The active site of chicken gizzard myosin was labeled by direct photoaffinity labeling with $[^3H]UDP$. $[^3H]UDP$ was stably trapped at the active site by addition of vanadate $(V_0)$ and Co$^{2+}$. The extraordinary stability of the myosin-Co$^{2+}$-[$^3H]UDP-V_0$ complex $(t_{90} > 5$ days at 0°C) allowed it to be purified free of extraneous $[^3H]UDP$ before irradiation began. Upon UV irradiation, >60% of the trapped $[^3H]UDP$ was photoincorporated into the active site. Only the 200-kDa heavy chain was labeled, confirming earlier results (Maruta, H., and Korn, E. (1981) J. Biol. Chem. 256, 499-502) using $[^3H]UTP$. Extensive tryptic digestion of photolabeled myosin subfragment 1 followed by high performance liquid chromatography separations and removal of nucleotide phosphates by treatment with alkaline phosphatase allowed two labeled peptides to be isolated. Sequencing of the labeled peptides and radioactive counting showed that Glu$^{185}$ was the residue labeled. Since UDP is a “zero-length” cross-linker, Glu$^{185}$ is located at the purine-binding pocket of the active site of smooth myosin and adjacent to the glycine-rich loop which binds the polyphosphate portion of ATP. This Glu residue is conserved in smooth and nonmuscle myosins and is the same residue identified previously by $[^3H]UTP$ photolabeling in Acanthamoeba myosin II (Atkinson, M. A., Robinson, F. A., Appella, E., and Korn, E. D. (1986) J. Biol. Chem. 261, 1844-1848).

A knowledge of the topology of the active site of myosin is a necessary first step to provide a molecular understanding of the mechanism of ATP hydrolysis and mechanical force generation in muscle. Labeling skeletal S1$^\dagger$ with photoaffinity analogs of ATP has proven to be a useful approach to identify active site amino acids (Cremo et al., 1989; Mahmood et al., 1989; Yount et al., 1987, Okamoto and Yount, 1985). It was of interest to extend these studies to smooth muscle myosin, because this myosin molecule is regulated by phosphorylation and may have an active site topology different from that found in skeletal myosin. Furthermore, previous work (Okamoto et al., 1986) has shown that elements of the 17-kDa essential light chains are located at the active site in smooth muscle myosin. In contrast, only residues of the 200-kDa heavy chain in skeletal myosin have been placed at the active site (Cremo et al., 1989; Mahmood et al., 1989; Sutoh, 1987; Sutoh et al., 1986; Okamoto and Yount, 1985; Szilagyi et al., 1979). Thus, even though smooth and skeletal myosins are superficially the same (e.g. molecular mass, subunit composition, and gross morphology), the active site region where ATP is bound and cleaved, appears to be different.

Although ATP photoaffinity analogs have been useful tools to identify amino acids at the active site of myosin, the photoreactive group is often bulky and may identify residues outside the active site. One approach to remedy this problem is to use native photoreactive nucleoside triphosphates as “direct” photoaffinity probes. This type of direct photoaffinity labeling using radioactive nucleotide substrates has been utilized to examine a broad variety of nucleotide binding proteins including aminoacyl-tRNA synthetases (Yue and Schimmel, 1979), DNA polymerases (Pandey et al., 1987; Biswas and Kornberg, 1984, Abraham and Modak, 1984; Hillel and Wu, 1978), ribonucleotide reductase (Kierszuk and Eriksson, 1988), recA binding protein (Banks and Sedgwick, 1986), and $\beta$-tubulin (Linse and Mandelkow, 1988; Nuth and Himes, 1983).

Uracil contains a photoreactive 5,6 double bond and will form photoproducts with nearby amino acids when exposed to UV radiation (Smith, 1969). This property makes UTP a useful photoaffinity analog of ATP and allows the structure of the purine binding pocket of the active site of myosin and other nucleotide binding proteins to be investigated. $[^3H]UTP$ has been used effectively to label myosin from turkey gizzard and Acanthamoeba myosin II to show that elements of the heavy chain provide part of the active site (Maruta and Korn, 1981). Subsequent work with Acanthamoeba myosin II (Atkinson et al., 1986) has identified Glu$^{185}$ of the heavy chain as a residue at the active site. An inherent problem with these studies was that the high energy UV light necessary for activating UTP (or other nucleotides) rapidly inactivates myosin so that it was not possible to correlate inactivation with the covalent photolabeling of the enzyme. These conditions make it difficult to rule out possible nonspecific labeling. One method to alleviate this possible complication is to “trap” a nucleoside diphosphate molecule at the active site with orthovanadate $(V_0)$ and divalent metals (Goodno, 1979). Replacing Mg$^{2+}$ with Co$^{2+}$ in the trapped complex will suppress the known photooxidation of active site residues by vanadate (Cremo et al., 1988; Grammer et al., 1988). The vanadate*
trapping approach allows the myosin-Co\(^{2+}\)-NDP-V complex to be isolated before irradiation begins, thus insuring specific photolabeling of only active site residues.

In this work we report that \(^{[3]H}\)UDP, when trapped at the active site with vanadate and Co\(^{2+}\) and irradiated with UV light, appears to react exclusively with Glu\(^{185}\) of the gizzard myosin heavy chain. The results place Glu\(^{185}\) in the adenine binding pocket of the active site, adjacent to the glycine-rich loop which is thought to bind the polyphosphate portion of ATP. This particular Glu residue is conserved in a variety of smooth and nonmuscle myosins, yet is replaced by Val in skeletal myosin. These results, in combination with other photофилиосор labeling studies of the active site of myosin, provide further evidence for differences in the structure and composition of the active site in smooth and skeletal myosins.

**EXPERIMENTAL PROCEDURES**

**Materials**—The commercial compounds and their sources were as follows: \(^{[3]H}\)UDP, Amersham Corp.; \(^{[3]C}\)ADP, Du Pont-New England Nuclear; papain, trypsin, bacterial alkaline phosphatase, Sephadex G-50, Sigma; ultrapure urea, Schuer-Mann; sodium orthovanadate, Na\(_2\)VO\(_4\), Fisher. Vanadate stock solutions (75-100 mM) were prepared as described by Goodno (1982). All buffers contained 0.025% sodium azide (Sigma) as a preservative.

**Enzyme Preparations**—Phosphorylated smooth muscle myosin was isolated from fresh chicken gizzards as described by Ebashe (1976) and stored in 50% glycerol at -20°C. K\(^{+}\)EDTA ATPase activities were quantified as previously described by Wells et al. (1977) except that the release of P\(_i\) was measured after 2 and 8 min. Protein concentrations were quantified by the dye binding assay of Bradford (1976) using a 2 mg/ml gizzard myosin standard (ε\(_{280}\) = 5.6).

**Trapping and Irradiation of \(^{[3]H}\)UDP on Gizzard Myosin**—Trapping procedures followed those of Goodno (1979). Myosin (120 mg in 45 ml, 0.5 M KCl, 20 mM Tris, pH 8.0) was incubated with 2 mM Co\(^{2+}\) and 30 nM \(^{[3]H}\)UDP (14,800 cpm/mg, at 25°C and 0.5 M Na\(_2\)SO\(_4\), \(0.025\) M Tris buffer, pH 10), and 50 mM L-cysteine, 1 mM EDTA, 1 mM Na\(_2\)EDTA, pH 8.0, to 20 mM and the solutions immediately placed on ice. Unbound \(^{[3]H}\)UDP was removed by centrifugation through approximately 5 ml Sephadex G-50 columns (Penesfksy, 1977) equilibrated in 0.5 M NaCl, 20 mM Tris, pH 8.0. \(^{[3]H}\)UDP-trapped at the active site of the recovered myosin was quantified by dissolving aliquots (50 µl) in 5 ml of ACS (Amersham Corp.) and counting with a Packard 1900CA scintillation counter. The purified myosin-Co\(^{2+}\)-\(^{[3]H}\)UDP-V complex (2 mg/ml) was irradiated in 5-cm samples with an unfiltered G.E. 30-watt germicidal lamp which produces predominantly 254 nm radiation. The flux at the plane of irradiation was 30 W/m\(^2\), as measured with a Blak-Ray UV meter (Ultra Violet Products). Photoincorporation of \(^{[3]H}\)UDP was quantified by precipitation of the myosin (0.1-ml samples) with 0.2 ml of cold 10% trichloroacetic acid. The precipitate was collected by centrifugation, washed twice with cold 5% trichloroacetic acid, and dissolved in approximately fifty 5-ml Sephadex G-50 columns (Penesfksy, 1977) equilibrated in 0.5 M NaCl, 20 mM Tris, pH 8.0. The purified complex was irradiated as described under "Experimental Procedures" (Fig. 2). It was important to irradiate the complex for less than 20 min, since longer times produced extensive protein cross-linking. For larger scale photolysis, 12 min of UV irradiation (21.6 kJ/m\(^2\)) produced the maximal amount of photoincorporation with the smallest amount of protein photocrosslinking. Approximately 0.59 mol/mol of active site or 63% of the trapped \(^{[3]H}\)UDP was found to be covalently attached to the protein after 20 min of irradiation.

The stability of myosin-Co\(^{2+}\)-\(^{[3]H}\)UDP-V complex was investigated to confirm that vanadate decreases the off-rate of HPLC, Capillary Electrophoresis, and Peptide Sequencing—HPLC hardware and software consisted of two Waters 501 pumps, a Waters 490E multiwavelength detector, a Waters 840 data station, interface module, and professional software, and a Pharmacia LKB Biotechnology Inc. Fracta-Frac collection system. HPLC separations of crude \(^{[3]H}\)UDP-labeled tryptic peptides were performed on a Brownlee Aquapore RP 300 semipreparative column (7 mm × 25 cm, 10 µm packing). Solvent A was 0.11% trifluoroacetic acid in H\(_2\)O, pH 2; solvent B was 0.10% trifluoroacetic acid in 60% CH\(_3\)CN, and the flow rate was 2.0 ml/min (fraction sizes = 2.0 ml).

Preparative HPLC purification steps were performed with a Brownlee Sphero-5 RP-18 column (4.6 mm × 10 cm, 5 µm packing) in either trifluoroacetic acid/H\(_2\)O/CH\(_3\)CN solvent system described above or a phosphate system consisting of 2 or 5 mM KH\(_2 PO\(_4\), pH 6.0 (solvent A) and 65% CH\(_3\)CN (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 a Bio-Rad HPE-100 capillary electrophoresis unit (Bio-Rad) using a 25 µm × 20-cm coated capillary cartridge and 0.1 M sodium phosphate buffer, pH 2.5. Peptide sequence analysis was performed on an Applied Biosystems model 470A gas phase sequencer with Pulse Liquid\(^{\text{TM}}\) update and model 120A phenylthiohydantoin analyzer (Applied Biosystems, Inc.) according to protocols supplied by the manufacturer.

**RESULTS**

Trapping and Irradiation of \(^{[3]H}\)UDP—It has been shown (Goodno, 1979) that ADP can be stably trapped at the active site of skeletal myosin when incubated with Mg\(^{2+}\) and vana
date (V). The vanadate ion (V) is thought to mimic the γ-phosphate of ATP and form a stable trigonal bipyramidal transition state-like structure with ADP. This approach leads to a 10\(^{-4}\) decrease in the off-rate of ADP and produces a complex with a lifetime of several days at 0°C. Here, we have replaced ADP with \(^{[3]H}\)UDP and Mg\(^{2+}\) with Co\(^{2+}\) to form a stable gizzard myosin-Co\(^{2+}\)-\(^{[3]H}\)UDP-V complex. UDP was selected because of all the common nucleoside diphosphates, it gives the highest photo
crosslinking into gizzard myosin (Maruta and Korn, 1981). Co\(^{2+}\) was used because it quenches the photo
crosslinking of photoreaction of vanadate itself with active site residues which occurs in the presence of Mg\(^{2+}\) (Gramer et al., 1988). Fig. 1 shows the loss of K\(^{+}\)EDTA ATPase activity as a function of vanadate trapped \(^{[3]H}\)UDP. The stoichiometry of trapped \(^{[3]H}\)UDP is nearly 2 mol/mol of myosin. To verify that \(^{[3]H}\)UDP was binding at the active site, competitive vanadate trapping experiments were performed with [\(^{3}C\)]ADP and [\(^{3}H\)UDP. As the mol fraction of [\(^{3}C\)]ADP was increased relative to the mol fraction of [\(^{3}H\)UDP, more [\(^{3}C\)]ADP was trapped, but the total amount of diphosphate bound remained constant (data not shown). These data indicate that \(^{[3]H}\)UDP binds at the active site in a manner similar to ADP.

The purified complex was irradiated as described under "Experimental Procedures" (Fig. 2). It was important to irradiate the complex for less than 20 min, since longer times produced extensive protein cross-linking. For larger scale photolysis, 12 min of UV irradiation (21.6 kJ/m\(^2\)) produced the maximal amount of photoincorporation with the smallest amount of protein photocrosslinking. Approximately 0.59 mol/mol of active site or 63% of the trapped \(^{[3]H}\)UDP was found to be covalently attached to the protein after 20 min of irradiation.

The stability of myosin-Co\(^{2+}\)-\(^{[3]H}\)UDP-V complex was investigated to confirm that vanadate decreases the off-rate of...
Regulatory light chains nor the l7-kDa essential light chains associated with the 200-kDa heavy chains. Neither the 20-kDa peptide nor the labeled subunits (Fig. 3). All the radioactivity was associated with the 200-kDa heavy chain, photolabeled Sl (see below) was partially digested with trypsin and analyzed on a 12% SDS-polyacrylamide gradient gel (17-20%) with a 5% stacking gel. Gel lanes were sliced, solubilized, and counted as described in Fig. 3.

**Isolation of Peptide I**—Peptide I was very hydrophilic, as it eluted in the void volume on the reverse phase semipreparative column (Fig. 5). Peptide I did not bind to an ODS (C18) column at pH 2 or 6 and was irreversibly retained on an anion exchange column (AX-10, Varian; 5 mM KH2PO4, pH 5.0, and a gradient of 0-0.5 M KCl). In order to make peptide I more hydrophobic, the phosphate groups of the covalently bound [3H]UDP were removed with bacterial alkaline phosphatase (see "Experimental Procedures") and the digest was purified on an ODS column at pH 2. Radioactive peptides eluting in the void volume (Fig. 6) were collected and further purified at pH 6.0 (Fig. 7) followed by a desalting step (Fig. 8) to prepare the peptide for sequencing.

**Isolation of Peptide II**—Peptide II, which eluted at 25 min on the reverse phase semipreparative column (Fig. 5), was further purified first at pH 6.0 (Fig. 9), then at pH 2 (Fig. 10). The desalting step (Fig. 10) was necessary to remove inorganic phosphate, which is known to be an inhibitor of alkaline phosphatase (Reid and Wilson, 1971). Radioactive peptide II was isolated (see "Experimental Procedures") and the digest was extensively digested with trypsin and peptides separated on a Brownlee RP-300 semipreparative column (see "Experimental Procedures" and Fig. 5). Two radioactive peaks (I and II) were independently pooled for further purification.

**Analysis of the Location of [3H]UDP**—The photolabeled gizzard myosin was digested with papain, and photolabeled Sl was isolated (see "Experimental Procedures") and the digest was purified first at pH 6.0 (Fig. 7) followed by a desalting step (Fig. 8) to prepare the peptide for sequencing.

**Isolation of Peptide I**—Peptide I was very hydrophilic, as it eluted in the void volume on the reverse phase semipreparative column (Fig. 5). Peptide I did not bind to an ODS (C18) column at pH 2 or 6 and was irreversibly retained on an anion exchange column (AX-10, Varian; 5 mM KH2PO4, pH 5.0, and a gradient of 0-0.5 M KCl). In order to make peptide I more hydrophobic, the phosphate groups of the covalently bound [3H]UDP were removed with bacterial alkaline phosphatase (see "Experimental Procedures") and the digest was purified on an ODS column at pH 2. Radioactive peptides eluting in the void volume (Fig. 6) were collected and further purified at pH 6.0 (Fig. 7) followed by a desalting step (Fig. 8) to prepare the peptide for sequencing.
was then treated with bacterial alkaline phosphatase (see "Experimental Procedures") and purified at pH 2 (Fig. 11) to prepare the peptide for sequencing.

Sequence Analysis of Peptides I and II—The amino acid sequence of peptide I was found to be T(X)NTK (Fig. 12). The residue at cycle 2 corresponds to Glu in the sequence of the gizzard myosin heavy chain (Yanagisawa et al., 1987). Radioactivity was first released at cycle 2, further indicating that Glu contained the [3H]uridine label.

The sequence of peptide II was TGESGAGKT(X)NTK (Fig. 12B), where the residue at cycle 10 corresponds to Glu in the amino acid sequence of the gizzard myosin heavy chain (Yanagisawa et al., 1987). The radioactivity detected after cycle 10 was most likely due to the continued partial extraction of the [3H]uridine-glutamic acid-phenylthiohydantoin adduct from the polybrene coated membrane during each subsequent cycle.

**DISCUSSION**

The goal of this work was to identify and locate active site amino acid residues of gizzard myosin which are photolabeled by [3H]UDP. Vanadate trapping methodology (Goodno, 1979, 1982) was employed to stably bind [3H]UDP at the active site and allow the myosin-Co^2+ complex to be purified away from free [3H]UDP. The addition of Co^2+ in the trapped complex is known to stabilize the photooxidation of active site residues by vanadate (Gramer et al., 1988; Cole and Yount, 1989). Subsequent irradiation of the purified complex resulted in approximately 60% incorporation of trapped [3H]UDP into the heavy chain (Fig. 2). Following extensive trypsin digestion of the labeled S1, two major [3H]UDP-labeled peptide pools were each dephosphorylated with bacterial alkaline phosphatase and purified further by reverse phase HPLC (Scheme I). This procedure allowed the effective separation of labeled peptides from contaminating peptides in one or two additional HPLC steps. In addition, the absence of phosphate groups on the photoincorporated [3H]uridine allowed the labeled residue, Glu, to be positively identified by its radioactivity during the appropriate cycle of peptide sequencing. The phenylthiohydantoins of phosphorylated amino acids, e.g. phosphoserine (Murakami et al., 1990) are known to be poorly extracted by the organic solvents used in automated peptide sequencing. Hence, phenylthiohydantoins of amino acids photolabeled with nucleotides or nucleotide derivatives will likely never be identified because of the strong affinity of the phosphates for the positively charged polybrene coating on the filters. Peptide I had the sequence Thr-Asn-Thr-Lys which corresponds to residues 144-188 of the gizzard myosin heavy chain (Yanagisawa et al., 1987). Peptide II was similar to peptide I except that it contained an eight residue NH2-terminal extension giving a peptide corresponding to residues 176-188. In sequencing both peptides, the cycle corresponding to Glu gave no identifiable phenylthiodydantoin derivative and contained the [3H]uridine label (Fig. 12, A and B).

Photoaffinity labeling of a variety of myosins has provided useful comparative information about active site structure. Previous direct photoaffinity labeling studies with [3H]UTP have identified Glu of the heavy chain in the active site of Acanthamoeba myosin II (Atkinson et al., 1986). Here we have shown that [3H]UDP labels the analogous residue in gizzard myosin. In addition, no labeling of light chains is observed in either gizzard or Acanthamoeba myosins when [3H]UDP is used as a photoprobe. These data indicate that myosins from gizzard and Acanthamoeba bind [3H]UDP in a similar way and have comparable subsite structures in the area where the 5,6 double bond of [3H]UDP is located. These results are unexpected based on inspection of the amino acid composition around Glu. This residue is conserved in all smooth and non-muscle myosins and is surrounded by a predominance of hydrophilic residues (Table I). The nature of this peptide region suggests it is located at the surface of the myosin head. In contrast, previous photoaffinity labeling studies with skeletal myosin (Okamoto and Yount, 1985; Sutoh, 1987) have implicated the hydrophobic region around Trp as providing the adenine binding site. It may be that the methylene groups of the side chain of Glu lie next to the adenine ring with the γ-carboxyl group hydrogen-bonded elsewhere.

[3H]UDP does not label skeletal S1 when trapped and irradiated as described above. These results may be explained.

| Table I |
|----------------------------------|
| **Sequences of myosin heavy chains** |
|----------------------------------|
| Peptide I (gizzard)          | T E N T K |
| Peptide II (gizzard)         | T G E S G A G K T E N T K |
| Acanthamoeba (myosin II)     | T G E S G A G K T E N T K |
| Acanthamoeba (myosin II)'    | T G E S G A G K T E N T K |
| Yeast*                       | T G E S G A G K T E N T K |
| Slime mold (Dictyostelium)   | T G E S G A G K T E N T K |
| Nematode (Caenorhabditis elegans) | T G E S G A G K T E N T K |
| Drosophila*                  | T G E S G A G K T E N T K |
| Avian brush border*          | T G E S G A G K T E N T K |
| Chicken (skeletal)*          | T G E S G A G K T V N T K |
| Rat (skeletal)               | T G E S G A G K T V N T K |
| Rabbit (skeletal)*           | T G E S G A G K T V N T K |

*The sequence of the gizzard heavy chain indicates that the residue immediately preceding the NH2-terminal Thr of peptide II is Cys. This finding suggests that the specificity of trypsin has been altered, possibly by exposure to 2 M urea during digestion.
* Jung et al. (1987).
* Haussner et al. (1987).
* Warrick and Spudich (1987).
* Warrick et al. (1986).
* Karn et al. (1985).
* Wassernberg et al. (1987).
* Shohet et al. (1989).
* Molina et al. (1987).
* Streher et al. (1986).
* Tong and Elzinga (1983).
that in skeletal myosin a Val replaces Glu\(^{85}\) (Table I), and Val may simply be less reactive towards phototoactivated UDP than Glu. Alternatively, it may be the composition of the active sites of skeletal and smooth muscle myosins are different, reflecting their different modes of regulation.

Photoaffinity labeling studies with the photoprobe NAPD provide additional evidence that suggest the active site topologies of smooth and skeletal myosins are different. NAPD contains a photoreactive nitroaryl-azido group which binds in the adenine subsite of the active site. The azido group of NAPD is located on the end opposite the diphosphate and should label residues deep within the adenine binding pocket. NAPD, when trapped and photoincorporated at the active site of skeletal S1, labeled Trp\(^{102}\) of the heavy chain (Okamoto and Yount, 1985). No labeling of the light chains was observed. Gizzard myosin, however, is labeled by NAPD in both the heavy chain and the 17-kDa essential light chain (Okamoto et al., 1986). This finding demonstrates further differences in active site topology between smooth and skeletal myosins.

In contrast to the above results, photoaffinity labeling studies with the photoprobe Bz\(^{88}\)ATP indicate that the heavy chain conformation near the ribose binding site for ATP in both smooth and skeletal myosins are essentially identical. Bz\(^{88}\)ATP contains a photoreactive benzamidro group esterified to the 2' or 3' hydroxyl of the ribose ring of ATP and will label residues near the ribose binding subsite of the active site. Bz\(^{88}\)ATP has been shown to label Ser\(^{95}\) of the heavy chain in skeletal myosin (Mahmood et al., 1989) and Pro\(^{94}\) of the heavy chain in gizzard myosin (Cole and Yount, 1990). Both of these residues are located within the central 50-kDa trypic fragment of their respective S1 heavy chains and suggest that the gross morphology and folding of this part of the heavy chain around the ATP binding site is similar in these two myosins.

The photochemistry of nucleotide bases with proteins remains largely unknown. However, we expect that the 5,6 double bond of uracil is the photoreactive center of UDP. The 5,6 double bond of pyrimidine bases is known to undergo a \(\pi \rightarrow \pi^*\) transition when exposed to UV light and form covalent photoadducts with nearby molecules (Wang, 1976; Smith, 1969). In a few cases, the photoproduits of direct photoaffinity labeling have been isolated and characterized. For example, photolabeling studies of diaphorase toxin and \textit{Pseudomonas exotoxin} with \(^{14}\text{C}\)NAD have identified glutamic acid at the active sites of these proteins (Carroll and Collier, 1987; Carroll et al., 1985). It is also not known if amino acids add preferentially at the C\(_6\) or C\(_5\) position of the pyrimidine ring. Kierdaszuk and Eriksson (1988) have isolated and characterized the major photoadduct of ribonucleotide reductase and dTTP and have shown it to contain a covalent bond between the Apyrimidine C\(_6\) position and the 5-carboxyanhydride group of thymidine. \[^{32}\text{P}]\text{TTP} is only one-sixth as efficient as UDP in photoaffinity labeling gizzard myosin, a result which suggests that the C\(_6\) position of uracil may be the major site of reaction with Glu\(^{85}\).

The confirmation of \[^{3}H]\text{UDP} bound at the active site may explain why a Glu residue next to the comparable glycine rich loop of gizzard myosin becomes labeled. The orientation of pyrimidine nucleic acids about the glycosyl bond is predominantly anti (Saenger, 1984). This conformation exhibits minimal steric hindrance between the ribose and uracil rings and places the photoreactive 5,6 double bond over the ribose, closer to the diphosphates. In contrast to NAPD, UDP should label an area in the adenine pocket that is closer to the phosphate binding region. Hence, even though Glu\(^{85}\) is directly adjacent to the GESGAGKT sequence known to be at the triphosphate binding site (Cremo et al., 1989), it could still be close enough to add to the 5,6 double bond of the trapped UDP. Whether Glu\(^{85}\) also plays an important role in binding to the adenosine ring of ATP awaits the solution of the crystal structure of the gizzard S1-ADP complex at atomic resolution.

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Supplemental Material to
Direct Photoaffinity Labeling of Gizzard Myosin with [3H]Uridine Diphosphate Places G105 of the Heavy Chain at the Active Site.

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Figure 5. HPLC of tryptic digest of photolabeled S1. Photolabeled S1 (15 nmol) was hydrolyzed and concentrated to 10 ml of 0.1 M urea, 0.1 M NH4HCO3 (pH 8.5), 5 mM DTE. The solution was diluted to 2 M urea with 0.1 M NH4HCO3 and CaCl2 was added to 0.1 mM. Aliquots of trypsin were added (1:100 w/w) at 0, 20, and 40 min, and the mixture was allowed in digest overnights at 17°C. Peptides were then separated on a Brownlee RP300 semipreparative column (Brownlee Labs) using the TFA/H2O/CH3CN solvent system (see Materials and Methods) with a linear gradient of 0.7% B per minute. Two radioactive peptide fractions were independently isolated: Peptide I (15 nmol) eluted in the void volume along with urea and other denaturing materials. Peptide II (20 nmol) eluted at 25 min (15% B).

Figure 6. HPLC of alkaline phosphatase treated peptide I. The area concentration of the peptide fractions were reduced from 6.5 M to 0.8 M (as determined by peaks of elution and comparison with area standard solutions) by dilution with 0.1 M Tris-Cl (pH 8.0). After bacterial alkaline phosphatase treatment (see Materials and Methods), the digest was separated using an ODS column (Brownlee Labs) and the TFA/H2O/CH3CN solvent system (see Materials and Methods) with a linear gradient of 0.33% B per minute. Peptides eluting at about 45 mm (3 nmol) were pooled and further purified (Figure 7).

Figure 7. Second HPLC purification of peptide I. Peptide I was further purified on an ODS column using the 2 nm KH2PO4 (pH 6.0) solvent system (see Materials and Methods) with a linear gradient of 0.25% B per minute. The peptide eluting at 42 mm (1.5 nmol) was prepared for sequencing as described in Figure 8.
Direct Photoaffinity Labeling of Gizzard Myosin with UDP

Figure 8. High-performance liquid chromatography (HPLC) analysis of peptide I. Peptide I was injected onto a C18 octyl column in the TFA/CH3CN/CH2O/H2O solvent system using a linear gradient of 0.1% TFA per minute. Pure peptide I eluting at 34 min was collected and subjected for radioactivity analysis (Figure 12 A).

Figure 9. High-performance liquid chromatography (HPLC) analysis of peptide II. Peptides eluting at 50 min on semipreparative HPLC (Figure 8) were pooled and further purified using the same C18 column. After elution, the peptides were fractionated using a gradient of 0.1% TFA per minute. Radioactive peptide eluting at 34 min (5 nmol) was collected and subjected for radioactivity analysis (Figure 12 B).

Figure 10. Second HPLC purification of peptide II. Peptide II (5 nmol) was injected onto a C18 column in the TFA/CH3CN/CH2O/H2O solvent system using a gradient of 0.1% TFA per minute. Peptides eluting at 34 min were collected and subjected for radioactivity analysis (Figure 11).

Figure 11. High-performance liquid chromatography (HPLC) analysis of alkaline phosphatase treated peptide. Peptide II (5 nmol) was digested with bacterial alkaline phosphatase (see Materials and Methods) and purified on an ODS column in the TFA/CH3CN/CH2O/H2O solvent system using a gradient of 0.1% TFA per minute. Pure peptide II eluting at 34 min was collected and subjected for radioactivity analysis (Figure 12 B).

Figure 12. Amino acid sequence analysis of peptides I and II. During sequencing, fractions were collected and subjected for radioactivity analysis. A: sequence analysis of peptide I. The radioactive peak eluting at 34 min (Figure 8) corresponded to the radioactivity peak (Figure 12 B). B: sequence analysis of peptide II. The appearance of radioactivity and the absence of radioactivity at the corresponding amino acid position indicated that this amino acid contains the radioisotope. As in panel A, this radioactivity peak corresponds to Tris. The amino acid sequence is shown in Figure 12 C.