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UV-Induced Vanadate-Dependent Modification and Cleavage of Skeletal Myosin Subfragment 1 Heavy Chain. 1. Evidence for Active Site Modification†

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ABSTRACT: Ultraviolet irradiation above 300 nm of the stable MgADP–orthovanadate (V_i)–myosin subfragment 1 (S1) complex resulted in covalent modification of the S1 and in the rapid release of trapped MgADP and V_i. This photomodified S1 had Ca^{2+}ATPase activity 4–5-fold higher than that of the nonirradiated control S1, while the K^+EDTA-ATPase activity was below 10% of controls. There was a linear correlation between the activation of the Ca^{2+}ATPase and the release of both ADP and V_i with irradiation time. Analysis of the total number of thiols and the ability of photomodified S1 to retrap MgADP by cross-linking SH1 and SH2 with various bifunctional thiol reagents indicated that the photomodification did not involve these reactive thiols. Irradiation of the S1–MgADP–V_i complex caused a large increase in absorbance of the enzyme at 270 nm which was correlated with the release of V_i from the active site, suggesting an aromatic amino acid(s) was (were) involved. However, analysis by three different methods showed no loss of tryptophan. All the irradiation-dependent phenomena could be prevented by replacing Mg^{2+} with either Co^{2+}, Mn^{2+}, or Ni^{2+}. Unlike previous irradiation studies of V_i–dynein complexes [Lee-Eiford, A., Ow, R. A., & Gibbons, I. R. (1986) *J. Biol. Chem. 261*, 2337–2342], no peptide bonds were cleaved in photomodified S1. Photomodified S1 was able to retrap MgADP–V_i at levels similar to unmodified S1. Upon irradiation of the photomodified S1–MgADP–V_i complex, MgADP and V_i were again released from the active site, resulting in heavy chain cleavage to form NH_2-terminal 21-kDa and COOH-terminal 74-kDa peptides. All evidence indicates that this new photomodification and subsequent chain cleavage occur specifically at the active site.

We recently reported the photoaffinity labeling of gizzard myosin (Okamoto et al., 1986) by the photoreactive ADP analogue NANDP[1] (Nakamaye et al., 1985; Okamoto & Yount, 1985). In these experiments, NANDP was trapped at the active site as the stable noncovalent S1–MgNANDP–V_i complex which mimics the MgADP·P_i transition state for ATP hydrolysis (Goodno, 1982). In the early stages of these photoaffinity labeling experiments using NANDP, and in other experiments using 2- and 8-N_3ADP analogues, it was found that, in both skeletal and gizzard myosin, Mg–nucleotide diphosphate–V_i complexes were unstable to ultraviolet irradiation. In the dark, a typical half-life of a S1–Mg–nucleotide–V_i complex is about 2 days at 0 °C. This value decreases to minutes when the complex is irradiated with UV light above 300 nm. Fortunately, it was possible to prevent NANDP release in our earlier photolabeling studies of gizzard myosin by filtering out light below 400 nm (Okamoto et al., 1986). Although light above 400 nm will activate the azide of NANDP (Nakamaye et al., 1985), it does not cause V_i and nucleotide to be released from the active site. However, for other photoprobes which do not absorb radiation above 400 nm, the release of nucleotide from the active site caused by light >300 nm hindered specific photoaffinity labeling experiments.

It was of interest then to find conditions in which the binding of photoprobes could be stabilized and, at the same time, to investigate the properties of S1 to see if it was modified during the irradiation-dependent release of V_i and nucleotide. The latter possibility was suggested by the studies of Gibbons and co-workers (Lee-Eiford et al., 1986; Gibbons et al., 1987), who have found that, in attempts to use V_i to stabilize binding of 8-N_3ATP to dynein during photolabeling studies, both the α

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[1] Abbreviations: S1, myosin subfragment 1; PAR, 4-(2-pyridylazo)-resorcinol; MES, 2-(*N*-morpholino)ethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); pPDM, *N,N′-p*-phenylenedimaleimide; Co-Phen, $[Co^{III}phen_2CO_3]Cl$; V_i, orthovanadate; NANDP, diphosphate ester of (4-azido-2-nitroanilino)ethanol; NBS, *N*-bromosuccinimide; SDS, sodium dodecyl sulfate; A_1 and A_2, alkali light chains of skeletal myosin.

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and β heavy chains were cleaved in specific locations which they referred to as the V1 sites. Here we report that, when MgADP–V_i trapped at the active site of S1 was irradiated, no polypeptide chains were cleaved but the active site was modified as evidence by stable changes in the ATPase activities and in the absorption spectrum. Photomodified S1, however, could still form the MgADP–V_i complex at the active site. Upon irradiation of the photomodified S1–MgADP–Vi complex, the heavy chain was now specifically cleaved to an NH_2-terminal 21-kDa and a COOH-terminal 74-kDa fragment. These results suggested that the photomodification and subsequent chain cleavage were specific to the active site and absolutely dependent on V_i and light. This is the first report of a vanadate-promoted photomodification of an enzyme which does not cleave the polypeptide chain. The evidence suggests that vanadate-dependent photocleavage of the peptide chain is at least a two-step process in which vanadate must bind twice in order to effect chain cleavage. The photomodified S1 described in this paper is a stable intermediate in the vanadate-dependent UV-induced cleavage pathway. The accompanying paper (Cremo et al., 1988) shows that the first photomodification is most likely the oxidation of the β-hydroxyl of a serine to an aldehyde.

Materials and Methods

The commercial compounds used and their sources were as follows: Li_3ADP (Pharmacia P-L Biochemicals); [8-^{14}C]ADP (New England Nuclear/Du Pont); trypsin, chymotrypsin, and DTNB (Sigma); pPDM and PAR (Aldrich); ultrapure ammonium sulfate and ultrapure guanidine hydrochloride (Schwarz/Mann); SDS (Pierce); MES (Research Organics); sodium orthovanadate (Na_3VO_4) (Fischer). We routinely prepared 100 mM working stock vanadate solutions as described by Goodno (1982). [$Co^{III}phen_2CO_3$]Cl and [^{14}C]-pPDM were synthesized as described previously (Wells et al., 1979a; Wells & Yount, 1982).

Enzyme Preparations. Myosin was isolated from rabbit leg and back muscles as previously described (Wagner & Yount, 1975) and stored in 50% glycerol at −20 °C. Chymotryptic S1 (115 000 g/mol) was prepared as described by Okamoto and Sekine (1985) except that the myosin was dialyzed overnight into 0.12 M NaCl–10 mM phosphate (pH 7.0 at 25 °C) and made 1 mM in EDTA just prior to addition of chymotrypsin. To prepare the S1–MgADP–V_i complex, 2 mM $MgCl_2$, 1 mM V_i, and a 1.5 molar excess of ADP (25–51 μM) over S1 (17–34 μM) were incubated for 25 min at 25 °C (Goodno, 1982) in 50 mM Tris, pH 7.4, 0.1 M KCl, and 0.01% NaN_3 (S1 buffer). Excess V_i and MgADP were removed by precipitation of the complex with 2.5 volumes of cold saturated $(NH_4)_2SO_4$ containing 20 mM EDTA, pH 8.0, and centrifugation at 25000*g* for 10 min. The pellet was dissolved in S1 buffer, and residual $(NH_4)_2SO_4$ was removed by centrifugation through a 5-mL column of Sephadex G-50 fine equilibrated in S1 buffer (NaCl replaced KCl in some experiments) as described by Penefsky (1977). To prepare photomodified enzyme, the purified complex in a Pyrex Petri dish was cooled on an ice bath and irradiated with a Hanovia 450-W medium-pressure Hg lamp (Ace Glass) at a distance of 9 cm. A Pyrex filter was used to remove radiation below 300 nm.

Analytical Procedures. Modified S1 protein concentrations were determined by a dye binding assay (Bradford, 1976) using unmodified S1 as the standard as peviously described (Wells et al., 1979a); $\epsilon_{280}^{1\%} = 7.5$ cm^{-1} (Wagner & Weeds, 1977). ATPase assays were performed as previously described (Wells et al., 1979b) except that the release of P_i was measured after 2 and 8 min. Radioactivity was determined in a Beckman LS 9000 scintillation counter using ACS (Amersham) as the scintillant. Absorbance spectra were recorded on a Varian 2200 spectrophotometer.

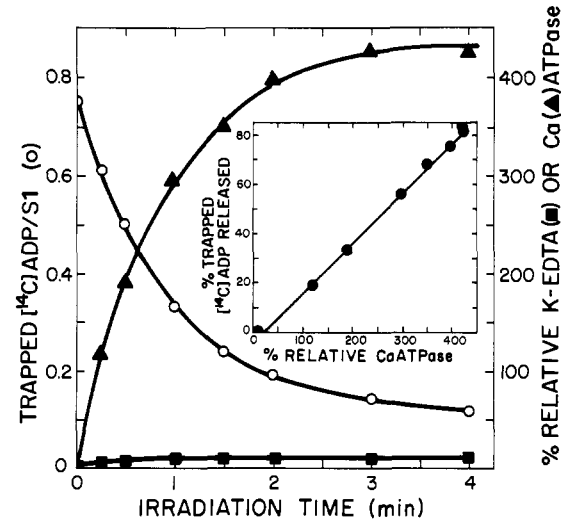


FIGURE 1: Loss of trapped [^{14}C]ADP vs activation of the Ca^{2+}ATPase activity during irradiation of S1–Mg[^{14}C]ADP–V_i. Samples of S1–Mg[^{14}C]ADP–V_i were removed at the indicated irradiation times and assayed for K^+EDTA- and Ca^{2+}ATPase activities (specific activity of contol S1: K^+EDTA-ATPase = 3.8 μmol of P_i mg^{-1} min^{-1}; Ca^{2+}ATPase = 1.0 μmol of P_i mg^{-1} min^{-1}). After the addition of EDTA to 5 mM final concentration, the samples were centrifuged through Sephadex G-50 columns, and the [^{14}C]ADP and protein concentrations were determined as described under Materials and Methods. The inset shows a replot of the data. ATPase activities are expressed relative to a control sample treated exactly like the S1–MgADP–V_i sample except without V_i.

Results

V_i and MgADP are known to form a stable complex with S1 (Goodno; 1979, 1982). This complex is considered to be a stable analogue of the S1–MgADP–P_i transition state intermediate and, in our hands, has a half-life of approximately 2 days at 0 °C. S1 preparations complexed with MgADP and V_i routinely had K^+EDTA- and Ca^{2+}ATPase activities less than 5% of unmodified S1. V_i and ADP were trapped at the active site in equimolar amounts at ~0.75 mol/mol of S1 (Figure 1). UV irradiation of this stable complex caused a rapid, time-dependent release of nucleotide from the enzyme (Figure 1). After 4 min of irradiation, less than 10% of the [^{14}C]ADP initially trapped remained at the active site. At the same time, the Ca^{2+}ATPase activity increased ~4–5-fold over the activity of S1 irradiated in the absence of V_i. The K^+EDTA-ATPase activities remained below 10% of that of the control, indicating further that the enzyme had been covalently modified by the irradiation.

Figure 2 shows that V_i is also released from the active site over the same irradiation time course as ADP release. Of the V_i originally trapped in the complex, 87% was released after 4 min of irradiation. The activation of the Ca^{2+}ATPase correlated in a direct manner with the release of both [^{14}C]-ADP and V_i (Figures 1 and 2, insets). These changes in ATPase activities with concomitant release of nucleotide and V_i could be prevented by filtering out light below 408 nm. Under these conditions, ATPase activities and the level of V_i and MgADP trapped did not change over the irradiation time course (data not shown).

The activation of the Ca^{2+}ATPase and the concomitant inactivation of the K^+EDTA-ATPase suggested a modification of either SH1 or SH2 (Sekine & Kielley, 1964). If these thiols were modified, it should not be possible to trap [^{14}C]ADP at

Table I: Total Thiols and Trapping of [^{14}C]ADP Using Thiol Cross-Linking Reagents[a]

	[^{14}C]ADP/S1			total thiols/S1[b]		
cross-linking reagent	control[c]	complex	photomodified	control	complex	photomodified
none	0.04			9.3 ± 0.3	9.5 ± 0.2	9.5 ± 0.1
pPDM	0.67	0.01	0.47			
CoPhen	0.74	0.03	0.60			
DTNB	0.80	0.17	0.64			

[a] The S1–MgADP–V_i complex and photomodified S1, prepared with unlabeled ADP, were repurified by adding EDTA to 5 mM and passage through a Sephadex G-50 column as described under Materials and Methods. Control, complex, or photomodified S1 (22–27 μM) was then treated with pPDM, CoPhen, or DTNB in the presence of Mg[^{14}C]ADP and then quenched, as previously described (Wells & Yount, 1982). A 25-fold excess of ATP and EDTA to 20 mM final concentration was added to all samples followed by centrifugation through a Sephadex G-50 column. Protein and [^{14}C]ADP concentrations were determined as described under Materials and Methods. [b] Thiols were determined by the method of Ellman (1959) as described in detail by Wells et al. (1979b). Values are the average and standard deviation of four measurements from two independent experiments. [c] The control was treated exactly as the photomodified sample except that V_i was not added.

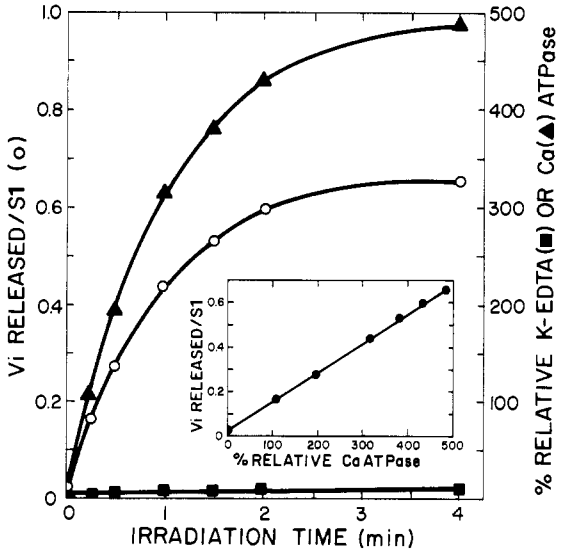


FIGURE 2: Release of trapped V_i vs the activation of the Ca^{2+}ATPase activity during irradiation of S1–MgADP–V_i. The conditions were the same as Figure 1 except that the S1–MgADP–V_i complex was not repurified after irradiation. The concentration of free V_i was determined colorimetrically by the PAR reaction as described by Goodno (1982) except that MES replaced imidazole as the buffer. The inset shows a replot of the data.

the active site by cross-linking with the bifunctional thiol cross-linking reagents pPDM, CoPhen, or DTNB, as described by Wells and Yount (1982). In Table I, the amounts of [^{14}C]ADP trapped by control S1, the purified S1–MgADP–V_i complex, and the photomodified S1 (irradiated complex) are compared. With control S1, these cross-linking reactions typically trap 0.65–0.80 mol of [^{14}C]ADP/mol of S1. As expected, the S1–MgADP–V_i complex did not trap [^{14}C]ADP to a significant level (<5%) with either pPDM or CoPhen since nucleotide is already trapped at the active site by V_i. Disulfide formation by DTNB did result in 0.17 mol of [^{14}C]ADP trapped per mole of S1–MgADP–V_i complex. However, this was most likely due to partial release of MgADP and V_i from the enzyme over the 24-h DTNB reaction, thus regenerating some active S1. In contrast, photomodified S1 trapped [^{14}C]ADP at levels approaching that of control S1, indicating that SH1 and SH2 were unmodified and available to react with the cross-linking reagents. In other experiments, analysis of radioactivity in SDS–polyacrylamide gels indicated that [^{14}C]pPDM reacted predominantly with the 20-kDa COOH-terminal tryptic peptide which contains SH1 and SH2 in both photomodified S1 and in control S1 (data not shown). This result rules out the possibility that SH1 was modified by the irradiation and that cross-linking between SH2 and another thiol in the 50-kDa tryptic peptide was responsible for trapping [^{14}C]ADP in the photomodified sample (see Table I) as has been observed by Chaussepied et al. (1986). Furthermore, no differences in the total thiol content could be detected between control S1, the S1–MgADP–V_i complex, and photomodified S1 (Table I), indicating that thiol modification, in general, did not occur.

To study further the nature of the photomodification, the effects of irradiation upon the UV absorbance spectrum of the S1–MgADP–V_i complex were examined (Figure 3A). After 4 min of irradiation, the absorbance at the wavelength maximum (278 nm) increased by 5% over that of the S1–MgADP–V_i complex. The absorbance tail extending from ~300 to 360 nm present in the complex decreased essentially to control S1 levels (control spectrum not shown). The absorbance above 300 nm was not seen (data not shown) if ADP, V_i, and Mg^{2+} were added in equivalent amounts to that found in trapped samples (conditions under which trapping does not occur at a measureable rate). The difference spectra in Figure 3B, with the S1–MgADP–V_i complex as the reference, shows an isosbestic point at 292 nm. The increase in absorbance at 278 nm and the decrease at 310 nm were linearly correlated with the release of trapped V_i (Figure 4). The isosbestic point indicates that both changes in absorbance are associated with the same product formation. The maximum change in the absorbance spectrum was at 270 nm, corresponding to an increase of 7.4% over the absorbance of the complex.

It was of interest to see if the photoinduced difference spectrum depended upon the tertiary structure of S1. Accordingly, Figure 5A shows the effects of irradiation upon the absorption spectrum of the S1–MgADP–V_i complex measured after the addition of 4 M guanidine hydrochloride. This concentration of denaturant was found to release all nucleotide from the enzyme (data not shown). The difference spectrum in guanidine hydrochloride (Figure 5B) showed only increasing absorbance at 270 nm and no changes in absorbance in the 300–350-nm region. Therefore, the absorbance decrease with irradiation time in the 300–350-nm region (Figure 3A) was due to release of the MgADP–V_i complex from the enzyme. However, the increase in absorbance at 270 nm (Figure 3B) was not due to regeneration of the spectrum of unmodified S1 upon MgADP and V_i release during irradiation because this increase was still present in the denatured sample (Figure 5). The increase in absorbance at 270 nm appears to be due to covalent modification of the enzyme. In the presence of denaturant, the maximum difference at 270 nm became even larger, corresponding to 15% of the absorbance of the unirradiated sample.

This large increase in the absorbance at 270 nm suggests that an aromatic amino acid has been modified. Since S1 has only five to seven tryptophans in the heavy chain (depending upon the site of chymotrypsin cleavage) and no tryptophans in the light chains, modification of tryptophan, as measured

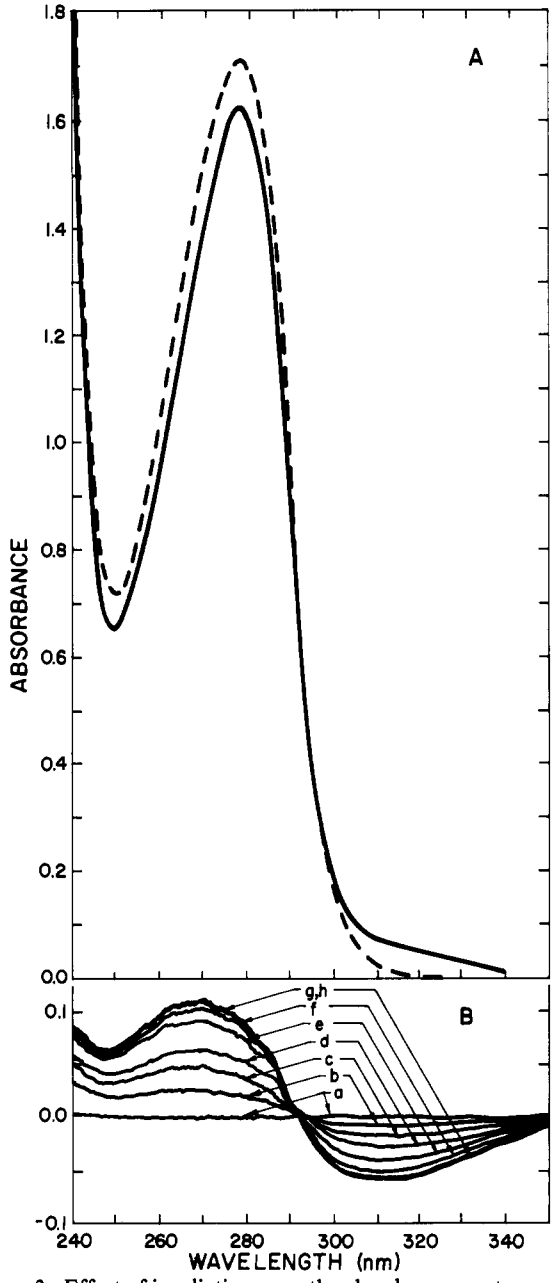


FIGURE 3: Effect of irradiation upon the absorbance spectrum of the S1–MgADP–V_i complex. (Panel A) The UV absorbance spectrum of 2.13 mg/mL S1–MgADP–V_i complex in S1 buffer was taken before (—) and after (--) 4 min of irradiation through a Pyrex filter in a 3-mL quartz cuvette. The base-line absorbance of S1 buffer is represented by the x axis. (Panel B) Difference spectra were taken at the indicated irradiation times with the nonirradiated S1–MgADP–V_i complex as the reference (a): (b–h) 20 s, 40 s, 1 min, 2 min, 3 min, 4 min, and 5 min, respectively.

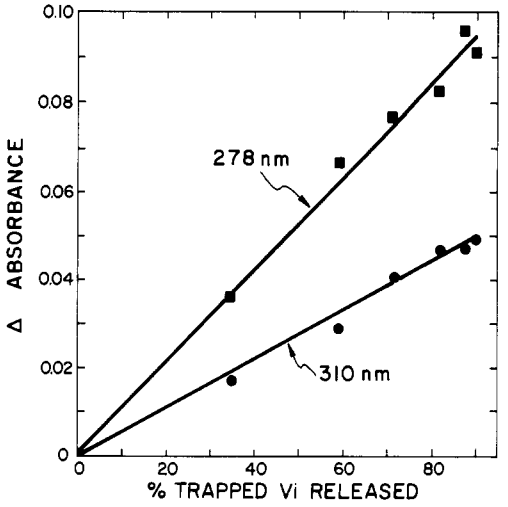


FIGURE 4: Percent of trapped V_i released vs changes in the absorption upon the irradiation of the S1–MgADP–V_i complex. At 0, 0.5, 1, 2, 3, and 4 min of irradiation, the absorbance at 310 (●) and 278 (■) were measured. The absolute value of the difference was plotted. The free V_i was determined as described in Figure 2. The initial amount of trapped V_i was determined after the addition of 1% sodium dodecyl sulfate to the PAR reaction mixture as described by Goodno (1982).

Table II: Tryptophan Content of Photomodified S1[a]

sample	method: amino acid analysis (mol of Trp/mol of S1)	method: NBS assay (mol of Trp/mol of S1 ± SD)	relative fluoresence intensity
S1–MgADP–V_i	4.9	7.6 ± 0.3	1.0
photomodified S1	4.6	7.6 ± 0.1	1.0

[a] The S1–MgADP–V_i complex and photomodified S1 were prepared as described under Materials and Methods. For amino acid analyses, samples (5.0 nmol) were dialyzed against 0.1% trifluoroacetic acid, lyophilized, and hydrolyzed in vacuo with 50 μL of 3 M mercaptoethanesulfonic acid at 110 °C for 22 h (Penke et al., 1974). Analyses were carried out on a Beckman Model 121 MB amino acid analyzer using a gradient system designed for high resolution of Trp, Arg, and Lys. The Trp per mole of S1 values were calculated by normalizing to the Arg content and assuming that 91% of the Trp was recovered. This latter value was based on the percent recovery of Trp determined after a parallel hydrolysis of lysozyme (6 mol of Trp/mol of enzyme). The NBS assay (three experiments) was performed according to Spande and Witkop (1967). The relative fluorescence intensities were compared in 8 M guanidine hydrochloride–50 mM Tris, pH 8.0, at identical protein concentrations on an SLM 4800 fluorometer. The excitation wavelength was 295 nm, and the emission was measured at 340 nm. Absorbances at 295 nm were less than 0.02.

by a change in tryptophan content, should be detectable. Table II presents a comparison of the tryptophan content of S1 and photomodified S1 by amino acid analysis, colorimetric assay with NBS, and relative tryptophan fluorescence. Amino acid analysis after hydrolysis in mercaptoethanesulfonic acid (to avoid destruction of tryptophan) showed no difference in tryptophan content before or after irradiation. Likewise, analysis with NBS indicated that tryptophan content did not change upon irradiation. NBS reacts with the pyrrole portion of the indole ring, oxidizing tryptophan to an oxindole (Spande & Witkop, 1967). Thus, this part of the indole ring remained unchanged by photomodification. For both samples, the NBS assay gave higher apparent levels of tryptophan than the amino acid analysis. This is probably due to the side reaction of NBS with the large number of tyrosines present in S1. Finally, the fluorescence intensity, which is probably the most sensitive assay for relative levels of tryptophan, did not change upon irradiation of the complex. Hence, it is unlikely that the large increase in UV absorbance in photomodified S1 involves the specific modification of tryptophan.

In an attempt to stabilize the S1–MgADP–V_i complex during irradiation and to prevent the photomodification, we replaced Mg^{2+} with Co^{2+}. Figure 5B shows that the spectrum of the S1–CoADP–V_i complex denatured in guanidine hydrochloride did not change appreciably after 4-min irradiation. In Table III, the effects of irradiation upon trapping and Ca^{2+}ATPase activity of complexes formed with various divalent metal ions are compared. Although Mn^{2+}, Ni^{2+}, and Co^{2+} trapped ADP to a similar extent as Mg^{2+}, the transition metal complexes were stable to irradiation. In contrast to the Mg^{2+} complex, irradiation had little effect upon the Ca^{2+}-ATPase activities of the Mn^{2+}, Ni^{2+}, or Co^{2+} complexes.

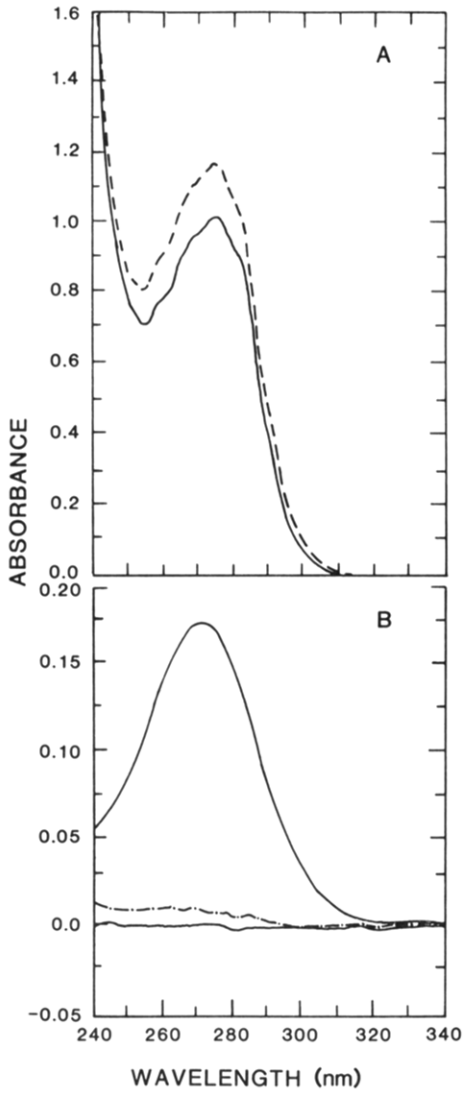


FIGURE 5: Effects of irradiation upon the absorbance spectra of guanidine hydrochoride denatured S1–MgADP–V_i and S1–CoADP–V_i complex. (Panel A) Irradiation was as described in Figure 3. Before (—) and after irradiation (– –), the S1–MgADP–V_i complex was denatured by addition of guanidine hydrochloride to a final concentration of 4 M before collecting spectral data. (Panel B) Difference spectrum [irradiated minus unirradiated (—)] corresponding to spectra in panel A, with the unirradiated sample as the reference (baseline). Difference spectrum of a corresponding experiment with an irradiated S1–CoADP–Vi complex denatured in 4 M guanidine hydrochloride (–·–) with the unirradiated sample as the reference (baseline). Final protein concentrations were 1.45 mg/mL.

These results taken together indicate that the enzyme was not significantly photomodified if Mg^{2+} was replaced with Mn^{2+}, Co^{2+}, or Ni^{2+}.

It was possible to retrap Mg[^{14}C]ADP and V_i on photomodified S1 at levels comparable to that seen with control S1 as shown in Table IV. Upon retrapping, the elevated Ca^{2+}-ATPase activity decreased to 13% of that of photomodified S1. MgADP and V_i were rapidly released from the active site upon irradiation of the purified photomodified S1–MgADP–V_i complex. After 4 min of irradiation, only 18% of the [^{14}C]-ADP originally trapped remained at the active site. The Ca^{2+}ATPase activity increased very slightly upon irradiation, most likely due to a small amount of residual S1 undergoing photomodification for the first time as only 75% of the S1 forms the complex and becomes photomodified initially. Gel electrophoretic analysis (Figure 6, upper) revealed that no polypeptide chain cleavage occurred in the absence of irradiation (lanes a, c, and e), in the photomodified S1 (lane d),

Table III: Effects of Irradiation upon [^{14}C]ADP Trapping and Ca^{2+}ATPase Activity of Various Metal–S1–ADP–V_i Complexes

metal[a]	[^{14}C]ADP/S1		Ca^{2+}ATPase activity (% of control)	
	$-h\nu$	$+h\nu$	$-h\nu$	$+h\nu$
Mg^{2+}	0.78	0.08	2.1	406.0
Co^{2+}	0.76	0.75	2.6	6.6
Mn^{2+}	0.68	0.66	17.3	28.0
Ni^{2+}	0.74	0.71	2.2	19.6

[a] Inactivations were performed as described under Materials and Methods except $MgCl_2$ was replaced with either $CoCl_2$, $MnCl_2$, or $NiCl_2$. After purification of the complex, the sample was split, and half was irradiated for 4 min. All samples were then repurified by centrifugation through a Sephadex G-50 column. Protein and [^{14}C]-ADP concentrations were determined as described under Materials and Methods. The control sample was untreated S1.

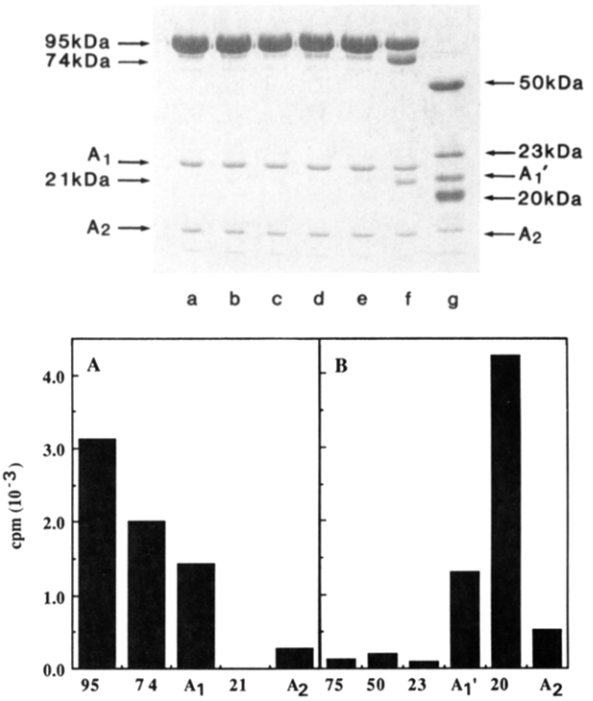


FIGURE 6: (Upper) SDS–PAGE analysis of S1, S1–MgADP–V_i complex, and photomodified S1–MgADP–V_i complex before and after UV irradiation: (Lane a) S1; (lane b) irradiated S1; (lane c) S1–MgADP–V_i complex (unirradiated); (lane d) irradiated S1–MgADP–V_i complex (photomodified); (lane e) photomodified S1–MgADP–V_i complex (unirradiated); (lane f) irradiated photomodified S1–MgADP–V_i complex (photocleaved); (lane g) tryptic S1. The photomodified S1–MgADP–V_i complex was prepared, purified, and irradiated as described for the S1–MgADP–V_i complex (see Materials and Methods). Samples (50 μg) were run on a 12% SDS–polyacrylamide gel (Laemmli, 1970) and stained with 0.05% Coomassie Blue R in methanol/water/acetic acid (5:5:1) and destained in the same solvent. (Lower) Localization of [^{14}C]pPDM on photocleaved S1 before (A) and after (B) trypsinization. The numbers on the x axis refer to the molecular weight of the peptides (kDa). A_1' is A_1 which has been tryptically clipped. Photocleaved S1 was reacted with equimolar [^{14}C]pPDM and quenched after 30 min by the addition of a 100-fold excess of β-mercaptoethanol. Non-protein-bound [^{14}C]pPDM was removed by centrifugation through a 5-mL column of Sephadex G-50. [^{14}C]pPDM-labeled photocleaved S1 was trypsinized at 25 °C with 1/100 (wt/wt) trypsin for 10 min before the reaction was quenched with 3/100 (wt/wt) soybean trypsin inhibitor. Samples (80 μg) were analyzed by gel electrophoresis as described above. The protein bands were excised and dissolved in 0.75 mL of 30% H_2O_2/NH_4OH (99:1) at 70 °C followed by the addition of 14 μL of concentrated acetic acid and 10 mL of ACS and counted as described under Materials and Methods.

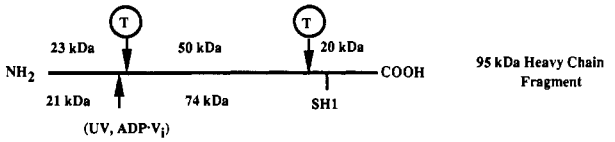
or in irradiated control S1 (lane b), whereas irradiation of photomodified S1–MgADP–V_i complex (lane f) resulted in specific cleavage of the 95-kDa polypeptide to yield two

Table IV: Trapping and Ca^{2+}ATPase Activity of Photomodified S1 Complex before and after Irradiation[a]

sample	trapped [^{14}C]ADP/S1 (mol/mol)	Ca^{2+}ATPase (% of photomodified S1)
photomodified S1 complex	0.74	13.0
photocleaved S1	0.13	20.0

[a] Photomodified S1 was prepared and purified after irradiation to remove released MgADP and V_i as described under Materials and Methods. The complex of photomodified S1 with V_i and [^{14}C]ADP was prepared and purified as described for the S1–MgADP–V_i complex (see Materials and Methods). Half of the sample was irradiated for 4 min to effect photocleavage and purified by a centrifuge column. Protein and [^{14}C]ADP concentrations and Ca^{2+}ATPase assays were performed on the purified samples as described under Materials and Methods.

Scheme I



peptides migrating at 21 and 74 kDa. Approximately 50% of the heavy chains were cleaved within 4 min, and further irradiation did not cause additional cleavage (data not shown). This level of cleavage is the maximum expected as only 75% trapping was observed for each trapping and irradiation cycle. Occasionally, our photomodified samples contained trace amounts of the cleaved heavy chain. We have found that if the irradiation was performed at high S1 concentrations (>4 mg/mL), small amounts of the released MgADP and V_i could rebind during the time course of the irradiation and promote photocleavage. Because of this effect, it was important to remove all excess MgADP and V_i added during the complex formation by gel filtration before irradiation.

It was of interest to determine which of the two peptides, 74 and 21 kDa, generated by photocleavage, corresponded to the NH_2-terminal of the heavy chain. The photocleaved S1 was reacted with [^{14}C]pPDM which reacts primarily with SH1 and SH2 in the COOH-terminal 20-kDa tryptic peptide of S1 (Reisler et al., 1974) and less readily with the single SH group of the A_1 and A_2 light chains (J. Grammer, unpublished observations). Separation of the peptides by gel electrophoresis in a manner similar to that in Figure 6 (upper) followed by analysis for radioactivity (Figure 6A) showed that the 21-kDa photocleavage fragment was not labeled while the uncleaved 95-kDa heavy chain and 74-kDa fragment were both labeled. As expected, some labeling of A_1 and A_2 was also seen. Upon trypsinization, all of the radioactivity originally seen in the 95- and 74-kDa peptides went to the 20-kDa tryptic peptide (Figure 6B), showing that the 21-kDa cleavage product came from the NH_2 terminus of the 95-kDa peptide and the 74-kDa fragment, which contained SH1 and SH2, from the COOH terminus, as shown in Scheme I. Thus the site of photocleavage is near the COOH-terminal end of the NH_2-terminal 23-kDa tryptic peptide.

Discussion

Specific photolabeling of the ATP binding site of skeletal myosin and its active subfragments (Okamoto & Yount, 1985; Cremo & Yount, 1987; Mahmood et al., 1987) has been achieved by stabilizing (trapping) nucleotide analogues at the active site by cross-linking SH1 and SH2 with bifunctional thiol reagents [reviewed in Wells and Yount (1982)]. Nucleotides trapped in this manner have been shown to remain stably trapped during the UV irradiation required to covalently label the enzyme. The thiols of other myosins, e.g., from smooth muscle and non-muscle sources, do not react with the same specificity and an alternative method of trapping was sought. We found that the binding of a variety of ADP photoaffinity analogues could be stabilized at the active site by forming a transition state complex with V_i (Okamoto et al., 1986; J. Grammer, R. Mahmood, D. Cole, unpublished results) as reported by Goodno (1979) for ADP–V_i. However, upon UV irradiation of the S1–Mg–analogue–V_i complex, nucleotide and V_i were found to be rapidly released from the active site. This same rapid dissociation also occurred with S1–MgADP–V_i complexes in both smooth and skeletal S1, implicating V_i as the essential component of the photoreaction.

The data presented here indicate that S1 is covalently modified when the S1–MgADP–V_i complex is irradiated with light in the 300–400-nm range. Upon irradiation, the Ca^{2+}-ATPase was activated 4–5-fold over control activity, while the K^+EDTA-ATPase activity remained very low (Figures 1 and 2). These results indicate that the irradiated enzyme is modified in some manner and that the release of MgADP and V_i does not simply regenerate active S1. The activated Ca^{2+}ATPase activity and loss of K^+EDTA-ATPase activity suggested SH1 or SH2 modification (Sekine & Kielley, 1964; Reisler et al., 1974). However, the absence of any change in total thiol content and the ability of photomodified S1 to trap ADP by cross-linking SH1–SH2 indicated that sulfhydryls had not been modified (Table I).

The results of spectral studies indicate that excitation of the S1–MgADP–V_i complex leads to the photomodification reaction. Upon formation of the complex, there was an increase in absorbance in the 290-350-nm range (Figure 3A). MgADP–V_i must be complexed at the active site to have the increased chromophoric properties. Irradiation with light above 335 nm, where protein does not absorb (Figure 3), did not prevent the photocatalyzed release of the MgADP and V_i (data not shown). However, the use of a 400-nm filter did prevent the photopromoted release, and it did protect the enzyme from photomodification as determined by trapping stability and unchanged ATPase activities (data not shown). Therefore, irradiation at wavelengths above 335 nm but lower than 400 nm causes the photomodification, thus demonstrating that the S1–MgADP–V_i complex per se, which absorbs in this region (Figure 3A), is the photoactivatable species. The V_i promoted photolytic cleavage of dynein heavy chains also occurs with irradiation in the 320–400-nm range (Gibbons et al., 1987).

It was possible to stabilize the ADP–V_i complex at the active site during irradiation by replacing Mg^{2+} with one of the divalent metal ions, Mn^{2+}, Co^{2+}, or Ni^{2+} (Table III). The Ca^{2+}ATPase activity remained essentially unchanged upon irradiation of S1–ADP–V_i complexed with Mn^{2+}, Co^{2+}, or Ni^{2+}, and therefore it appears that no photomodification of the S1 had occurred. This conclusion was further supported by spectral studies of the S1–CoADP–V_i complex. Difference spectra of the complex during the irradiation time course (Figure 5B) did not show the increased absorbance at 270 nm that occurred when the comparable Mg complex was irradiated. By use of Co^{2+}, Mn^{2+}, or Ni^{2+}, rather than Mg^{2+}, to form the complex, photoaffinity labeling experiments are now feasible without the problem of nonspecific labeling from released nucleotide. For example, the active site of smooth muscle myosin in the 6S conformation has been specifically photolabeled by stabilizing 3′(2′)-*O*-(4-benzoylbenzoyl)-ADP as the Co^{2+}–V_i complex (Cole & Yount, 1988).

Difference spectra of the S1–MgADP–V_i complex taken during the irradiation time course (Figure 3B) show that a new chromophore with a maximum absorbance at 270 nm is generated. The native conformation of the enzyme was not required for the spectral change, as the peak was still present in guanidine hydrochloride denatured S1 (Figure 5B). The contribution of the new chromophoric species to the total absorbance of the protein was appreciable. In guanidine hydrochloride, the absorbance of the new chromophore alone corresponded to an extinction coefficient of approximately 12 000 M^{-1} cm^{-1} at 270 nm. This large spectral change implicated an aromatic amino acid(s) in the photomodification reaction. However, as shown in Table II, tryptophan does not appear to be modified. In addition, in other experiments (J. Grammer and C. Cremo, unpublished results) the presence of an oxidized tyrosine in photomodified S1 was ruled out by the absence of increased absorbance above 300 nm in basic solutions as would be expected for dihydroxyphenylalanine residues. As shown in the following paper (Cremo et al., 1988), it is likely the increased absorbance is due to the formation of an enolate anion of a serine aldehyde.

Gel electrophoretic analysis gave no evidence that any polypeptide chain of the photomodified enzyme had been cleaved (Figure 6, upper). However, the heavy chain was rapidly and specifically cleaved upon irradiation of the photomodified S1–MgADP–V_i complex (Figure 6, upper). The cleavage occurred near the COOH terminus of the tryptic 23-kDa peptide which has previously been labeled by a series of photoaffinity analogues (Szilagyi, 1979; Nakamaye et al., 1985; Atkinson et al., 1986), thus implicating this portion of the heavy chain with both the purine and phosphate binding sites. These results further suggest photocleavage is a two step reaction which requires that MgADP and V_i be complexed at the active site and irradiated twice to effect chain cleavage. Formation of either Co^{2+}ADP–V_i complex (i.e., on S1 or on photomodified S1) prevented either photomodification or photocleavage from occurring [Figure 5 and J. Grammer (unpublished observation)]. Thus, the photomodified S1 described here likely represents a stable intermediate formed in the first step of the photocleavage pathway.

Vanadate-promoted photocleavage has been used extensively to investigate the structure of the α and β heavy chains of dynein (Lee-Eiford et al., 1986; Gibbons & Gibbons, 1987; Tang & Gibbons, 1987; King & Witman, 1987). Gibbons and co-workers have shown that cleavage of the dynein heavy chains occurs at a site termed V1 with micromolar concentrations of V_i and MgATP after irradiation with UV light in the 254–400-nm range. This cleavage is inhibited by Mn^{2+}, Co^{2+}, or Ni^{2+} and appears to cause a parallel loss of ATPase activity (Gibbons et al., 1987). These aspects of the dynein V1 cleavage are similar to those reported here for the specific vanadate-dependent photocleavage of the heavy chain of S1, suggesting that S1 and the V1 site of dynein are cleaved by a common mechanism. In the following paper (Cremo et al., 1988), we propose that a serine at the active site is oxidized to a serine aldehyde by photoreaction with V_i. This is likely the first step in the photocatalyzed cleavage of the heavy chain. The second step may be the photooxidation of serine aldehyde which leads to cleavage of the polypeptide chain. We have been able to isolate the photomodified enzyme as an intermediate in the cleavage pathway for two reasons. First, the S1–MgADP–V_i complex is so stable that excess MgADP and V_i can be removed prior to irradiation. Second, the re-formation of the MgADP–V_i complex with photomodified S1 is very slow at low ADP and V_i concentrations. Thus, even though V_i and ADP are released during the 4-min irradiation of S1–MgADP–V_i, neglible new complex is formed, and no cleavage is observed.

By appropriate modification of the dynein–V_i photoreaction conditions, it may be possible to obtain similar intermediates for this enzyme as well. However, specific photomodification of dynein will be more difficult because the V_i–ADP complex is much less stable ($t_{1/2}$ for dissociation ~4 min, 25 °C; Omoto & Moody, 1988) than S1 ($t_{1/2}$ ~ 2 days, 0 °C; this paper; Goodno, 1979). For this reason, intermediate uncleaved form(s) would probably not accumulate because the formation of the dynein–MgADP–V_i complex is so rapid (Shimizu & Johnson, 1983). It is known that dynein heavy chains are cleaved by substoichiometric amounts of V_i (Gibbons et al., 1987), indicating that V_i is serving as a catalyst. Thus it is possible that V_i may react more than once with a single site to cleave the dynein heavy chain. In contrast, under our conditions used to photomodify S1, V_i can only react one time with the enzyme. An understanding of the chemistry of the photomodification of S1 will be an important first step to understand the general mechanism of vanadate-dependent photochemical cleavage of polypeptides.

In summary, the irradiation-dependent release of MgADP and V_i, the activation of the Ca^{2+}ATPase, and the increase in the 270-nm absorption all correlated in a linear manner (Figures 1, 2, and 4). These data suggest that all three phenomena are the result of the same photochemical reaction of V_i with the enzyme. Retrapping V_i and MgADP on photomodified S1 and reirradiating, caused specific cleavage of the heavy chain. The site of cleavage, 21 kDa from the NH_2-terminal end, is near the consensus sequence believed to provide part of the polyphosphate binding site for ATP (Walker et al., 1982; Fry et al., 1986). Future work is aimed at identifying the specific site of cleavage and the chemical nature of the cleavage products.

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Registry No. V_i, 14333-18-7; MgADP, 7384-99-8; ATPase, 9000-83-3.

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UV-Induced Vanadate-Dependent Modification and Cleavage of Skeletal Myosin Subfragment 1 Heavy Chain. 2. Oxidation of Serine in the 23-kDa NH_2-Terminal Tryptic Peptide†

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ABSTRACT: Myosin subfragment 1 (S1) can be specifically photomodified at the active site without polypeptide chain cleavage by irradiating the stable MgADP–orthovanadate–S1 complex with UV light above 300 nm [Grammer, J. C., Cremo, C. R., & Yount, R. G. (1988) *Biochemistry* (preceding paper in this issue)]. Here, the UV spectral properties of photomodified S1 were used to determine the nature and location of the photomodified residue(s) within S1. By comparison of the unusual pH dependence of the UV absorption spectrum of the photomodified S1 to that of the S1–MgADP–V_i complex as a control, the photomodified residue(s) was (were) localized to the 23-kDa NH_2-terminal tryptic peptide of the heavy chain. $NaBH_4$ reduced the photomodified S1, but not the control, to regenerate the original spectral properties and ATPase activities of the unmodified S1. Amino acid analysis of photomodified S1 reduced with NaB^3H_4 gave only [^{3}H]serine, suggesting the hydroxyl group of serine had been oxidized to a "serine aldehyde". The pH dependence of the absorption spectrum of the photomodified enzyme can be explained by an equilibrium between a chromophoric enolate anion of the serine aldehyde (favored in base) and less chromophoric keto and enol forms (favored in acid). The oxidized serine(s) was (were) shown to be directly involved with the vanadate-dependent photocleavage of the S1 heavy chain previously described by Grammer et al. (1988). This serine(s) is (are) likely to be important to the binding and hydrolysis of the γ-PO_4 of ATP at the active site of S1.

In the previous paper (Grammer et al., 1988) we have shown that irradiation with UV light above 300 nm of the stable MgADP–orthovanadate (V_i)[1]–myosin subfragment 1 (S1) complex covalently modified the enzyme and rapidly released trapped MgADP and V_i from the active site. This photomodified S1 had Ca^{2+}ATPase activity 4–5-fold higher than that of nonirradiated control S1, while the K^+EDTA-ATPase activity was below 10% that of control. Analysis of the total number of thiols indicated that the photomodification did not involve SH1, SH2, or any other thiols. Irradiation of the

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[1] Abbreviations: serine aldehyde, form of serine in which the hydroxyl group has been oxidized to the level of aldehyde; V_i, orthovanadate; S1, myosin subfragment 1.

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