Stability and Photochemical Properties of Vanadate-Trapped Nucleotide Complexes of Gizzard Myosin in the 6S and 10S Conformations: Identification of an Active-Site Serine†

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ABSTRACT: The properties of divalent metal·ADP·vanadate (V_i) complexes of the 6S extended and 10S folded conformations of gizzard myosin before and after UV irradiation have been studied. The half-lives of both 6S and 10S myosin·MgADP·V_i complexes in the dark at 0 °C are on the order of 2 weeks. Brief irradiation with UV light, however, photomodified the enzyme as suggested by changes in the NH_4^+-, K^+-, and Ca^{2+}-ATPase activities, and destabilized the complexes. The 6S complex, when irradiated, released ADP and V_i rapidly ($t_{1/2} \leq 1$ min) as has been observed in comparable experiments with skeletal myosin subfragment 1 (S1) [Grammer et al. (1988) *Biochemistry 27*, 8408–8415]. The irradiated 10S complex released ≈20% of the ADP and V_i rapidly ($t_{1/2} \leq 1$ min), but the remainder stayed trapped, possibly as the vanadyl (VO^{2+})·ADP complex, for much longer times ($t_{1/2} \approx 8$ h). The site of photomodification was sought by reducing both photomodified 6S and 10S myosin with NaB^3H_4. Amino acid composition analyses identified [^{3}H]serine as the only labeled residue(s), suggesting that the hydroxymethyl group of serine had been oxidized to an aldehyde as shown previously for photomodified skeletal myosin S1 [Cremo et al. (1989) *J. Biol. Chem. 264*, 6608–6611]. The 29-kDa NH_2-terminal tryptic peptide from the heavy chain was found to contain essentially all of the [^{3}H]serine. Preparations of 6S and 10S [^{3}H]myosin were digested exhaustively with trypsin. An identical [^{3}H]peptide was purified from each preparation and its sequence determined to be Glu_{169}-Asp-Gln-Ser-Ile-Leu-(Cys)-Thr-Gly-Glu-[^{3}H]Ser-Gly-Ala-Gly-Lys_{183}. These results place Ser-179 at the γ-phosphate-binding site for ATP and are direct evidence that this glycine-rich region provides critical elements to bind the phosphates of ATP.

The comparative structures of myosins from smooth and skeletal muscle are of interest because although both proteins are superficially similar in their size, shape, and subunit composition, they differ notably in other respects. First, contraction of smooth muscle is regulated primarily by the specific phosphorylation of the regulatory light chains of myosin [reviewed in Stull (1989) and Trybus (1991)] while skeletal muscle is regulated by Ca^{2+} binding to troponin in the thin filaments (Ebashi & Ebashi, 1964). Second, under physiological conditions, unphosphorylated smooth muscle myosins, unlike skeletal myosins, interconvert between a folded 10S form (the α-helical coiled-coil tail folds into thirds and appears to attach to the base of the heads) and an extended 6S form (Suzuki et al., 1982; Onishi & Wakabayashi, 1982; Trybus et al., 1982). This interconversion may play a role in the regulation of contraction in smooth muscle and nonmuscle cells [reviewed in Cross (1988) and Trybus (1991)]. Finally, Cross et al. (1986, 1988) have shown that unphosphorylated smooth muscle (gizzard) and nonmuscle myosins trap the products of ATP hydrolysis in the folded 10S form with a half-life (100 min, 25 °C) about 100–200-fold greater than that for the extended 6S form. This dramatically slower off-rate of ADP and P_i suggests that the 10S form somehow affects the active site to stabilize binding of the hydrolysis products.

One way to compare smooth muscle myosin with skeletal myosin is to map their active sites with photoreactive analogues of ADP or ATP [reviewed in Yount et al. (1992)]. These photolabeling studies have been aided by the ability to trap ADP (or ADP analogues) at the active site (Cole & Yount, 1990; Garabedian & Yount, 1990, 1991) as a complex with vanadate and a divalent metal (Goodno, 1979). These complexes, which are believed to mimic the transition state of ATP hydrolysis, are stable for days at 4 °C and thus can be purified free of extraneous nucleotides before irradiation. This step improves both the specificity and the extent of photolabeling. In the course of these studies, the simple skeletal myosin·MgADP·orthovanadate (V_i)[1] complex was found to be photoreactive (Grammer et al., 1988; Cremo et al., 1988). Thus, irradiation with light between 300 and 400 nm promoted the specific oxidation of the side chain of Ser-180 of the heavy chain to an aldehyde (Cremo et al., 1989) with concomitant release of ADP and V_i (Grammer et al., 1988).[2] Because V_i is believed to bind in the γ-phosphoryl portion of the ATP-binding site, Ser-180 was postulated to be part of the phosphate-binding region. Further experiments irradiating the MgADP·V_i complex of photomodified skeletal myosin (the side

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[1] Abbreviations: S1, myosin subfragment 1; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TCA, trichloroacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; V_i, orthovanadate; DTE, dithioerythritol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LC_{17}, essential light chain; LC_{20}, regulatory light chain.

[2] This photoreaction and subsequent decrease in stability of the vanadate complex can be prevented by replacing Mg^{2+} with Mn^{2+} or Co^{2+} (Grammer et al., 1988). These transition metals quench the activated state of the M^{2+}ADP·vanadium complex in an unknown manner. Thus, photolabelling studies with purine- or ribose-modified ADP analogues have used Co^{2+} in place of Mg^{2+}.

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chain of Ser-180 was previously oxidized to an aldehyde) led to cleavage of the heavy chain at Ser-180 and to oxidation of a second serine, Ser-243, a residue which also appears to be near the phosphate-binding site (Grammer & Yount, 1991, and in preparation).

In this work, we have used the vanadate-promoted photomodification reaction to probe the nature of the active site of gizzard myosin in both the 6S and 10S conformations. We found that Ser-179 of the heavy chain of gizzard myosin was specifically photomodified in both forms of myosin, suggesting the γ-phosphoryl sites are similar in both conformations. No other modified residues were detected. The photomodified 10S form, however, retained MgADP and vanadium (oxidation state unknown) with a half-life of several hours while photomodified 6S myosin released ADP and V_i immediately ($t_{1/2} \leq 1$ min). It is suggested that the unusually stable MgADP·vanadium complex with 10S photomodified myosin may be vanadyl (VO^{2+})·ADP which remains trapped by the 10S conformation.

Materials and Methods

Materials. The sources of commercial compounds used were as follows: NaATP (P-L Biochemicals); [2,8-^{3}H]ATP (ICN Pharmaceuticals); NaB^3H_4 (Du Pont–New England Nuclear); TPCK-trypsin, papain, cysteine, TFA, iodoacetic acid (Sigma); HEPES (Research Organics, Inc.); sodium orthovanadate (Na_3VO_4) (Fisher Scientific Co.); Sephadex G-50-80 (fine) (Pharmacia); 4-(2-pyridylazo)resorcinol (Aldrich); and cobalt(II) chloride (Mallinckrodt). Vanadate solutions (100 mM) were prepared as described by Goodno (1982).

Enzyme Preparations. Gizzard myosin was prepared from fresh chicken gizzards by a minor modification of the method of Ebashi (1976) and stored on ice in 0.5 M KCl, 20 mM Tris, pH 8.0, 0.1 mM DTE, 0.2 mM EDTA, and 0.2 mM EGTA for up to 2 weeks. For longer storage, myosin solutions were made to 50% in glycerol and stored at −20 °C. Gizzard myosin S1 was prepared from myosin (15 mg/mL) with papain (23 units/mg of papain, 3 μg of papain/mg of myosin) by the method of Seidel (1980). Papain digestion was carried out at 25 °C in 0.5 M KCl and 20 mM Tris, pH 8.0, and was terminated after 12 min with the addition of iodoacetate (pH 6) to 5 mM. The digest was dialyzed overnight against 40 mM KCl, 10 mM $MgCl_2$, and 10 mM HEPES, pH 7.5 at 4 °C. Insoluble material was removed by centrifugation at 50000*g* for 1 h at 4 °C. Skeletal myosin was isolated from rabbit leg and back muscles as described by Wagner and Yount (1975) and stored in 50% glycerol at −20 °C. Chymotryptic skeletal myosin S1 was prepared essentially by the procedure of Okamoto and Sekine (1985) was described by Cremo et al. (1989). Myosin ATPase assays were performed as previously described (Wells et al., 1979) except that the release of P_i was measured after 2 and 8 min.

Vanadate Trapping and Irradiation. Gizzard myosin (0.5 M KCl and 20 mM Tris, pH 8.0) and skeletal myosin S1 (0.1 M KCl and 50 mM Tris, pH 8.0) were incubated with 2-fold excess over sites of either [^{3}H]ATP or ATP with either 1 mM $MgCl_2$ or 1 mM $CoCl_2$ for 20–30 min at 25 °C to hydrolyze the nucleotides to the diphosphate forms. Na_3VO_4 (100 mM, pH 10) was added to a final concentration of 1 mM, and the solution was incubated 20–30 min at 25 °C to trap nucleotide at the active site (Goodno, 1982). Vanadate trapping was quenched with the addition of 10 mM EDTA, pH 8.0, before addition of a 50-fold excess over sites of ATP to reduce nonspecific binding of radioactive nucleotide to the protein. The 6S myosin·vanadate and S1·vanadate complexes were purified by centrifugal gel filtration (Penefsky, 1977) as described by Cole and Yount (1990) through columns of Sephadex G-50-80 equilibrated in 0.5 M KCl/10 mM NaP_i, pH 7.5, and in 0.15 M KCl/10 mM NaP_i, pH 7.5, respectively. 10S myosin·MgADP·V_i complexes were produced by passing 6S myosin·MgADP·V_i complexes through centrifugal gel filtration columns equilibrated in 0.15 M KCl/10 mM NaP_i, pH 7.5. Myosin solutions on ice were irradiated with a Hanovia 450-W medium-pressure Hg lamp (Ace glass) at a distance of 9 cm. Two pyrex filters were used to remove radiation below 300 nm. The concentration of free V_i was determined colorimetrically with 4-(2-pyridylazo)resorcinol as described by Goodno (1982) except that 2-(*N*-morphilino)ethanesulfonic acid (Research Organics) replaced imidazole as the buffer.

HPLC Equipment and Columns. HPLC was carried out at room temperature using a microprocessor-controlled Altex/Beckman dual-pump setup connected to a Beckman 165 dual-wavelength detector. Peptides and whole myosin were monitored at 214 and 280 nm, respectively. Myosin conformation was verified using a 7.5 × 600 mm TSK 4000 SW gel filtration column with a 7.5 × 75 mm guard column (Phenomenex) as described by Trybus and Lowey (1988). Peptide separations utilized a semipreparative (7.0 × 250 mm) column and an analytical (4.6 × 220 mm) 300-Å pore C8 reversed-phase column (Aquapore RP-300, Brownlee Labs/Applied Biosystems Inc.). The gel filtration, semipreparative, and analytical columns were run at flow rates of 1.0, 2.0, and 1.0 mL/min, respectively. All samples were passed through 0.45-μm Nylon-66 (Rainin) syringe filters prior to being loaded. A 7.5-mL sample loop was used to load large volumes. Larger samples were concentrated on a Speedvac (Savant Instruments, Inc.) prior to loading on the columns.

Results

Myosin Conformation. A major goal of this study was to compare the active-site composition of the 6S and 10S conformations of myosinMgADP·V_i complexes. Therefore, it was important to demonstrate that vanadate can replace phosphate without altering the nature of the 6S–10S transition. Since the conformation of gizzard myosin depends on ionic strength, myosin·MgADP·P_i and myosin·MgADP·V_i complexes were compared at various KCl concentrations (Figure 1). The 6S extended and 10S folded conformations of smooth muscle myosin were identified by HPLC gel filtration (Trybus & Lowey, 1988). The 6S myosin (≥0.40 M KCl) eluted at 11.4 ± 0.2 mL, and the 10S myosin (0.15 M KCl) eluted at 14.1 ± 0.2 mL (Figure 1A). Myosin eluted at intermediate volumes at salt concentrations between 0.20 and 0.40 M KCl, which reflects the rapid equilibrium between the 6S and 10S conformations (Figure 1B). The myosin species that eluted at approximately 12 mL in 0.05 M KCl was probably the dimer of 10S myosin which is known to form at low ionic strength (Trybus & Lowey, 1984). No significant difference in the elution volume profile was observed between the myosin·MgADP·V_i complex and myosin·MgADP·P_i. Therefore, on the basis of gel filtration assays, V_i complexation does not appreciably affect the salt dependence of the 6S–10S transition.

Stability of Myosin·MgADP·V_i Complexes in the Dark and under UV Light. Gizzard myosin·MgADP·V_i complexes are very stable when stored in the dark at 4 °C. The half-lives of gel-purified 6S (0.5 M KCl) and 10S (0.1 M KCl) myosin·Mg[^{14}C]ADP·V_i complexes as determined by HPLC gel filtration were approximately 2 and 2.5 weeks, respectively (data not shown). The TSK SW 4000 HPLC gel filtration column cleanly separated free [^{14}C]ADP (trailing peaks in

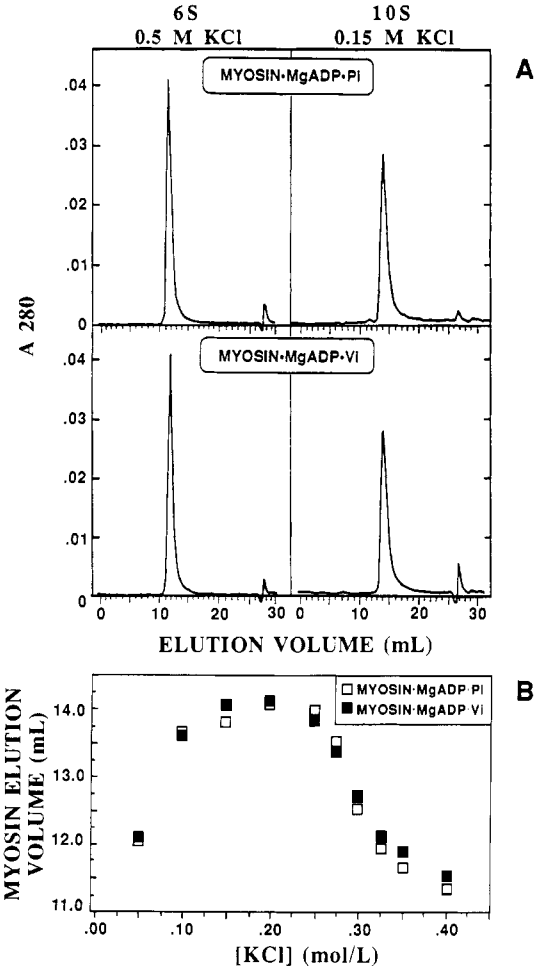

FIGURE 1: Myosin conformation vs KCl concentration. Myosin (1 mg/mL) in 0.05–0.5 M KCl, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.3 (25 °C), was incubated with either 20 μM ATP for 5 min or 20 μM ADP and 1 mM V_i for at least 20 min. The solutions were then passed through 0.45-mm Nylon-66 (Rainin) syringe filters prior to being analyzed by HPLC gel filtration. The TSK SW 4000 column was equilibrated in each buffer (0.05–0.5 M KCl, 20 μM ATP, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.3) prior to being loaded with 100 μg of protein. The flow rate was 1 mL/min. (A) Elution profiles of myosin at 0.15 and 0.50 M KCl. (B) Myosin elution volume at varying KCl concentrations.

Figure 1A; elution time ≈27.5 min) from the protein and allowed the accurate measurement of small increases in free [^{14}C]ADP concentration over long periods of time.

The instability of V_i complexes under UV light has been observed previously with gizzard myosin (Cole & Yount, 1990) and has been characterized extensively with skeletal myosin S1 (Grammer et al., 1988; Cremo et al., 1988, 1989). The release of [^{3}H]ADP from gizzard myosin·metal^{II}[^{3}H]ADP·V_i complexes during UV irradiation is shown in Figure 2. More than 90% of the [^{3}H]ADP was released after 120 s of irradiation of 6S myosin·Mg[^{3}H]ADP·V_i, while substitution of Mg^{2+} with Co^{2+} completely prevented the loss of [^{3}H]ADP (Figure 2, top curve). These results are essentially identical to those observed for skeletal myosin S1 (Grammer et al., 1988). In contrast, only 15–20% of the [^{3}H]ADP (Figure 2) and vanadate (data not shown) was released from 10S myosin·Mg[^{3}H]ADP·V_i complexes following 120-s irradiation (based on four separate experiments). The remaining 80% of the trapped [^{3}H]ADP and vanadate was released with a half-life on the order of 8 h (data not shown). These results suggest that photomodification of gizzard myosin does not significantly affect the phenomena of 10S trapping.

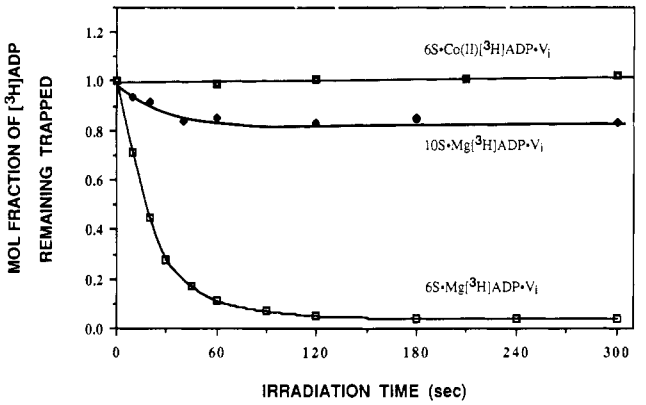

FIGURE 2: Effect of UV irradiation on myosin-metal^{II}ADP·V_i complexes. Myosin (2 mg/mL; 0.5 M KCl/10 mM HEPES, pH 7.3) was incubated with 17 μM [^{3}H]ATP (21 000 cpm/nmol), 1 mM V_i, and either 1 mM $MgCl_2$ or 1 mM $CoCl_2$ for 30 min at 25 °C. The myosin complexes were applied to centrifugal gel filtration (G-50-80) columns equilibrated in either 0.5 M KCl/10 mM HEPES, pH 7.3 (6S conditions), or 0.15 M KCl/10 mM HEPES, pH 7.3 (10S conditions), to remove untrapped [^{3}H]ATP and V_i. The purified 6S and 10S complexes were then irradiated at 4 °C as described under Materials and Methods. Aliquots were removed during irradiation and repurified by centrifugal gel filtration to remove any released [^{3}H]ADP. The protein and [^{3}H]ADP concentrations in the eluant were determined by A_{280} ($\epsilon^{1\%}_{280nm}$ = 5.6) and scintillation counting, respectively.

Table I: Effect of Photomodification on ATPase Activities (%) of 6S Gizzard Myosin[a]

sample	NH_4^+-EDTA	K^+-EDTA	Ca^{2+}
control	100	100	100
vanadate complex	<1 ± 0.5	2 ± 1	6 ± 2
photomodified	5 ± 2	8 ± 3	440 ± 30

[a] The myosin·vanadate complex and photomodified myosin were prepared and ATPase activities were determined as described under Materials and Methods. The control myosin was irradiated for 4 min with no V_i present.

Characterization of Photomodified Gizzard Myosin. It is known that UV irradiation of skeletal myosin S1·MgADP·V_i drastically alters the S1 ATPase activities (Grammer et al., 1988). It was of interest than to examine the ATPase activities of UV-irradiated gizzard myosin·MgADP·V_i. Prior to irradiation, the myosin·MgADP·V_i complex was essentially inactive in three assay conditions (Table I). After irradiation, the photomodified myosin remained essentially inactive in both the NH_4^+-EDTA and the K^+-EDTA assays. In contrast, the Ca^{2+}-ATPase activity increase 4–5-fold over the native enzyme (based on five separate experiments), a response similar to that observed with skeletal S1 (Grammer et al., 1988), suggesting the photomodification was identical in both cases.

Na[^{3}H]$_4$ Reduction of Photomodified Myosin. Treatment of photomodified skeletal S1 with Na[^{3}H]$_4$ is known to result in the covalent incorporation of ^{3}H into the protein (Cremo et al., 1989), as a serine "aldehyde" is reduced to a [^{3}H]serine. To test for a similar reaction in smooth muscle myosin, the covalent incorporation of ^{3}H into 6S or 10S gizzard myosin and skeletal S1 during a photomodification time course was compared (Figure 3). All three samples appeared to incorporate similar levels of radioactivity which reached saturation at approximately 120 s.[3] This time course parallels the release of [^{3}H]ADP seen in Figure 2. As expected, no incorporation of ^{3}H was observed after Na[^{3}H]$_4$ reduction of irradiated 6S

[3] In order to reduce nonspecific incorporation of ^{3}H, we used only freshly prepared myosin. Myosin that was stored in 50% glycerol at −20 °C often became partially oxidized during storage and was nonspecifically labeled by Na[^{3}H]$_4$ in the absence of irradiation.

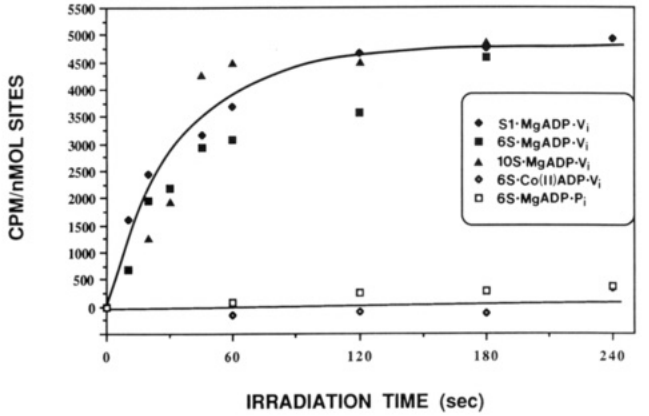

FIGURE 3: NaB^3H_4 reduction of irradiated myosin complexes. ADP was trapped with V_i on gizzard myosin (2 mg/mL) or skeletal myosin S1 (2 mg/mL) with either Mg^{2+} or Co^{2+} as described under Materials and Methods. The 10S and S1 complexes were purified by centrifugal gel filtration into 0.15 M KCl/10 mM NaP_i, pH 7.5 (at 4 °C), and the 6S complexes were purified by centrifugal gel filtration into 0.5 M KCl/10 mM NaP_i, pH 7.5. The control myosin was made 1 mM in $MgCl_2$ and 20 μM in ADP. All myosin solutions were irradiated as described under Materials and Methods. Following irradiation, 3 M KCl was added to the 10S samples to achieve a final concentration of 0.5 M, and guanidine hydrochloride (2 M final concentration) was added to the 6S myosin·Co^{II}ADP·V_i samples to release trapped nucleotide. All of the samples (0.2 mg) were incubated with NaB^3H_4 (4-fold over sites, 40 000 cpm/nmol) for 30 min at 25 °C. Unreacted NaB^3H_4 was destroyed with ice-cold 5% trichloroacetic acid for an additional 30 min. All work with NaB^3H_4 was carried out in a radioactive hood. The protein was pelleted, washed with 5% trichloroacetic acid, and then dissolved in 0.4 mL of 4% SDS (in 1 M Tris, pH 10) prior to scintillation counting.

myosin·MgADP·P_i and 6S myosin·Co^{II}ADP·V_i (Figure 3). Amino acid composition analyses of [^{3}H]myosin from both 6S and 10S preparations showed that all of the covalent ^{3}H was contained in serine (data not shown). Limited trypsin digestion of 6S and 10S [^{3}H]myosin revealed that essentially all of the radioactivity was associated with the 29-kDa NH_2-terminal peptide from the heavy chain (Figure 4). These data were consistent with the photomodification being a specific oxidation of the side chain of an active-site serine to an aldehyde as shown for skeletal S1 (Grammer et al., 1988; Cremo et al., 1988).

Purification and Sequencing of a [^{3}H]Peptide. A single [^{3}H]peptide was isolated from 6S myosin as diagrammed in Figure 5. Gizzard myosin in the 6S conformation containing trapped MgADP·V_i was photomodified as described under Materials and Methods and then reduced with NaB^3H_4. [^{3}H]S1 was prepared from the [^{3}H]myosin and digested exhaustively with trypsin. The digest was run on a reversed-phase HPLC column equilibrated in 0.1% TFA (Figure 6). The single major peak of radioactivity was collected and rerun on the same column with a shallow gradient (0.1% B/min) of elution buffer. The radioactive peak was then loaded on the reversed-phase column equilibrated in 5 mM phosphate buffer, pH 6.9, and eluted with a shallow gradient of acetonitrile (Figure 7). The radioactive peptide was essentially pure at this stage. This was verified by first desalting the sample with the reversed-phase column equilibrated in 0.1% TFA and then analyzing the sample with capillary zone electrophoresis (Figure 7, inset). The radioactive peptide, estimated to be at least 95% pure, was determined to be E-D-Q-S-I-L-(C)-T-G-E-[^{3}H]S-G-A-G-K (Figure 8), corresponding to residues 169–183 in the gizzard myosin heavy chain (Yanagisawa et al., 1987). All of the radioactivity was associated with Ser-179. A similar analysis of photomodified 10S myosin produced a single pure peptide with a sequence

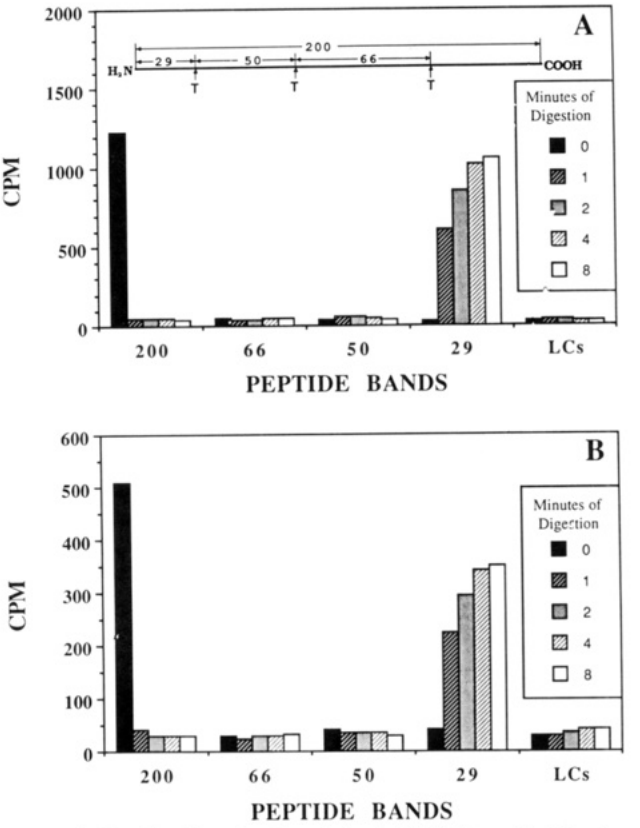

FIGURE 4: Localization of radioactivity in 6S [^{3}H]myosin following brief trypsin digestion. Myosin in the 6S and 10S conformations was photooxidized with vanadate and reduced with NaB^3H_4 as described in Figure 3. Unreacted NaB^3H_4 was removed by centrifugal gel filtration into 0.5 M NaCl/20 mM NaP_i, pH 7.5. The [^{3}H]myosins were digested with trypsin (1:100, w/w). Aliquots (6S, 100 μg; 10S, 50 μg) were removed at various times, and the trypsin was inactivated with soybean trypsin inhibitor (1:3, w/w). The aliquots were run on 12% polyacrylamide gels according to the method of Laemmli (1970). Protein bands were excised from the gels and solubilized by heating 2–3 h at 80 °C in 0.75 mL of 30% H_2O_2 prior to scintillation counting. The digestion pattern is summarized by the diagram in panel A (inset) where "T" represents the trypsin-sensitive sites. LCs refer to both light chains, LC_{20} and LC_{17}. LC_{20} is quickly degraded by trypsin to a polypeptide of approximately 17 kDa. (Panel A) 6S myosin. (Panel B) 10S myosin.

identical to that from the 6S preparation (bottom panel, Figure 8). Thus, regardless of the conformation of the trapped complexes, vanadate promoted the photooxidation of the same active-site serine.

Discussion

The purpose of this work was to use the V_i photomodification reaction to probe for possible differences in the γ-phosphoryl-binding site of the 6S and 10S forms of gizzard myosin. V_i is known to form very stable complexes with MgADP at the active site for both skeletal (Goodno, 1979) and smooth muscle myosin (Cole & Yount, 1990). Here we have extended these studies to show that both the 6S and 10S forms of gizzard myosin form extremely stable complexes with MgADP·V_i ($t_{1/2} \approx 14$ days, 4 °C) which have the same gel filtration properties as those formed with MgATP or its hydrolysis products.

As with skeletal myosin, irradiation with UV light (300–400 nm) of the 6S Mg[^{3}H]ADP·V_i gizzard myosin complex promoted the rapid release of [^{3}H]ADP (Figure 2) and V_i. In contrast, irradiation of the 10S gizzard myosin·Mg[^{3}H]ADP·V_i complex released a small amount of [^{3}H]ADP (Figure 2) and V_i but the large majority (80%) remained bound with a half-life of approximately 8 h. The 20% which was released

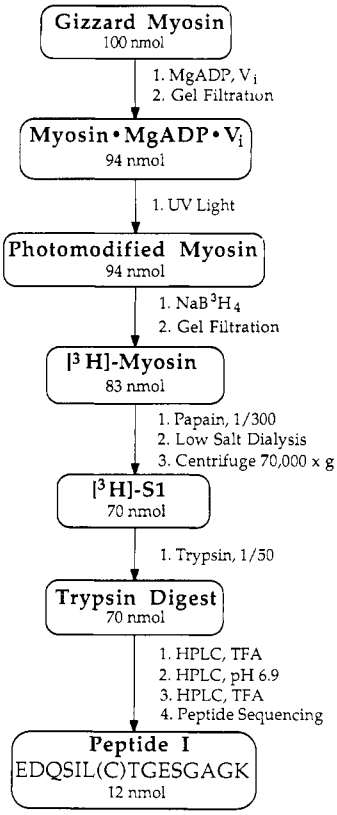

FIGURE 5: Flowchart for the isolation of a vanadate-photomodified peptide. Photomodified gizzard myosin was prepared by 5-min irradiation of 6S myosin·MgADP·V_i as described under Materials and Methods. The photomodified myosin was reduced with NaB^3H_4 (4 × sites, 30 min at 25 °C) and purified by centrifugal gel filtration (equilibrated in 0.5 M KCl/20 mM Tris, pH 8.0). The [^{3}H]myosin was digested with papain (1:300, w/w) for 12 min at 25 °C. The papain was inactivated with the addition of 5 mM iodoacetate, pH 6.0. The digest was dialyzed against 100 volumes of 10 mM $MgCl_2$, 40 mM KCl, and 12 mM Tris, pH 8.0, to precipitate rods and undigested myosin and clarified by centrifugation at 70000*g* for 1 h. The supernatant, essentially pure [^{3}H]S1, was digested exhaustively with trypsin (see Figure 6 legend). A [^{3}H]peptide was then purified using HPLC.

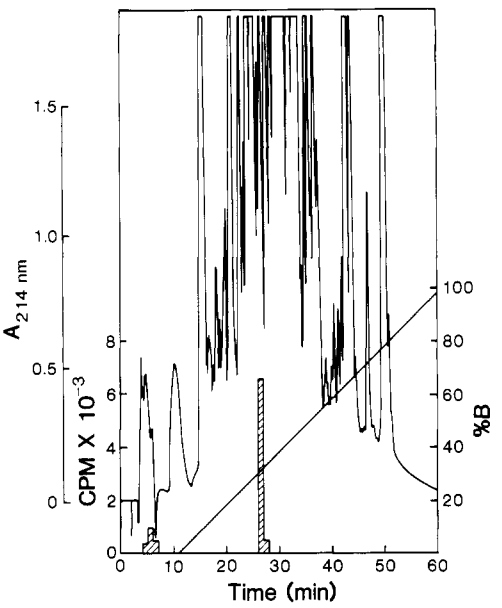

FIGURE 6: HPLC separation of the tryptic digest of [^{3}H]S1. [^{3}H]S1 (70 nmol, 55 000 cpm/nmol as determined by TCA precipitation) was prepared as described in Figure 5 and digested with trypsin (1:50, w/w) for 10 h at 25 °C. It was found important to keep reductant, e.g., β-mercaptoethanol, present during the trypsin digestions and during storage of the [^{3}H]peptides to prevent oxidation of Cys-175 to a disulfide (see Figure 8). The digest was separated in two runs (35 nmol each) on a semipreparative Aquapore RP-300 column equilibrated in 0.11% TFA and eluted with a 2%/min gradient of 0.10% TFA in 60% acetonitrile (solvent B). Radioactivity was determined from 0.02-mL aliquots taken from 1-min (2-mL) fractions.

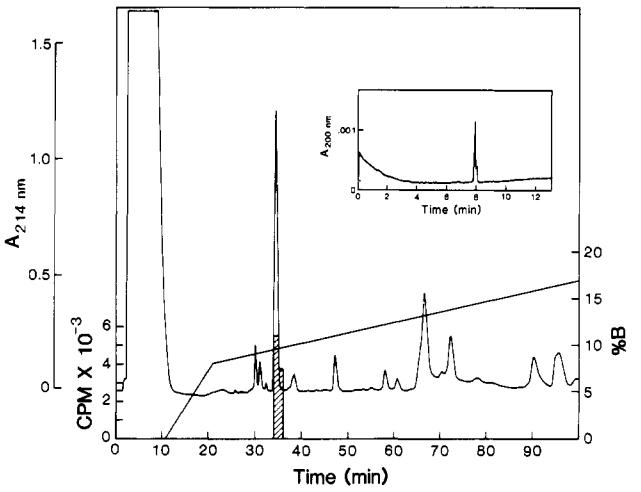

FIGURE 7: HPLC purification of the major radioactive peak. Radioactive fractions (Figure 6, elution time = 27 min) were pooled from two separate runs, concentrated to 1 mL, and rerun under the same conditions as in Figure 6 except that the radioactive peak was eluted with 0.1%/min gradient of solvent B. The radioactive peak was pooled, concentrated, then loaded on an analytical Aquapore RP-300 column equilibrated in 5 mM KP_i, pH 6.9, and eluted with a 0.8%/min gradient of 0–10% B (65% acetonitrile) followed by a 0.1%/min gradient of 8–17% B. The radioactive peak which eluted at 35 min was essentially pure. The radioactive peak was desalted on the analytical column using the TFA system (Figure 6), and its purity was verified with high-performance capillary-zone electrophoresis (Bio-Rad HPE 100). The sample was loaded for 8 s at 8 kV on a 20 cm × 25 μm coated cartridge equilibrated in 100 mM KP_i, pH 2.5, and run at 8 kV (see inset).

rapidly may be a different form of the 10S complex or it may represent partial inhomogeneity in our myosin preparations. Regardless, these results indicate the majority of the 10S form, even though photomodified, still prevented the release of [^{3}H]ADP and vanadium (oxidation state unknown). This result was surprising because reduction with NaB^3H_4 of irradiated MgADP·V_i complexes of skeletal and 6S and 10S myosins all indicated the same degree of photooxidation (see below). We assume that the photooxidation of the protein is accompanied by a concomitant photoreduction of the vanadium(V). Vanadium(V) is known to oxidize organic carbonyl compounds in the presence of UV light (Panwar & Guar, 1967) and is believed to undergo reduction to vanadyl(IV) ions in the process (Satapathy et al., 1963). This reduction should abolish the transition-state-like complex and promote a fast off-rate of ADP and vanadyl ions. Vanadyl ions could then be rapidly converted to vanadate by reaction with molecular oxygen. The conformation of the photomodified 10S form may simply retain both ADP and vanadyl(IV) ions (VO^{2+}) long enough for reoxidation of vanadyl to vanadate at the active site. The subsequent rapid re-formation of the MgADP·V_i·10S complex would explain the longer half-life observed.

Several results indicated that the V_i-promoted photomodification of 6S and 10S myosins was similar to that observed with skeletal myosin. First, as mentioned above, the degree of photomodification as revealed by NaB^3H_4 reduction was similar for all three complexes (Figure 3). Second, photomodification led to similar changes in ATPase activities. That is, as shown in Table I, the Ca-ATPase activity of photooxidized 6S myosin increased 440% while K^+- and NH_4^+-

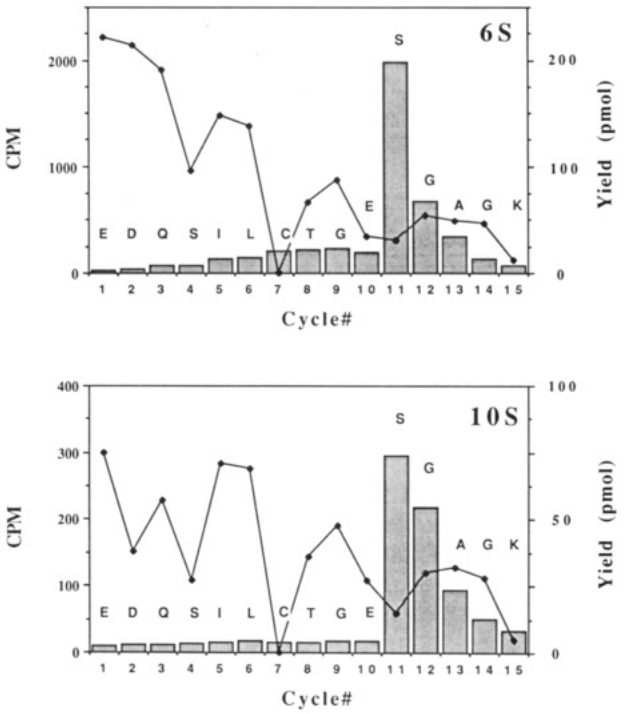

FIGURE 8: Amino acid sequences of the [^{3}H]peptides. The radioactive peptides (upper panel, 6S, 600 pmol; lower panel, 10S, 400 pmol) were sequenced with an ABI Model 470-A gas-phase sequencer with Pulsed Liquid uptake (Applied Biosystems, Inc.) and an ABI 120-A PTH analyzer. Data acquisition and analysis were performed with the standard program RUN470-1 (ABI). The yield of the predominant amino acid is plotted against the radioactivity released at each cycle. A phenylthiohydantoin–cysteine derivative was not detected in cycle 7 because cysteine was presumably destroyed during prior steps of the sequencing cycles. The apparent radioactivity of Gly in cycle 12 is the result of incomplete cleavage of [^{3}H]serine in cycle 11.

ATPase activities were very low. These results were highly analogous to those observed with photomodified skeletal myosin subfragment 1 (Grammer et al., 1988) and suggest that the photomodification was similar in all three cases. Further evidence came from amino acid analysis of both the 6S and 10S NaB^3H_4-reduced myosin. These studies showed that all of the radioactivity was associated with serine as was found previously with photomodified skeletal S1 (Cremo et al., 1988). Furthermore, SDS–PAGE analysis of a limited trypsin digest of both 6S and 10S [^{3}H]myosin (Figure 4) showed that the NH_2-terminal 29-kDa fragment of the heavy chain contained all of the radioactivity. Exhaustive trypsin digestion of 10S [^{3}H]myosin and of S1 derived from 6S [^{3}H]myosin produced single radioactive peptides in each case which gave the sequence E-D-Q-S-I-L-(C)-T-G-E-[^{3}H]S-G-A-G-K. This sequence corresponds to residues 169–183 in the gizzard myosin heavy-chain sequence (Yanagisawa et al., 1987). All of the radioactivity was associated with Ser-179 (Figure 8). No evidence for additional modified residues was found in the 10S preparations as has been observed after irradiation of the MgADP·V_i complex of photomodified skeletal myosin (Grammer & Yount, 1991, and in preparation). In these latter studies, in addition to photocleavage at Ser-180, an additional serine (Ser-243) was photooxidized. The absence of such additional reactions in these studies is taken as further evidence that the vanadium trapped with MgADP on 10S photomodified gizzard myosin was not in the V(V) state. Clearly, further study of the long-lived MgADP·vanadium complex of the photomodified 10S form of myosin should give better insight into the chemical mechanism of the photooxidation.

Table II: Comparison of Homologous Sequences of Enzymes Which Hydrolyze ATP or GTP

enzyme	sequence no.	sequence	ref
myosin (chicken gizzard)	178–186	GESGAGKTE	Yanagisawa et al. (1987)
kinesin (*Drosophila*)	92–100	GQTSSGKTH	Yang et al. (1989)
adenylate kinase (rabbit)	15–23	GGPGSGKGT	Kuby et al. (1984)
C-Ha-*ras* p21 (human)	10–18	GAGGVGKSA	Capon et al. (1983)
EF-1 (human)	14–22	GHVDSGKTT	Brands et al. (1986)
transducin α	36–44	GAGESGKST	Tanabe et al. (1985)
F-1 ATPase/β (bovine)	156–164	GGAGVGKTV	Walker et al. (1982)

Ser-179 of gizzard myosin and Ser-180 of skeletal myosin are in the sequence G-E-S-G-A-G-K-T which is conserved in all myosin heavy chains sequenced to date. Furthermore, this conserved sequence corresponds to the glycine-rich sequence G-X-X-X-X-G-K-(T/S) (where one or more X is glycine) which has been found in a large number of ATP- and GTP-binding proteins (Table II) (Walker et al., 1982). This sequence occurs as a flexible loop between a β-sheet and the first α-helix in the crystal structure of adenylate kinase (Sachsenheimer & Schulz, 1977; Pai et al., 1977; Dreusicke et al., 1988). The best evidence from recent X-ray studies of crystals of adenylate kinase with a variety of bound nucleotides (Egner et al., 1987; Diederichs & Schulz, 1990) combined with NMR studies (Vetter et al., 1991) places the glycine-rich loop over the β,γ-phosphates of ATP [for a critical review of the assignment of AMP- and ATP-binding site on adenylate kinase, see Tsai and Yan (1991)]. In addition, recent photochemical modification studies of cytoplasmic adenylate kinase (chicken muscle) with vanadate ions in the absence of nucleotides show the polypeptide chain is cleaved at Pro-17 (Cremo et al., 1992). This proline residue is at the top of the glycine-rich loop [see Dreusicke and Schulz (1986) for a detailed structural presentation] and is in the analogous position in the overall sequence as Ser-180 is in skeletal myosin. The large induced conformational change which occurs when ATP binds to adenylate kinase (up to 32 Å for some residues; Schulz et al., 1990) indicates that myosin may also undergo such large conformational changes as part of the force-generating cycle in muscle contraction. The fact that both smooth muscle and skeletal myosins contain the same sequence around the modified serine indicates their reaction intermediates are likely to be the same despite the marked difference in the rate of their contraction.

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