallographic information, photoaffinity-labeling studies using photoreactive analogues of ATP or the phosphate analogue vanadate have proved to be a useful approach to identify specific amino acid residues and peptide regions present at the active site of both skeletal muscle myosin (Szilagyi et al., 1979; Okamoto & Yount, 1985; Sutoh et al., 1986; Sutoh, 1987; Mahmood et al., 1989; Cremo et al., 1989) and smooth muscle myosin (Garabedian & Yount, 1990; Cole & Yount, 1990, 1991). To date, only residues in the 200-kDa[1] heavy chain in both skeletal and smooth muscle myosin have been identified at the active site. These results, however, do not eliminate the possibility of different active-site architecture and composition in smooth and skeletal muscle myosins due, possibly, to their different mechanisms of regulation.

Direct photoaffinity labeling using native, radioactive nucelotide substrates was originally developed to study the ATP-binding site of tRNA synthetases (Yue & Schimmel, 1977). This type of photoaffinity labeling has since been used to examine a diverse group of nucleotide-binding proteins including DNA polymerases (Hillel & Wu, 1978; Biswas & Kornberg, 1984; Pandey et al., 1987), ribonucleotide reductase (Kierdaszuk & Eriksson, 1988), β-tubulin (Nath & Himes, 1986; Linse & Mandelkow, 1988), and myosin (Maruta & Korn, 1981; Atkinson et al., 1986; Garabedian & Yount, 1990). In many cases, pyrimidine nucleotides (UTP, TTP, CTP) were used as photoprobes because they usually gave higher photoincorporation and their photochemistry is more clearly understood (Wang, 1976; Cadet & Vigny, 1990). Direct photolabeling studies using purine nucleotides, however, are less common (Yue & Schimmel, 1977; Stein et al., 1984; Schleicher et al., 1986; Banks & Sedgwick, 1986; Linse & Mandelkow, 1988; Stroop et al., 1989) due primarily to the low degree of covalent photoincorporation and to somewhat ambiguous photochemistry.

Since ATP is the native substrate of myosin, identification of photolabeled amino acid residues by ATP itself can provide useful information about the topology of the purine-binding subsite. [α-^{32}P]ATP has previously been shown to label only the heavy chains of gizzard myosin, *Acanthamoeba* myosin I, and *Acanthamoeba* myosin II (Maruta & Korn, 1981); however, the specific site of ATP attachment was not identified. A possible complication in these experiments was nonspecific labeling. During irradiation, the high-energy UV light necessary for activating the nucleotides rapidly inactivates myosin so that it was not possible to correlate inactivation with the covalent photolabeling. By using vanadate (V_i) and divalent metals, it is possible to trap nucleoside diphosphates at the active site of myosin (Goodno, 1979, 1982; Yount et al., 1987). Furthermore, by replacing Mg^{2+} with Co^{2+}, the known photoreaction of vanadate with the protein is suppressed (Grammer et al., 1988; Cremo et al., 1988, 1991). The myosin·Co^{2+}·[^{3}H]ADP·V_i complex can then be purified away from excess nucleotide before irradiation begins and thereby ensure that only active-site residues will be labeled.

In this work, we show that [^{3}H]ADP trapped at the active site with vanadate and Co^{2+} and irradiated with UV light reacts with Glu-185 located in the NH_2-terminal 29-kDa

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[1] Abbreviations: S1, myosin subfragment 1; BAP, bacterial alkaline phosphatase; NANDP, 2-[(4-azido-2-nitrophenyl)amino]ethyl diphosphate; Bz_2ATP, 3′(2′)-*O*-(4-benzoylbenzoyl)adenosine triphosphate; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; ODS, octyldecylsilane; DTE, dithioerythritol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; kDa, kilodalton; LC_{17}, 17-kDa essential light chain; LC_{20}, 20-kDa regulatory light chain.

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tryptic fragment of the gizzard myosin heavy chain. Glu-185 is located immediately following the glycine-rich loop which is implicated in binding the polyphosphate groups of ATP (Cremo et al., 1989). Since UDP also labels Glu-185 (Garabedian & Yount, 1990), these results suggest that the photoreactive bonds of both ADP and UDP are located near Glu-185.

Materials and Methods

Materials. [2-^{3}H]ADP was purchased from Amersham; bacterial alkaline phosphatase, papain, trypsin, and Sephadex G-50–80 were from Sigma; ultrapure urea was from Schwarz-Mann; trifluoroacetic acid was from Pierce; acetonitrile was from Baker; sodium orthovanadate, Na_3VO_4, was from Fisher. Vanadate stock solutions (75–100 mM) were prepared according to Goodno (1982).

Enzyme Preparations. Dephosphorylated smooth muscle myosin was isolated from fresh chicken gizzard according to Ebashi (1976) and stored in either a storage buffer [0.5 M KCl, 20 mM HEPES (pH 7.0), 1 mM DTE, 0.1 mM EDTA, 0.1 mM EGTA, 0.025% NaN_3] at 4 °C or in the same storage buffer in 50% glycerol at −20 °C. K^+EDTA ATPase activities were measured as described by Wells et al. (1979) except that the release of P_i was measured after 2 and 8 min. Protein concentrations were determined by the method of Bradford (1976) using a 2 mg/mL myosin standard ($\epsilon_{280}^{1\%} = 5.6$).

Trapping and Irradiation of [^{3}H]ADP on Gizzard Myosin. Gizzard myosin (300 mg at 3 mg/mL in 0.5 M KCl and 20 mM Tris, pH 8.0) was incubated with 2 mM $CoCl_2$ and 40 μM [^{3}H]ADP (34000 cpm/nmol) for 40 min at 25 °C. Vanadate (V_i) was added to 1 mM and the mixture incubated for another 40 min at 25 °C. The trapping reaction was terminated by addition of EDTA (pH 8.0) to 20 mM and ATP to 1 mM, and the solutions were immediately placed on ice. Excess [^{3}H]ADP was removed by centrifugation of 0.8 mL of solution through 5-mL Sephadex G-50-80 columns (Penefsky, 1977) equilibrated in 0.5 M NaCl and 20 mM Tris, pH 8.0. [^{3}H]ADP trapped at the active site of the recovered myosin was quantified by dissolving aliquots (50 μL) in 4 mL of BCS solvent (Amersham) and counting on a Packard 1900CA liquid scintillation counter. Irradiation of the purified myosin·Co^{2+}·[^{3}H]ADP·V_i complex (1–2 mg/mL) and determination of the amount of covalently incorporated [^{3}H]ADP was performed as previously described (Garabedian & Yount, 1990).

Preparation of [^{3}H]ADP-Labeled S1. Papain (1–2 mg/mL) was activated in 10 mM MOPS, pH 7.5, 1 mM EDTA, and 50 mM cysteine for 1 h at 37 °C (Seidel, 1980). Photolabeled gizzard S1 was prepared from photolabeled whole gizzard myosin using procedures previously described (Garabedian & Yount, 1990). For this study, however, a 60-min papain digestion was necessary because the gizzard myosin was more extensively cross-linked by longer UV irradiation and, hence, was less susceptible to proteolytic cleavage.

Bacterial Alkaline Phosphatase Treatment. In order to obtain reliable extraction of PTH amino acids during automated peptide sequencing and to assist in the purification of the labeled peptides, the phosphate groups of [^{3}H]ADP-photolabeled peptide fractions were removed with bacterial alkaline phosphatase. HPLC fractions containing radioactive peptides were frozen, concentrated under vacuum, and resuspended in 0.1 M Tris (pH 8.0). Bacterial alkaline phosphatase (1 unit) was added per 10 nmol of labeled peptide, and the mixture was allowed to digest at 37 °C for 16–24 h. This treatment was found to be satisfactory to remove both phosphate groups from the photoincorporated ADP.

HPLC, Capillary Electrophoresis, and Peptide Sequencing. HPLC hardware and software consisted of two Waters 501 pumps, a Waters 490E multiwavelength detector, a Waters U6K injector, a Waters 840 data station, interface module, and professional software, and a Pharmacia Frac-100 fraction collector. HPLC separations of crude [^{3}H]ADP-labeled tryptic peptides were performed on a Brownlee Aquapore RP-300 semipreparative column (7 mm × 25 cm, 10-μm packing) at room temperature. Solvent A was 0.11% aqueous TFA (pH 2), solvent B was 0.10% aqueous TFA in 60% CH_3CN, and the flow rate was 2.0 mL/min (fraction size was 2 mL). All subsequent HPLC separations were done on a Brownlee Spheri-5 RP-18 (ODS) column (4.6 mm × 10 cm, 5-μm packing) at room temperature in either the TFA solvent system described above or a solvent system consisting of 2 mM KH_2PO_4, pH 6.0 (solvent A), and 65% CH_3CN (solvent B). In all cases the flow rate was 1.0 mL/min and the fraction size was 1.0 mL. The purity of HPLC fractions was analyzed on an HPE-100 capillary electrophoresis unit (Bio-Rad Laboratories) using a 25 mm × 20 cm coated capillary cartridge and 0.1 M sodium phosphate buffer (pH 2.5). Peptide sequence analysis was performed on a model 470A gas-phase sequencer with Pulse Liquid update, a model 120A PTH analyzer, and a model 900A control module (Applied Biosystems, Inc.).

Molecular Modeling. Molecular models of ATP and UTP were produced on an Evans & Sutherland data station with the Macromodel[2] molecular modeling program (Still et al., 1990).

Results

Trapping and Irradiation of [^{3}H]ADP on Gizzard Myosin. It has been shown previously (Goodno, 1979, 1982) that MgADP can be stably trapped at the active site of skeletal myosin when vanadate ions (V_i) are added. Vanadate is thought to replace the γ-phosphoryl group of ATP and form a structure very similar to the proposed trigonal-bipyramidal transition state of phosphate during hydrolysis. Vanadate trapping of nucleotides on myosin results in a 10^4-fold decrease in the off-rate of ADP and produces a complex with a lifetime of several days at 0 °C. In this work, we have replaced Mg^{2+} with Co^{2+} and formed a stable gizzard myosin·Co^{2+}·[^{3}H]-ADP·V_i complex. Co^{2+} was used in place of Mg^{2+} to quench the photoreaction of vanadate with active-site residues that is known to occur when Mg^{2+} is present (Cremo et al., 1988, 1991; Grammer et al., 1988). The loss of K^+EDTA ATPase activity as a function of vanadate-trapped [^{3}H]ADP was demonstrated by incubating myosin (1.0 mg/mL) with 8.5 μM [^{3}H]ADP and 2 mM $CoCl_2$ for 10 min at 25 °C. V_i was added to 1 mM, and at various times, samples (0.5 mL) were removed, and the trapping reaction was quenched with 215 μM ATP and 20 mM EDTA (final concentrations). Untrapped [^{3}H]ADP was removed by gel centrifugation columns, and ATPase activities, protein concentrations, and trapped [^{3}H]ADP were quantified as described under Materials and Methods. The stoichiometry of [^{3}H]ADP bound to completely inactive gizzard myosin was found to be approximately 2 mol/mol of myosin (data not shown).

The purified gizzard myosin·Co^{2+}·[^{3}H]ADP·V_i complex was irradiated as described under Materials and Methods. After 40 min of irradiation, the protein became extensively cross-

[2] Computer graphics services were provided by the VADMS Center, a campus-wide computer resource supported by the National Institutes of Health, the Graduate School, and Academic Computing Services of Washington State University.

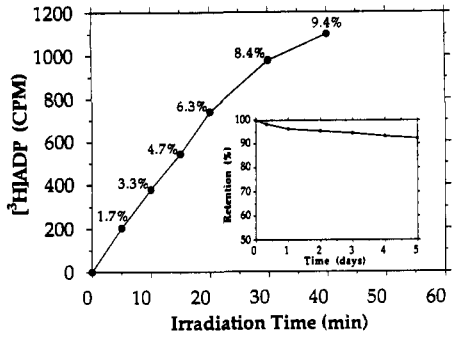


FIGURE 1: Time-dependent photolabeling of gizzard myosin by [^{3}H]ADP. Gizzard myosin (1.8 mg/mL) in 0.5 M KCl and 20 mM Tris (pH 8.0) was incubated with 2 mM $CoCl_2$, 1 mM V_i, and 15 μM [^{3}H]ADP for 20 min at 25 °C. Trapping was quenched by addition of ATP to 375 μM and EDTA to 20 mM, and the complex was purified as described (see Materials and Methods). At specific irradiation times, 0.3-mL samples were removed and analyzed for covalently bound [^{3}H]ADP as described previously (Garabedian & Yount, 1990). The percent of covalently incorporated [^{3}H]ADP at each time point is indicated (expressed as moles of [^{3}H]ADP per mole of active sites). Inset: Relative stability of gizzard myosin·Co^{2+}·[^{3}H]ADP·V_i complexes. Gizzard myosin·Co^{2+}·[^{3}H]ADP·V_i complexes were prepared as described above and stored at 0 °C in the dark. At appropriate times, 0.5-mL samples were purified by gel centrifugation and analyzed for protein concentration and radioactivity.

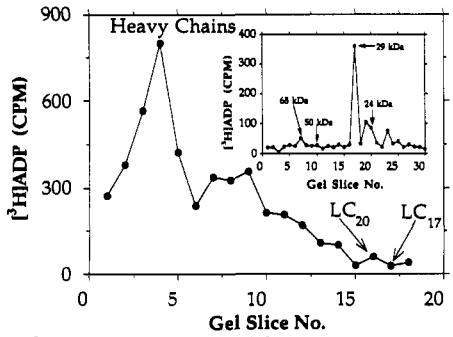


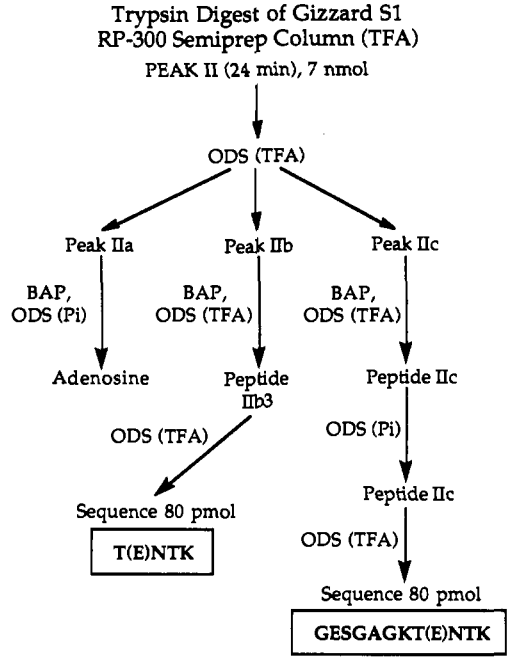
FIGURE 2: [^{3}H]ADP labels only the heavy chain in gizzard myosin. One milligram of photolabeled myosin was analyzed by SDS–PAGE using a 7–20% acrylamide separating gel and a 5% acrylamide stacking gel. The gel was stained with Coomassie Blue and sliced into 2-mm fragments. Thirty percent H_2O_2 (0.75 mL) was added to each fragment, and the mixture was heated to 90 °C for 3–4 h. Ten milliliters of BCS scintillant was added and each vial counted. Inset: [^{3}H]ADP labels the NH_2-terminal 29-kDa tryptic fragment of the heavy chain. Five milligrams of photolabeled S1 was digested with trypsin (1:100 w/w) for 8 min, and the digestion was terminated by addition of a 3-fold (w/w over trypsin) excess of soybean trypsin inhibitor. The partially digested S1 was analyzed in a 12% SDS–polyacrylamide gel with a 5% stack. Gel lanes were sliced, solubilized, and counted as described above.

linked (data not shown), so preparative-scale photolyses were limited to 15–18 min. This time of irradiation produced satisfactory photoincorporation with minimal photo-cross-linking. Figure 1 shows that the amount of [^{3}H]ADP photoincorporation increased linearly to a maximum of 0.094 mol/mol of active site or 10% of the trapped [^{3}H]ADP. To verify that vanadate produced a stable active-site complex with ADP and myosin, the retention time of vanadate-trapped [^{3}H]ADP was measured. Gizzard myosin·Co^{2+}·[^{3}H]ADP·V_i complexes have half-lives greater than 5 days at 0 °C (Figure 1, inset), indicating that virtually none of the bound [^{3}H]ADP dissociates from the active site during purification and irradiation.

Localization of [^{3}H]ADP on Gizzard Myosin. Whole gizzard myosin photolabeled with [^{3}H]ADP was analyzed by SDS gel electrophoresis to identify the labeled subunit(s) (Figure 2). Most of the radioactivity was found in the 200-kDa heavy chains as shown previously by Maruta and Korn (1981). Neither the 20-kDa or the 17-kDa light chains were labeled. Due to the damage by UV light and the length of the irradiation time, portions of the heavy chain have been photocleaved, resulting in the peaks of radioactivity immediately following the heavy chains. These results indicate that the photoreactive bond of [^{3}H]ADP is located close to the heavy chain only. To localize the site of [^{3}H]ADP attachment within the heavy chain, photolabeled S1 was briefly digested with trypsin and analyzed on a 12% SDS–polyacrylamide gel (Figure 2, inset). The majority of the radioactivity (>60%) comigrated with the 29-kDa NH_2-terminal tryptic fragment, a region previously identified to contain the purine-binding site for ATP in gizzard myosin (Garabedian & Yount, 1990).

Production and Isolation of [^{3}H]ADP-Labeled Peptides (Scheme I). Photolabeled S1 was isolated from photolabeled

Scheme I: Isolation of ADP-Labeled Peptides



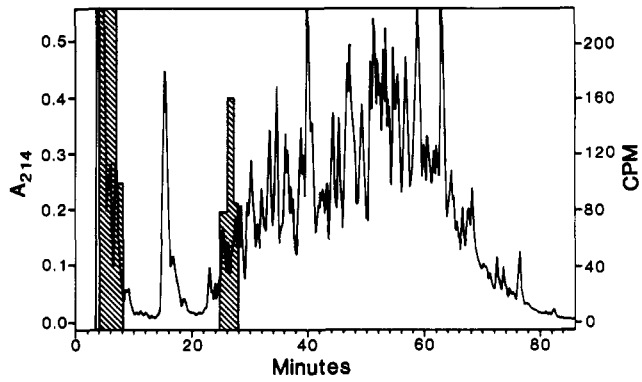


FIGURE 3: HPLC of crude trypsin digest of photolabeled S1. Photolabeled S1 (130 mg) was lyophilized and resuspended in 10 mL of 8 M urea, 0.1 M NH_4HCO_3 (pH 8.5), and 5 mM DTE. The solution was allowed to stir for 30 min and then was diluted to 2 M urea with 0.1 M NH_4HCO_3. $CaCl_2$ was added to 0.1 mM, followed by aliquots of trypsin (1:100 w/w) at 0, 20, and 40 min. The mixture was allowed to digest overnight at 37 °C and then was applied to a Brownlee RP-300 semipreparative column using the TFA/CH_3CN solvent system (see Materials and Methods) and a linear gradient of 1% solvent B/min. The major radioactive fractions were each pooled and further purified. Peak I (33 nmol) eluted in the void volume along with urea and other nonbinding materials and was later identified as [^{3}H]ADP after conversion to adenosine (see text). Peak II (7 nmol) eluted at 24 min (14% solvent B).

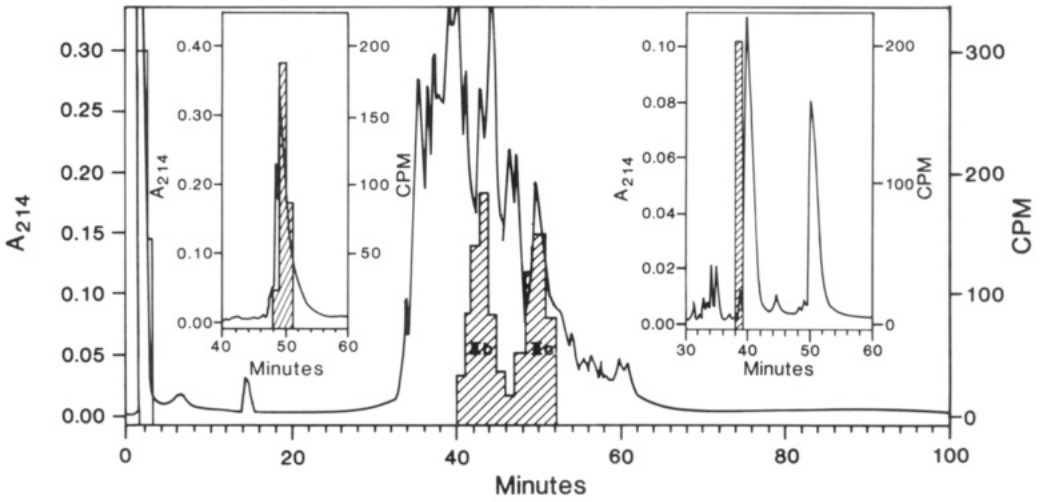


FIGURE 4: Purification of peak II. Peak II (7 nmol) was further purified on an ODS column (Brownlee Labs) in the TFA/CH_3CN solvent system (see Materials and Methods) using a linear gradient of 0.33% solvent B/min. Three radioactive peaks were collected and further characterized. Peak IIa was identified as [^{3}H]ADP after conversion to adenosine by alkaline phosphatase treatment (see text). Left inset: Purification of peptide IIc. Peptide IIc (1 nmol) was treated with alkaline phosphatase (see Materials and Methods) and purified on an ODS column in the TFA/CH_3CN solvent system using a linear gradient of 0.33% solvent B/min. Right inset: Second purification of peptide IIc. Peptide IIc (250 pmol) was next purified on an ODS column in the KP_i/CH_3CN solvent system (see Materials and Methods) using a gradient of 0.33% solvent B/min. Radioactive peptide IIc was collected and sequenced (Figure 6).

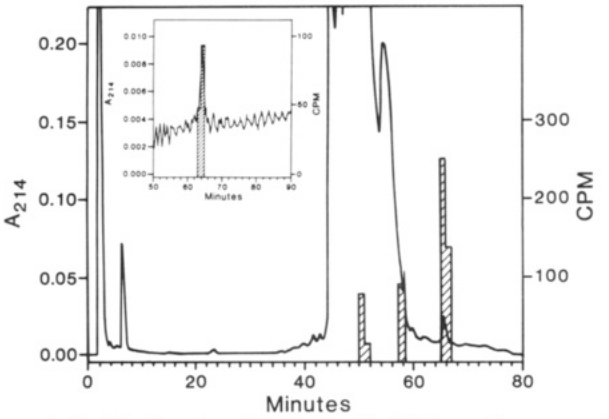


FIGURE 5: Purification of peptide IIb. Peptide IIb (1 nmol) was treated with alkaline phosphatase (see Materials and Methods) and purified on an ODS column in the TFA/CH_3CN solvent system using a gradient of 0.25% solvent B/min. Three labeled peptides (IIb1, IIb2, IIb3) were eluted. Peptide IIb3 (400 pmol) was further analyzed. Inset: Final purification of peptide IIb3. Peptide IIb3 (130 pmol) was further purified with the ODS column in the TFA/CH_3CN solvent system using a gradient of 0.25% solvent B/min. Peptide IIb3 was next subjected to peptide sequence analysis (Figure 6).

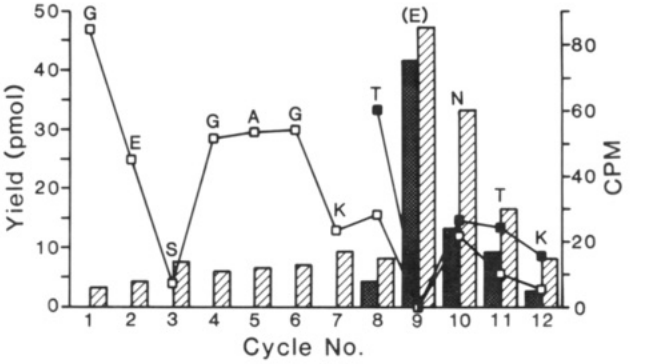


FIGURE 6: Sequence analysis of photolabeled peptides IIc and IIb3. During sequencing, fractions were collected and assayed for radioactivity and identity of the PTH amino acids by HPLC. Sequence analysis of peptide IIc: □, yield; ▨, cpm. Sequence analysis of peptide IIb3: ■, yield; ⊡, cpm. The peak of radioactivity and lack of an identifiable amino acid at cycle 9 indicated that this PTH derivative contained the radiolabel. The residue at cycle 9 (peptide IIc) corresponds to Glu-185 of the gizzard myosin heavy chain (Yanagisawa et al., 1987). The peak of radioactivity and the absence of a PTH derivative at cycle 2 in the sequencing of the related peptide IIb3 again indicated that this amino acid (Glu-185) was photolabeled.

myosin (see Materials and Methods) and was extensively digested with trypsin. Labeled peptides were first separated on a Brownlee RP-300 semipreparative column (Figure 3), and two radioactive peaks, I and II, were each pooled and further purified. Peak I was treated with bacterial alkaline phosphatase, analyzed by capillary zone electrophoresis (see Materials and Methods), and found to be free adenosine (data not shown). Peak II was further purified on an ODS (C_{18}) column at pH 2 (Figure 4), and the radioactive peaks (IIa, IIb, and IIc) were each pooled and further analyzed. Peak IIa was treated with alkaline phosphatase, analyzed as described above, and found to be free adenosine (data not shown). Peak IIc was treated with alkaline phosphatase and purified on an ODS column at pH 2 (Figure 4, left inset). The single peak of radioactivity was collected and purified further on an ODS column at pH 6.0 (Figure 4, right inset). The peptide eluting at 39 min was collected, and phosphate buffer was removed by a reversed-phase column to prepare the peptide for sequencing. Peak IIb was treated with alkaline phosphatase and purified on an ODS column at pH 2 (Figure 5). Peptide IIb3, eluting at 65 min, was purified on an ODS column at pH 2 one final time (Figure 5, inset).

Sequencing of Labeled Peptides. The amino acid sequence of peptide IIc was GESGAGKTXNTK (Figure 6, open squares and hatched bars). Residue X at cycle 9 corresponds to Glu-185 in the amino acid sequence of the gizzard heavy chain (Yanagisawa et al., 1987). The absence of an identified PTH amino acid and the peak of radioactivity at cycle 9 indicates that this residue contained the [^{3}H]adenosine label. The amino acid sequence of peptide IIb3 was TXNTK (Figure 6, solid squares and stippled bars). Residue X at the second cycle again corresponds to Glu-185 in the gizzard heavy-chain sequence.

Discussion

In this work, [^{3}H]ADP was used as a direct photoprobe for the purine-binding site of gizzard myosin. [^{3}H]ADP was chosen because ATP is the native substrate for myosin, and its binding within the active site will not be perturbed by ancillary photoreactive groups. In addition, ADP has been

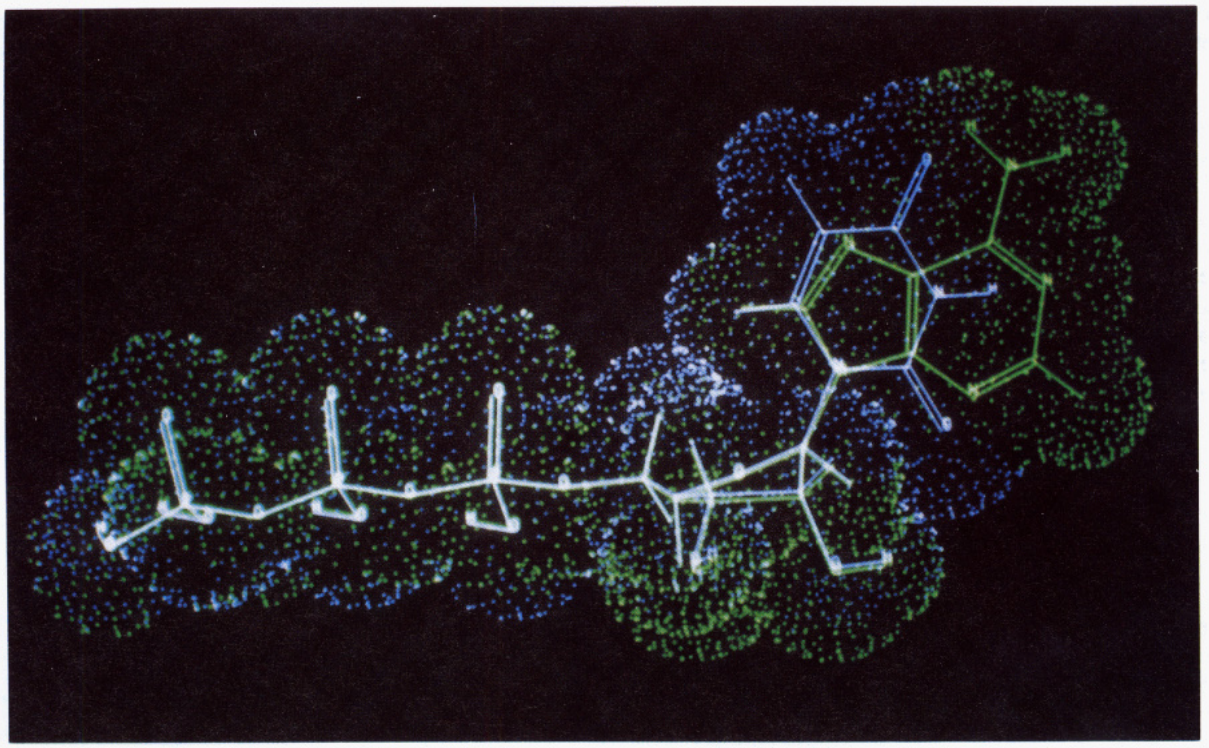


FIGURE 7: Superimposition of ATP and UTP. The Macromodel program (Still et al., 1990) was used to construct and superimpose models of ATP (green) and UTP (purple). Atoms and bonds common to both nucleotides are shown in white. When each nucleotide is in the anti conformation, the 7,8 double bond of the adenine ring is nearly coincident with the photoreactive 5,6 double bond of the uracil ring.

shown to photoincorporate into gizzard myosin (Maruta & Korn, 1981), but the precise residue of attachment was not identified. Trapping the [^{3}H]ADP at the active site with vanadate (Goodno, 1979, 1982) and Co^{2+} produced a very stable myosin·Co^{2+}·[^{3}H]ADP·V_i complex that could be readily separated from extraneous [^{3}H]ADP (Figure 1, inset), greatly reducing the possibility of nonspecific photolabeling. Irradiation of this complex with UV light resulted in approximately 10% covalent attachment of trapped [^{3}H]ADP into only the heavy chain of gizzard myosin (Figure 1). After extensive digestion of photolabeled S1 with trypsin, radioactive HPLC peptide peaks were treated with bacterial alkaline phosphatase to remove phosphate groups and purified (Scheme I). This procedure allowed the effective separation of labeled peptides from contaminating materials in one or two additional HPLC steps. Furthermore, since PTH derivatives of phosphorylated amino acids are known to be poorly extracted by butyl chloride during automatic peptide sequencing (Murakami et al., 1990), the absence of phosphate groups allowed Glu-185 to be unambiguously identified by its radioactivity during the appropriate cycle of peptide sequencing. Peptide IIb3 had the sequence Thr-X-Asn-Thr-Lys, which corresponds to residues 184–188 of the gizzard myosin heavy chain (Yanagisawa et al., 1987). Peptide IIc was similar, except that it contained a seven-residue NH_2-terminal extension consistent with residues 177–188. Upon sequencing each peptide, the cycle corresponding to Glu-185 gave no identifiable PTH amino acid and contained the [^{3}H]adenosine label (Figure 6). These results indicate that Glu-185 is the residue labeled and is located in the purine-binding pocket of the active site, close to the photoreactive bond of [^{3}H]ADP (see below).

Photoaffinity-labeling techniques have provided a useful way to assess active-site architecture in a variety of myosins. Direct photoaffinity-labeling studies with [^{3}H]UTP and [^{3}H]UDP have both identified Glu-185 of the heavy chain as located at the active site of *Acanthamoeba* myosin II (Atkinson et al., 1986) and gizzard myosin (Garabedian & Yount, 1990), respectively. This study shows that [^{3}H]ADP also labels Glu-185 in gizzard myosin. These results indicate that gizzard myosin binds both [^{3}H]UDP and [^{3}H]ADP in a manner such that the photoreactive bond of each base is very close to Glu-185. That Glu-185 becomes labeled by both [^{3}H]UDP and [^{3}H]ADP is an unexpected result, based on the nature of the amino acids adjacent to it (Table I). Glu-185 is conserved in all smooth and nonmuscle myosins and is surrounded by numerous hydrophilic residues. Residues of this nature are commonly found on the surface of proteins and suggest that this segment of the heavy chain is located at the surface of the myosin head. Photolabeling studies with NANDP on skeletal S1, on the other hand, have suggested that the hydrophobic region around Trp-130 constitutes part of the adenine-binding subsite (Ok-

Table I: Sequences of Myosin Heavy Chains

source	sequence	reference
peptide IIb3 (gizzard)	TENTK	this study
peptide IIc (gizzard)[a]	GESGAGKTENTK	this study
Acanthamoeba (myosin I)	GESGAGKTEASK	Jung et al., 1987
Acanthamoeba (myosin II)	GESGAGKTENTK	Hammer et al., 1987
yeast	GESGAGKTENTK	Warrick & Spudich, 1987
Dictyostelium	GESGAGKTENTK	Warrick et al., 1986
nematode (*C. elegans*)	GESGAGKTENTK	Karn et al., 1983
Drosophila	GESGAGKTENTK	Wassenberg et al., 1987
avian brush border	GESGAGKTEASK	Shohet et al., 1989
chicken (skeletal)	GESGAGKTVNTK	Molina et al., 1987
rat (skeletal)	GESGAGKTVNTK	Strehler et al., 1986
rabbit (skeletal)	GESGAGKTVNTK	Tong & Elzinga, 1983

[a] The sequence of the gizzard heavy chain indicates that the residue immediately preceding the NH_2-terminal Gly of peptide II is Thr. This finding suggests that the specificity of trypsin has been altered, possibly by exposure to 2 M urea during proteolysis.

amoto & Yount, 1985). A possible explanation may be that the methylene groups of the side chain of Glu-185 provide part of the inner surface of the adenine binding pocket, with the γ-carboxyl group hydrogen bonded to a purine or pyrimidine NH group or elsewhere.

As with UDP, ADP does not significantly label (<1%) skeletal S1 when V_i-trapped and irradiated as described here (T. Garabedian, unpublished results). In skeletal myosin, a valyl residue replaces Glu-185 in the heavy-chain sequence (Table 1). Valine may be less reactive to photoactivated [^{3}H]ADP, or the active sites of smooth and skeletal myosin may be fundamentally different, perhaps reflecting their different modes of regulation. Interestingly, scallop myosin contains a glutamyl residue immediately following the glycine-rich loop (Nyitray et al., 1990), even though this myosin is striated like skeletal myosin, but is regulated by binding of Ca^{2+} directly to the myosin.

The direct photolabeling results discussed above contrast with those obtained with the photoaffinity label Bz_2ATP. This photoprobe, which labels areas near the ribose ring, has been shown to label the same region of both skeletal and smooth muscle myosin, i.e., Ser-324 in skeletal S1 (Mahmood et al., 1989) and Pro-324 in gizzard myosin (Cole & Yount, 1990). Both Ser-324 and Pro-324 are located within the 50-kDa tryptic fragment of their respective heavy chains and indicate that the folding of the heavy chain near the ribose-binding subsite is essentially the same in both skeletal and gizzard myosins.

While the photochemical reactions of pyrimidine bases have been studied in detail, relatively little information is available concerning the photochemistry of purines. Purine bases form part of the light-absorbing system in DNA, but they are less sensitive to light than the pyrimidine bases (Elad, 1976). Nevertheless, some photochemical reactions of purine bases have been reported, primarily photooxidation reactions (Rahn, 1976; Gallagher & Duker, 1986). The 8-position of the purine ring has been claimed to have a unique character as a reactive site, particularly toward free radicals (Robins, 1967), but in no case has a photoproduct of a purine and a protein or peptide been explicitly characterized.

The likely conformation of the nucleotide bound at the active site may help explain why both UDP and ADP label the same residue in gizzard myosin. The crystal structures of several nucleotide-binding proteins in the presence of their substrates or cofactors have been reported, including actin (Kabsch et al., 1990), phosphofructokinase (Evans et al., 1981), p21 protein from Ha-*ras* (La Cour et al., 1985; De Vos et al., 1988; Pai et al., 1990; Milburn et al., 1990), phosphoglycerate kinase (Watson et al., 1982), and several tRNA synthetases (Blow & Brick, 1985; Brick et al., 1988; Rould et al., 1989; Brunie et al., 1990). In every case, the conformation of the nucleotide bound at the active site is anti.[3] In addition, recent NMR data have indicated that nucleotides bind in the anti conformation in adenylate kinase (Yan et al., 1990), pyruvate kinase (Rosevear et al., 1987), dihydrofolate reductase (Brito et al., 1991), methionyl-tRNA synthetase (Williams & Rosevear, 1991), and F_1ATPase (Garin et al., 1988). The anti conformation of a purine nucleotide allows for minimal steric interactions between the base and ribose and results in a structure with C-8 of the purine ring positioned over the ribose (Saenger, 1984). Similarly, the anti conformation of the pyrimidine ring of UTP places the photoreactive C-5, C-6 double bond of uracil over the ribose ring. Molecular modeling studies of UTP and ATP indicate that when both nucleotides are anti, the photoreactive 5,6 double bond of UTP can be superimposed on top of the N-7, C-8 double bond of ATP (Figure 7). This result suggests that the 7,8 double bond of adenine may participate in the photoreaction of [^{3}H]ADP into myosin and may also explain why Glu-185 becomes labeled by both [^{3}H]UDP and [^{3}H]ADP. C-8 has been shown to be a site of photoinduced attachment between alcohols and purine nucleosides in solution studies (Steinmaus et al., 1971); however, it is unclear which atoms in the adenine ring participate in the covalent attachment to Glu-185. The exact structural role of Glu-185 will most likely not be determined until the crystal structure of the gizzard S1·ATP complex is solved.

[3] X-ray crystallographic analysis has shown that cyclic 3′,5′-AMP (cAMP) binds in the anti conformation on the *E. coli* catabolite gene activator protein (McKay et al., 1982), but molecualr modeling indicates that cAMP likely binds in the syn conformation to the regulatory subunit of cAMP-dependent protein kinase (Weber et al., 1987). In addition, the crystal structure of fructose-1,6-bisphosphatase complexed with AMP has also been reported (Ke et al., 1990), but at the present level of resolution and refinement, the electron density of the AMP bound on the enzyme can fit either the syn or anti conformation.

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