Photocleavage of Myosin Subfragment 1 by Vanadate†

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ABSTRACT: The heavy chain of myosin's subfragment 1 (S1) was cleaved at two distinct sites (termed V1 and V2) after irradiation with UV light in the presence of millimolar concentrations of vanadate and in the absence of nucleotides or divalent metals. The V1 site cleavage appeared to be identical with the previously described active site cleavage at serine-180, which is effected by irradiation of a photomodified form of the S1–MgADP–V_i complex [Cremo, C. R., Grammer, J. C., & Yount, R. G. (1989) *J. Biol. Chem. 264*, 6608–6011]. The V2 site was cleaved specifically, without cleavage at the V1 site, first by formation of the light-stable S1–Co^{2+}ADP–V_i complex at the active site [Grammer, J. C., Cremo, C. R., & Yount, R. G. (1988) *Biochemistry 27*, 8408–8415] and then by irradiation in the presence of millimolar vanadate. By gel electrophoresis, the V2 site was localized to a region about 20 kDa from the COOH terminus of the S1 heavy chain. From the results of tryptic digestion experiments, the COOH-terminal V2 cleavage peptide appeared to contain lysine-636 in the linker region between the 50- and 20-kDa tryptic peptides of the heavy chain. This site appeared to be the same site cleaved by irradiation of S1 (not complexed with Co^{2+}ADP–V_i) in the presence of millimolar vanadate as previously described [Mocz, G. (1989) *Eur. J. Biochem. 179*, 373–378]. Cleavage at the V2 site was inhibited by Co^{2+} but was not significantly affected by the presence of nucleotides or Mg^{2+} ions. Tris buffer significantly inhibited V2 cleavage. From the results of UV–visible absorption, ^{51}V NMR, and frozen-solution EPR spectral experiments, it was concluded that irradiation with UV light reduced vanadate +5 to the +4 oxidation state, which was then protected from rapid reoxidation by O_2 by complexation with the Tris buffer. The relatively stable reduced form or forms of vanadium were not competent to cleave S1 at either the V1 or the V2 site. ^{51}V NMR titration experiments indicated that a tetrameric species of vanadium preferentially bound to S1 and to the S1–MgADP–V_i complex, whereas no binding of either the monomeric or dimeric species could be detected. These results suggest that the vanadate tetramer was responsible for the photocleavage of S1 which occurred at both the V1 and V2 sites in the absence of nucleotides or divalent metals.

Current interest in vanadium biochemistry (Chasteen, 1983) has been recently spurred by the finding that vanadate (V_i)[1] can mediate highly specific photooxidations of proteins. An exciting aspect of this discovery is that the oxidations often result in site-specific cleavage of polypeptides. The first example of this was discovered in the course of photoaffinity labeling experiments with the flagellar ATPase dynein, where V_i was used in an attempt to stabilize the binding of a nucleotide analogue at the ATP binding site (Lee-Eiford et al., 1986; Gibbons et al., 1987). Upon irradiation with 365-nm light, the polypeptide backbone was unexpectedly cleaved at one site (termed V1), which inactivated the enzyme. Another vanadate cleavage site on dynein (called the V2 site), which does not require nucleotide binding, has also been characterized (Tang & Gibbons, 1987; King & Witman, 1987). As dyneins are very large multisubunit proteins, the use of vanadate to cleave the high molecular weight chains, together with other data, has provided important structural information about the sizes of the polypeptides, the location of the ATPase sites (Tang & Gibbons, 1987; Gibbons et al., 1987; King et al., 1989), and the identity of related soluble dynein-like translocating proteins from many sources (Lye et al., 1989).

It is known that V_i and MgADP together form a stable complex at the ATP binding site of the muscle ATPase myosin (Goodno, 1982). Concurrent with the initial dynein photoaffinity labeling experiments described above, V_i was being used to stabilize a photoaffinity analogue at the ATP binding site in smooth muscle myosin (Okamoto et al., 1986). In this case, irradiation destabilized the tight myosin–Mg–nucleotide–V_i complex, unless a filter was used to block light below 400 nm. In subsequent studies it was found that irradiation of the S1–MgADP–V_i complex covalently modified skeletal myosin S1 by oxidizing a serine to a serine "aldehyde" (Cremo et al., 1988). A second V_i-dependent photooxidation step specifically cleaves the polypeptide chain (V1 cleavage) in a reaction that involves the serine (Grammer et al., 1988). The oxidized residue was later identified as serine-180 (Cremo et al., 1989), located in the glycine-rich consensus sequence for ATP binding proteins.

Recently, a serine in ribulose-1,5-bisphosphate carboxylase/oxygenase has also been oxidized with V_i and UV light (Mogel & McFadden, 1989). These studies suggest that V_i-dependent photomodifications may be specific for serine;

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[1] Abbreviations: V_i, orthovanadate; Tris, tris(hydroxymethyl)aminomethane; S1, myosin subfragment 1; A1 and A2, alkali light chains of skeletal myosin; pPDM, *N,N'-p*-phenylenedimaleimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate.

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but this and other aspects of the chemical mechanism of protein oxidations by V_i remain to be investigated. V_i is known to interact with many classes of proteins including ATPases, phosphatases, kinases, phosphorylases, nucleases, and enzymes of the citric acid cycle (Chasteen, 1983). Thus, photooxidations of proteins by V_i may prove to be a generally useful new method to identify amino acids at the active sites of phosphoryl transfer proteins.

As an extension of our mechanistic studies of the myosin active site cleavage, we have detailed the conditions required to cleave the protein at a second unique site, termed V2 in analogy to dynein photocleavages (Gibbons, 1987). In contrast to the specific oxidations described above, the specificity of cleavage at this site is not derived from the stability of a nucleotide–V_i–protein complex. It appears to be the same site cleaved by V_i recently reported by Mocz (1989). To effectively block the V1 site from cleavage and to study the cleavage at the V2 site alone, we have exploited our previous finding that the S1–Co^{2+}ADP–V_i complex is stable to UV irradiation (Grammer et al., 1988). In the course of these studies we have found that V_i, in the presence of Tris used as a buffer, can be reduced to a relatively stable vanadium(IV) species under the irradiation conditions used in the cleavage experiments. This reduced form of vanadium was not competent to photocleave S1, a finding of importance to others devising experiments to photocleave proteins in the presence of V_i. Finally, by using ^{51}V NMR methods, we have determined that the oligomer tetravanadate preferentially binds to the S1–MgADP–V_i complex and to free S1, suggesting that this species is responsible for cleaving S1 at the V2 site and at the active site in the absence of nucleotides, respectively.

Materials and Methods

The sources of commercial compounds were as follows: SDS (Pierce), $VOCl_3$ and pPDM (Aldrich), Li_3ADP (Pharmacia P-L Biochemicals), $CoCl_2$ (Mallinkrockdt), sodium orthovanadate (Fischer); Tris (Sigma). [^{14}C]pPDM was synthesized as described (Wells & Yount, 1982).

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) with the following modifications: the myosin was dialyzed overnight into 0.12 M NaCl–10 mM sodium phosphate (pH 7.0 at 25 °C) and made 1 mM in EDTA just prior to addition of chymotrypsin; the ammonium sulfate precipitated protein was resuspended in S1 buffer containing 0.7 mg/mL phenylmethanesulfonyl fluoride (PMSF) prior to gel filtration to inactivate traces of chymotrypsin which slowly reactivate after PMSF treatment. To prepare the S1–MgADP–V_i complex or the S1–CoADP–V_i complex, 2 mM $MgCl_2$ or $CoCl_2$, 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 TrisHCl, pH 8.0, 0.1 M KCl, and 0.01% NaN_3 (S1 buffer). Excess V_i and MgADP were removed by centrifugation through a 5-mL column of Sephadex G-50 fine equilibrated in the appropriate buffer as described by Penefsky (1977). The complexes typically contained 0.85–0.90 ADP and V_i per S1 as determined by the use of [^{14}C]ADP and by the 4-(2-pyridylazo)resorcinol assay for V_i (Goodno, 1982). To cleave specifically at the V2 site, V_i was added to the S1–CoADP–V_i complex to the specified final concentration from a 100 mM pH 10 aqueous stock solution prepared as described by Goodno (1982). The sample was then placed in a small Pyrex Petri dish, 10-mL Pyrex beaker, or 1.5-mL plastic microcentrifuge tube, cooled on an ice bath lined with aluminum foil to reflect radiation, and irradiated for various times with a Hanovia 450-W medium-pressure Hg lamp (Ace Glass) at a distance of 9 cm. A Pyrex filter (Petri dish cover) was used to remove radiation below 300 nm. The extent of cleavage varied slightly between experiments depending upon the distance of the sample from the lamp, the absorbance and path length of the sample, and the vessel in which the sample was irradiated.

SDS gel electrophoresis was as described by Laemmli (1970) with 12% acrylamide, 0.32% bis(acrylamide), and 0.1% SDS, on a 1.5 mm thick 10 × 10 cm preparative mold (Hoeffer). Gels were stained with 0.05% Coomassie Blue R in methanol/water/acetic acid (5:5:1 v/v/v), destained in the same solvent, and soaked in water prior to photographing and scanning. An LKB Ultroscan XL laser densitometer was used to determine the integrated intensity under each stained band. Modified S1 protein concentration were determined by a dye binding assay (Bradford, 1976) using unmodified S1 as the standard as previously described (Wells et., 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.

^{51}V NMR spectra were obtained at 52.6 MHz on a Nicolet NT-200 spectrometer. Spectra were acquired with 2K real points with a 20 000 Hz spectral width resulting in a 0.1-s repetition rate with a 30° pulse angle. A line broadening of 40 Hz was applied to all spectra. Integration of peak intensities was performed with the standard manufacturer-supplied software routine. Chemical shifts are reported relative to an external standard of $VOCl_3$ at 0 ppm. No deuterated solvents were used, and the magnetic field was not locked. No notable broadening occurred during the time required to acquire data for each sample (about 30 min). Samples were kept at 0 °C during and between spectral acquisitions.

Results

The conditions required for photocleavage of S1 by V_i are illustrated in Figure 1 (lanes a–f). In the absence of V_i, S1 (lane a) showed no detectable cleavage of either the heavy or light chains after irradiation for up to 1.5 h (lane b). However, irradiation for 20 min in the presence of 1 mM V_i (lane c) caused substoichiometric cleavage (49%) of the 95-kDa heavy chain at two sites, termed V1 and V2. The V1 peptides were estimated to be 21 and 74 kDa, whereas the V2 peptides were 20 and 75 kDa. Although the smaller peptides were clearly resolved, the two larger fragments comigrated in this gel system. A small amount of a 54-kDa peptide was formed by cleavage at both the V1 and V2 sites, suggesting that the sites were at opposite ends of the heavy chain. As a comparison, lane d shows S1 that has been cleaved only at the V1 site (46% cleavage), by irradiation of the photomodified S1–MgADP–V_i complex that has been gel filtered to remove excess free V_i (Grammer et al., 1988). In photomodified S1, serine-180 has been oxidized to the aldehyde form (Cremo et al., 1988), and cleavage at the V1 site is known to involve serine-180 (Cremo et al., 1989; Grammer et al., 1989). The heavy chain was cleaved at both the V2 and V1 sites if V_i was added to 1 mM in the presence of 1 mM $MgCl_2$ and 0.1 mM ADP (lane e; 39% cleavage). However if $MgCl_2$ was replaced by $CoCl_2$, only the V2 site was cleaved (lane f; 50% cleavage). Under the experimental conditions used for lanes e and f, both the S1–MgADP–V_i and S1–CoADP–V_i complexes formed rapidly (less than 5 min; Goodno, 1982). Thus, irradiation of S1–MgADP–V_i (lane e) caused photomodification (Grammer et al., 1988), which then led to cleavage at the V1 site by irradiation of the small amount of photomodified S1–MgADP–V_i

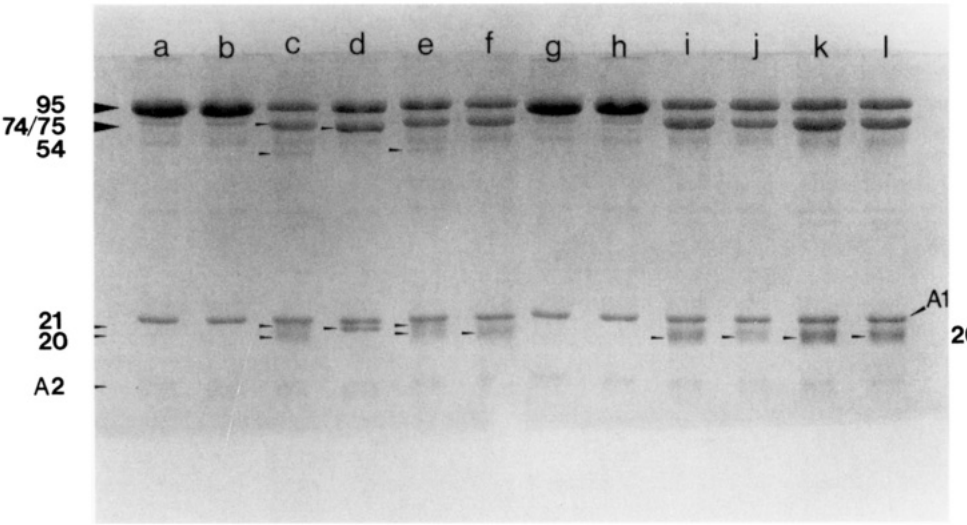

FIGURE 1: V_i-dependent cleavage of S1 and of the S1–CoADP–V_2 complex. S1 and the purified Si–CoADP–V_i complex were prepared as described under Materials and Methods. V_i was added to the final indicated concentrations, and samples were irradiated for 20 min in S1 buffer at 1.7 mg/mL (14.8 μM) protein. 25 μg of protein (except lane k; 29 μg) was applied to a 12% SDS–polyacrylamide gel as described under Materials and Methods. The numbers at the left represent the approximate molecular weights of the fragments indicated by the arrows. A1 and A2 are the alkali light chains. Control S1 before (a) and after (b) irradiation; (c) S1 irradiated in the presence of 1 mM V_i; (d) S1 specifically cleaved at the active site serine-180 (V1 site) as previously described (Grammer et al., 1988); (e) S1 incubated in the presence of 1 mM V_i, 2 mM $MgCl_2$, and 0.1 mM ADP for 10 min at 25 °C prior to irradiation; (f) S1 incubated in the presence of 1 mM V_i, 2 mM $CoCl_2$, and 0.1 mM ADP for 10 min at 25 °C prior to irradiation; (g and h) purified S1–CoADP–V_i complex before (g) and after (h) irradiation; (i) purified S1–CoADP–V_i complex irradiated in the presence of 2 mM $MgCl_2$ and 1 mM V_i; (j) same conditions as in (i) except with $CoCl_2$ replacing $MgCl_2$; (k) same conditions as in (i) except without added divalent metal ion; (l) same conditions as in (i) except with 0.1 mM ADP added.

complex that re-formed in the presence of 1 mM V_i during the irradiation. For the case of the S1–Co^{2+}ADP–V_i complex (lane f), the V1 site was effectively blocked from cleavage (Grammer et al., 1988), but cleavage at the V2 site was only partially inhibited by $CoCl_2$ (50% cleavage; see below).

To study the requirements for cleavage at the V2 site alone without cleavage at the V1 site, the effects of various treatments upon the purified S1–Co^{2+}ADP–V_i complex were investigated (lanes g–l, Figure 1). The S1–Co^{2+}ADP–V_i complex (lane g) did not photocleave after 20-min irradiation in the absence of any added free V_i (lane h). However, if V_i was added to 1 mM, the complex was cleaved specifically at the V2 site (lanes i–l), to generate a 20-kDa peptide which appeared to migrate at the same position as that observed in lanes c and e. This indicated that V_i cleaved at or near the same site whether or not the active site was occupied with nucleotide as the S1–Co^{2+}ADP–V_i complex. V2 cleavage of the S1–CoADP–V_i complex did not require divalent metal, as the extent of cleavage was similar in the presence of 2 mM $MgCl_2$ (lane i; 62% cleavage) or without added metal (lane k; 64% cleavage). However, the cleavage was inhibited in the presence of 2 mM $CoCl_2$ (lane j; 46% cleavage). In further experiments (data not shown), it was found that increasing $CoCl_2$ concentrations decreased the amount of V2 cleavage in a linear manner (75% inhibition at 10 mM). The presence of 2 mM $MgCl_2$ and 0.1 mM ADP (lane l; 60% cleavage) did not change the extent or apparent position of the V2 cleavage (compare with lane i; 62% cleavage). In summary, it appeared that the V1 site was associated with the nucleotide binding site. In contrast, the V2 site was cleaved irrespectively of nucleotide bound to the active site. Cleavage at both sites was inhibited by $CoCl_2$, but the V2 site required much higher concentrations that the V1 site.

To determine the approximate position of the V2 cleavage site within the 95-kDa heavy chain, we performed the experiment described in Figure 2. ADP and cobalt were trapped at the active site by cross-linking SH1 (Cys-707) and SH2 (Cys-697), in the COOH-terminal 20-kDa tryptic peptide, with [^{14}C]pPDM as previously described (Wells & Yount, 1982). Irradiation of the pPDM-cross-linked sample in the presence of 1 mM V_i (Figure 2) appeared to generate the same peptides generated by irradiation of the S1–Co^{2+}ADP–V_i complex (Figure 1, lane i). Analysis by gel electrophoresis showed that, in the control sample which was not irradiated, 77% of the radioactivity comigrated with the 95-kDa heavy chain as expected for cross-linking of SH1 and SH2. After irradiation to cleave 50% of the heavy chains at the V2 site, the level of radioactivity in the 95-kDa heavy chain decreased to 45% of the total. At the same time, the radioactivity comigrating with the V2 cleavage band at approximately 20 kDa increased from background level to 26% of the total counts. The 75-kDa fragment showed no significant increase in radioactivity upon irradiation. These results indicated that the V2 cleavage site was approximately 20 kDa from the COOH terminus of the 95-kDa heavy chain. In an effort to define the position of V2 cleavage further, the size of the smaller V2 peptide was compared with the 20-kDa tryptic fragment on polyacrylamide gels. The V2 peptide migrated slightly higher than the 20-kDa fragment (data not shown), in agreement with the results of Mocz (1989). Furthermore, the V2 peptide was cleaved by trypsin to a peptide that exactly comigrated with the 20-kDa tryptic peptide (data not shown). These results suggested that the V2 peptide contained Lys-636, the predominant tryptic cleavage site in the linker region between the 50- and 20-kDa tryptic peptides (M. Elzinga, personal communication).

Aqueous solutions of V_i are known involve rapid equilibria between monovanadate and various polyvanadates. At higher V_i concentrations, appreciable concentrations of di- and tetravanadate oligomers form (Baes & Mesmer, 1976; Habayeb & Hileman, 1980; Heath & Howarth, 1981). As a way to determine which predominant V_i species was responsible for cleavage at the V2 site, we examined the amount of V2 cleavage versus V_i concentration (Figure 3). To cleave specifically at the V2 site, the V1 site was blocked by forming the light-stable S1–Co^{2+}ADP–V_i complex (Grammer et al., 1989). The complex was then purified by gel filtration from

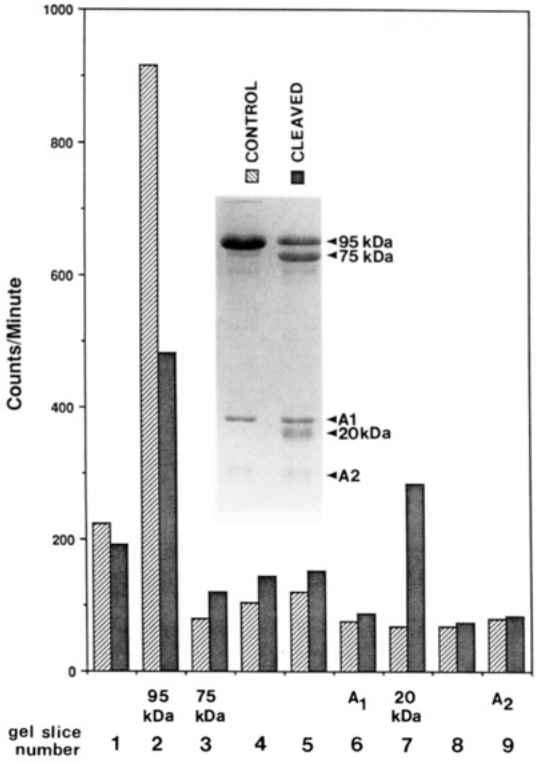

FIGURE 2: Localization of SH_1 and SH_2 in V2-cleaved S1. To covalently cross-link SH1 and SH2, S1 (17 μM) was incubated on ice for 1 h in the presence of 2 mM $CoCl_2$, 0.1 mM ADP, and 20 μM [^{14}C]pPDM (sp act. 3400 cpm/nmol). After removal of noncovalently bound [^{14}C]pPDM by rapid gel filtration, the specific activity of the protein corresponded to 1.0 mol of [^{14}C]pPDM/mol of S1. V_i was then added to 1 mM prior to irradiation for 20 min as described under Materials and Methods. The control was not irradiated. Samples (100 μg) were applied to a 12% SDS–polyacrylamide gel as described under Materials and Methods. The Coomassie Blue stained protein bands were excised and dissolved in 0.75 mL of 30% H_2O_2/NH_4OH (99:1) at 70 °C. Concentrated acetic acid (14 μL) and ACS scintillation fluid (10 mL) were added prior to counting. 50% of the radioactivity applied to the gel was recovered. Gel slice number: (1) stacking gel to above 95 kDa; (2) 95 kDa; (3) 75-kDa cleavage peptide or equivalent position in the control sample; (4 and 5) between 75 kDa and A1 light chain; (6) A1 light chain; (7) 20-kDa cleavage peptide or equivalent position in control sample; (8) between 20-kDa peptide and A2 light chain; (9) A2 light chain.

the remaining untrapped V_i, so that V_i could be added back to specified concentrations. The plot of the amount of V2 cleavage of the S1–Co^{2+}ADP–V_i complex versus V_i concentration is shown in Figure 3. Less than 5% cleavage was observed at V_i concentrations less than 0.25 mM whereas at 3 mM V_i approximately 90% of the heavy chains were cleaved. Half-maximal cleavage was observed at about 0.6 mM V_i. At all V_i concentrations tested, the 95-kDa heavy chain was cleaved only once to give two peptides (75 and 20 kDa) after 20-min irradiation. At V_i concentrations above 2 mM, there was a small but increasing loss in the intregrated intensity for A1 (data not shown). The time course of V2 cleavage at 0.8 mM V_i (Figure 3, inset) demonstrated that the appearance of the 75- and 20-kDa peptides was correlated directly to the cleavage of the 95-kDa peptide. The total absorbance from the 20-kDa, 75-kDa, and the remaining uncleaved 95-kDa heavy chain was 92 ± 6% of that of the unirradiated control sample, indicating that V_i cleaved specifically at this site.

During the above experiment (Figure 3), it was noticed that irradiation of the buffer (Tris) and V_i, even without the protein, caused the solution to turn amber. This suggested that irradiation was changing the concentration of the vanadium species involved in the photochemical reaction with the protein. It

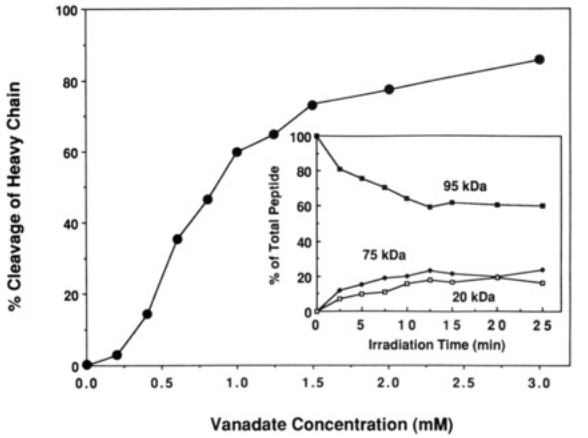

FIGURE 3: Dependence of V2 cleavage upon V_i concentration. The purified S1–CoADP–V_i complex (1.8 mg/mL) was prepared in S1 buffer as described under Materials and Methods. V_i was then added to the indicated final concentrations, and samples were irradiated for 30 min. 25 μg was applied to a 12% SDS–polyacrylamide gel as described under Materials and Methods. Gels were stained and scanned as described under Materials and Methods to determine the area under the 95-, 75-, and 20-kDa peptides. The percent cleavage of the 95-kDa heavy chain was calculated as the area of the 95-kDa peptide divided by the combined area of all three peptides. A1 and A2 were not scanned. The inset shows the time course of cleavage of the purified S1–CoADP–V_i complex in the presence of 0.8 mM V_i from a separate experiment. The percent of the total peptide was calculated by dividing the area for that peptide by the total area of all three peptides: 95 kDa (■), 75 kDa (◆), and 20 kDa (□).

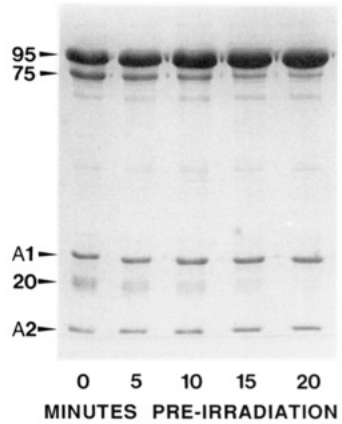

FIGURE 4: Effect of preirradiation of V_i–Tris solutions upon ability of V_i to photocleave at the V2 site. Purified S1–CoADP–V_i complex was prepared in S1 buffer as described under Materials and Methods. S1 buffer containing 1 mM V_i was preirradiated (in the absence of protein) for the indicated times. The S1–CoADP–V_i complex and the preirradiated V_i solutions were then mixed prior to irradiation of all samples for an additional 20 min. Final concentrations during the irradiation were 2 mg/mL protein and 0.9 mM V_i. 25 μg of protein was applied to each lane and electrophoresed as described under Materials and Methods. The number of minutes that the V_i–Tris solutions were preirradiated is shown at the bottom of the gel. The bands labeled 75 and 20 are the molecular masses (kDa) of the V2 fragments from cleavage of the 95-kDa heavy chain.

was of interest then to examine the potential of the preirradiated Tris–V_i solutions to effect photocleavage of the heavy chains at the V2 site (Figure 4). If preirradiated solutions of Tris–V_i were incubated with S1 without further irradiation, no cleavage at any site was observed (data not shown), indicating that the protein must be present during the irradiation for cleavage to be observed. However, if the Tris–V_i solution was preirradiated for increasing times, mixed with the protein, and irradiated, the level of V2 cleavage successively dropped from 37% without V_i preirradiation to less than 5% after a 20-min preirradiation. These results suggested that the com-

pound or compounds that were formed by irradiation of Tris–V_i were not competent to cleave S1 at the V2 site, or were inhibiting the cleavage reaction directly.

The direct effects of Tris during V2 cleavage (data not shown) were examined by irradiation of the S1–Co^{2+}ADP–V_i complex in either 0.1 M KCl (pH adjusted to 8.0) or S1 buffer (50 mM Tris, 0.1 M KCl, pH 8.0). After 55-min irradiation the sample with Tris was maximally cleaved to about 25%, whereas in the absence of Tris the sample was cleaved to nearly 80%. Half-maximal cleavage in the absence of Tris occurred at about 0.3 mM V_i, compared to 0.6 mM in the presence of 50 mM Tris (Figure 3). Tris concentrations up to 5 mM did not significantly diminish the cleavage of the heavy chain, but increasing the Tris concentration to 50 mM inhibited V2 cleavage to 40%. These results suggested that Tris was reacting with vanadium to cause the inhibition of cleavage and was not just a catalyst in the formation of the inhibiting species.

The effect of irradiation upon the UV–visible absorption spectrum of V_i in the presence of Tris at pH 8.0 is shown in Figure 5A. Unirradiated Tris-V_i did not absorb above 400 nm. However, irradiation caused the absorbance to increase above and decrease below an isosbestic point at 354 nm. The inset to Figure 5A shows the time dependence of the spectral change. The spectrum of the 30 min irradiated sample did not change after further incubation without irradiation on ice for 30 min. A similar spectrum with a broad maximum at 450 nm was observed if $NaBH_4$ (0.5 mM) was added to V_i (1 mM) in the presence of Tris (50 mM) or if vanadyl sulfate [$(VO^{2+})SO_4^{2-}$] was added to S1 buffer and the pH was adjusted to 8.0 [data not shown; initially a precipitate formed, $VO(OH)_2$(s) (Chasteen, 1983), which then dissolved to form a clear amber solution]. Solutions of irradiated Tris–V_i remained amber for days if stored on ice but returned to colorless if stored overnight at room temperature (data not shown). Irradiation of V_i in 0.1 M KCl (pH adjusted to 8.0) without Tris caused no change in the absorption spectrum (data not shown). Frozen-solution EPR spectra of all three solutions tested (irradiated, $NaBH_4$ treated, or vanadyl sulfate in Tris at pH 8.0) gave a signal which upon acidification to pH 1 was converted to the characteristic eight-line spectra for VO^{2+} (Chasteen, 1981) whereas no VO^{2+} could be detected if Tris was omitted (data not shown). These results together suggested that irradiation in the presence of Tris was necessary to form the amber species. The species was probably in the +4 oxidation state as the effect of irradiation could be mimicked by vanadyl sulfate.

To further characterize the role of Tris in the light-induced reaction, the effect of irradiation upon the ^{51}V NMR spectrum of 1 mM V_i in Tris, pH 8.0, was examined (Figure 5B). The detectable V_i species present under these conditions were monovanadate and the oligomers di-, tetra-, and pentavanadate. Figure 5B shows a plot of the calculated concentrations for each species obtained by integration of the normalized spectra and the total known V_i concentration. Irradiation caused a time-dependent loss in signal of all four detectable V_i species. After 30-min irradiation, 19.5% of the total integrated signal remained. No new V_i signals between −300 and −711 ppm were generated, indicating that the species generated did not contain vanadium(V). Treatment of an identical unirradiated V_i solution with $NaBH_4$ (0.5 mM) decreased the total vanadium signal by 70%. Without irradiation, or with irradiation in the absence of Tris (1 mM V_i, 0.1 M KCl, adjusted to pH 8.0 with HCl), there was no detectable change in the integrations of the ^{51}V NMR signals of any species (data not shown). These results suggested that

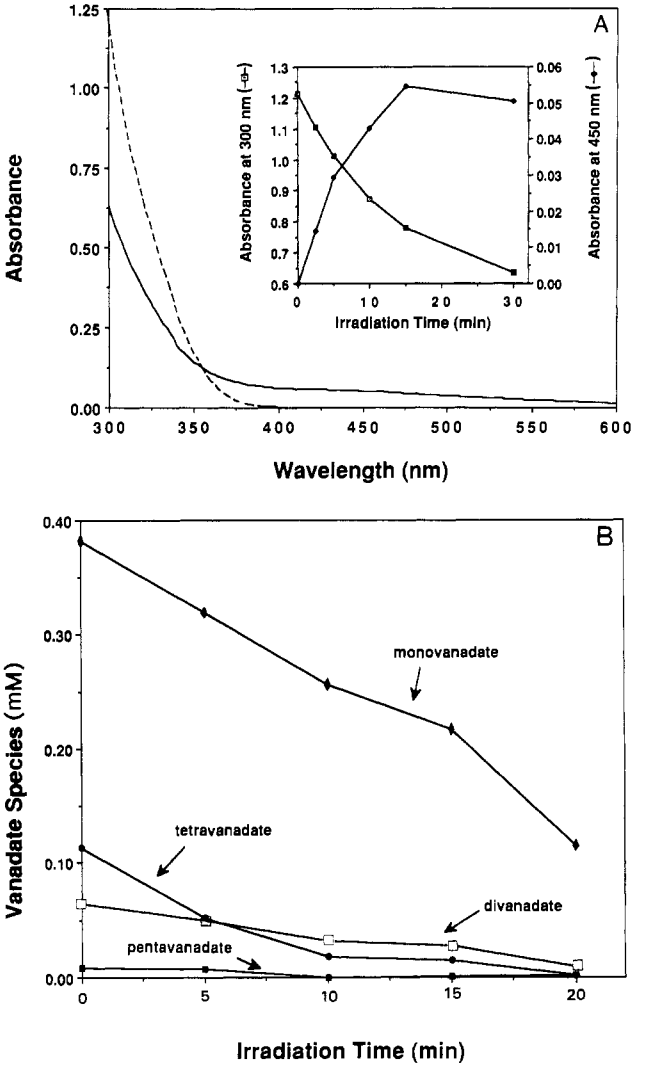

FIGURE 5: Effects of irradiation upon V_i in the presence of Tris at pH 8.0. Solutions of 1 mM V_i, 50 mM Tris, and 0.1 M KCl, pH 8.0, were irradiated for the indicated times as described under Materials and Methods. (A) The absorbance spectra before (--) and after (—) 30- min irradiation are shown. S1 buffer was used to establish the baseline. All spectra were recorded at 4 °C on a Varian 2200 dual-beam spectrophotometer on line to a Varian DS-15 computer within 30 min of irradiation. The inset shows a plot of the absorbance values at both 300 and 450 nm versus irradiation time. (B) ^{51}V NMR spectra were acquired at 0 °C as described under Materials and Methods immediately after each irradiation to minimize a change in signal during the time of the data acquisition (about 30 min). The concentration of each species was calculated from the peak integration, the known number of vanadium nuclei for each species, and the known total added concentration of V_i (1 mM).

irradiation caused a decrease in the concentration of vanadium(V) (observable by NMR) and an increase in the concentration of paramagnetic vanadium(IV), which is not detected under these conditions. This further suggested that the colored species observed in Figure 5A did not contain vanadium in the +5 oxidation state. Results of the absorption (Figure 5A), the ^{51}V NMR (Figure 5B), and the EPR spectral studies (data not shown) all indicated that the observed photochemical reaction of V_i required Tris to be stably reduced to a vanadium(IV) species.

To determine which of the predominant V_i species present under these conditions was responsible for cleavage at the V2 site, ^{51}V NMR was used to follow the binding of the various species to the protein. Figure 6 shows the titration of 0.8 mM V_i in S1 buffer with increasing concentrations of the S1–MgADP–V_i complex. The spectrum at the far right of the

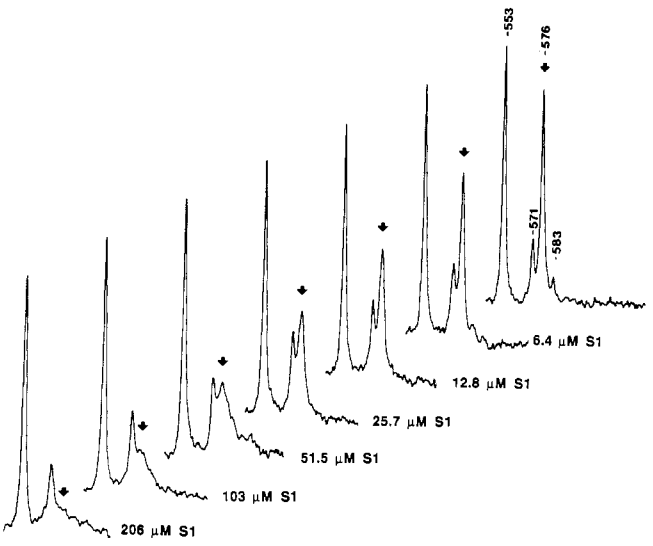

FIGURE 6: ^{51}V NMR spectra of the titration of V_i with the S1–MgADP–V_i complex. The S1–MgADP–V_i complex was prepared in S1 buffer as described under Materials and Methods. The protein was then precipitated with 2.5 volumes of saturated ammonium sulfate and centrifuged, and the pellet was resuspended in S1 buffer and rapidly gel filtered as described under Materials and Methods. The final protein concentration was 23.7 mg/mL (210 μM). V_i was added to a final concentration of 0.8 mM, and the ^{51}V NMR spectrum was acquired at 0 °C (left trace). The remaining data (from left to right) show the spectra of the sample after sequential dilutions to the indicated final protein concentrations with a solution of 0.8 mM V_i in S1 buffer. The spectrum at the extreme right is of 0.8 mM V_i in S1 buffer without protein. The calculated concentration of tetravanadate in this sample was 70 μM. The arrows indicate the position of tetravanadate (−576 ppm). Monovanadate, divanadate, and pentavanadate are indicated at −553, −571, and −583 ppm, respectively. Assignments are based upon those of Tracey et al. (1987). For all spectra, a standard solution of 1 mM V_i adjusted to pH 11 with NaOH in water (all V_i is monomeric at this pH) was placed into a 4-mm NMR tube, which was then inserted into the 12-mm NMR tube containing the sample (2.5 mL). The integrated signal of the standard (resonance at −536 ppm; not shown) was used to normalize the integrations between each spectra.

figure shows the composition of the V_i solution in the absence of protein. The resonance at −553 ppm is monomeric tetrahedral vanadate (Heath & Howarth, 1981). At −571 ppm is the resonance for divanadate ($V_2O_7^{4-}$, $HV_2O_7^{3-}$, $H_2V_2O_7^{2-}$; Heath & Howarth, 1981) and at −576 ppm is that for tetravanadate (Habayeb & Hileman, 1980; Heath & Howarth, 1981; Gresser et al., 1986; Tracey et al., 1988). The small, poorly resolved signal at −583 ppm is attributed to pentavanadate. The concentrations of each species, calculated from the signal integrations, were as follows: monovanadate, 340 μM; divanadate, 60 μM; pentavanadate, <10 μM. Not shown is a broad signal at −533 ppm (<20 μM) which corresponds to the bipyramidal Tris–V_i complex containing two vanadium nuclei and two Tris ligands described by Tracey and Gresser (1988). Decavanadate was not detected in this solution. Each successive spectrum proceeding to the left shows the effects of adding the S1–MgADP–V_i complex to the final indicated protein concentrations. There was no detectable signal from the V_i in the S1–MgADP–V_i complex under these conditions, as it is tightly bound to the protein (data not shown). Thus the only observable signals were from the free V_i (0.8 mM) added to the S1–MgADP–V_i complex. As the protein concentration was increased, the signal for tetravanadate broadened, indicating that this species was in rapid exchange and thus interacting with the protein. The mono- and divanadate signals showed no consistent decrease in peak integration (±2%) or width with increasing protein concentration. The addition of protein did not generate any new signals in the −330 to −711 ppm range. The signal for pentavanadate also appeared to broaden, but it was possible that this effect was due to the broadening of the larger tetravanadate signal. A significant decrease in the total integrated signal between −560 and −600 ppm (includes di-, tetra-, and pentavanadate) was not observed until the protein concentration was 103 μM. Collection of more extensive data for a binding curve was not feasible as it is difficult to prepare more concentrated solutions of S1. These results indicated that tetravanadate bound preferentially to the S1–MgADP–V_i complex.

Discussion

Previously we have shown that irradiation, with UV light above 300 nm, of the stable S1–MgADP–V_i complex covalently modifies S1 and rapidly releases trapped MgADP and vanadium from the active site (Grammer et al., 1988). This photomodified S1 has been oxidized specifically at serine-180 to the serine aldehyde (Cremo et al., 1988). Although the photomodified S1 has altered ATPase activities, it is like unmodified S1, capable of stably trapping MgADP and V_i at the active site. If the excess V_i (1 mM) used to drive the formation of the photomodified complex is removed by gel filtration prior to irradiation, the heavy chain is cleaved at one site to form an NH_2-terminal 21-kDa and a COOH-terminal 74-kDa peptide (Grammer et al., 1988). This cleavage, termed V1, appears to involve serine-180 (Cremo et al., 1988; Grammer et al., 1989). In these experiments, the only V_i present during irradiation is tightly bound at the active site as the photomodified S1–MgADP–V_i complex. Upon irradiation and cleavage, the complex is destabilized again (as it is upon photomodification; Grammer et al., 1989), and the concentration of the released V_i remains very low, never exceeding about 20 μM. In contrast, if the photomodified complex is formed with excess V_i by the usual procedure, but not gel filtered prior to irradiation, so that the excess free V_i is 1 mM, a second specific cleavage (V2) is observed. It is this second cleavage site that is the focus of this paper.

In the course of irradiation experiments to investigate this second site, we found that irradiation of S1 with millimolar concentrations of V_i alone, in the absence of nucleotide or divalent metal, cleaved S1 at two sites (Figure 1). The first site appeared to be identical with the one described above that is cleaved by irradiation of the photomodified S1–MgADP–V_i complex (Grammer et al., 1988, 1989; Cremo et al., 1988). Thus V_i alone, without stabilization as a complex with MgADP, interacted specifically with the active site to effect photocleavage at the V1 site. This V1 photocleavage was slightly enhanced by 1 mM $MgCl_2$ but was not appreciably inhibited by 1 mM $CoCl_2$ (data not known). This is in contrast to V_i bound as the S1–Co^{2+}ADP–V_i complex, which is stable to irradiation (Grammer et al., 1988). Mocz (1989) has reported that, under conditions similar to those reported here, V_i will cleave S1 at a site which is NH_2 terminal to but near the 23–50-kDa tryptic cleavage site (lysine-204; Tong & Elzinga, 1983). As this position is consistent with cleavage at or near serine-180, it is likely that our V1 site is the same site described by Mocz (1989).

The second photocleavage site termed V2 was also promoted by millimolar concentrations of V_i, did not require Mg^{2+} or nucleotide, and was inhibited by relatively high concentrations of $CoCl_2$. The V2 site was specifically cleaved without cleavage at the V1 site, first by formation of the light-stable S1–Co^{2+}ADP–V_i complex and then by irradiation in the presence of V_i at millimolar concentrations (Figure 3). We have localized the V2 cleavage site to a region about 20 kDa

from the COOH terminus of the S1 heavy chain (Figure 2). The 20-kDa peptide appears to contain Lys-636 in the linker region between the 50- and 20-kDa tryptic peptides. The position of this site agrees well with a cleavage site described by Mocz (1989) to be slightly NH_2 terminal of the junction between the central 50- kDa and COOH-terminal 20-kDa tryptic fragments. V_i (1 mM) did not slow the rate of trypsinolysis at the 50/20-kDa trypsin cleavage site (data not shown), suggesting that it was not binding to the same site as trypsin or that the V_i binding was weak (see below) in comparison to trypsin binding.

V2 cleavage was not inhibited by NaN_3 (5 mM, data not shown). Azide inhibits V_i-mediated photocleavage of the F_1-ATPase (Kao & Gresser, 1988). Although the mechanism for the inhibition is not known, it has been proposed that azide may quench singlet oxygen generated during the irradiation (Kao & Gresser, 1988).

The dependence of V2 cleavage upon V_i concentration (Figure 3) was reproducibly sigmoid-like with half-maximal cleavage occurring at about 600 μM V_i, suggesting that a weakly bound species favored at higher V_i concentrations was responsible for cleavage. A similar sigmoidal dependence has also been observed by Mocz for cleavage at an apparently equivalent site on S1 (Mocz, 1989; half-maximal cleavage rate observed at about 600 μM V_i in 10 mM hepes, pH 7.0) and for the cleavage of dynein heavy chains at the V2 site (Tang & Gibbons, 1987). These data have beren interpreted as evidence that oligomers such as trivanadate are responsible for cleavage. However, we noticed that irradiation caused the protein solutions to turn amber. In further experiments, it was found that only the buffer (Tris) and the V_i were required to form the color upon irradiation. V_i irradiated in H_2O, or in 0.1 M KCl at pH 8.0, did not change color, which suggested that the V_i was reacting with the buffer during the irradiation. If so, the concentrations of the photoreactive vanadium species responsible for cleaving the protein may change during the irradiation and the shape of the plot of V2 cleavage versus V_i concentration would be affected by the buffer.

To test for a photodependent interaction between V_i and Tris, we assayed preirradiated Tris–V_i solutions for their ability to cleave the protein at the V2 site. As predicted, preirradiated solutions of Tris–V_i were less effective in cleaving S1 at the V2 site than unirradiated solutions (Figure 4). This suggested that a relatively stable compound was generated by irradiation in the absence of protein, which either was not competent to cleave the protein or inhibited protein cleavage. Because of this complication, we did not pursue further an analysis of the rate of cleavage versus V_i concentration. Instead, the direct effects of Tris upon the cleavage reaction were investigated (data not shown). Tris inhibited the cleavage in a concentration-dependent manner, where 50 mM Tris decreased the amount of cleavage by more than 2-fold compared to the absence of Tris. Tracey and Gresser (1988) have shown that Tris forms monoesters with vanadium(V) oxyanions in aqueous solutions, much as other simpler alcohols such as ethylene glycol (Gressor & Tracey, 1986) and ethanol (Gresser & Tracey, 1985) and as with aldoses and nucleosides (Geraldes & Castro, 1989). Tris may also act as a tridentate ligand, binding to V_i through two hydroxyl groups and an amino group (Tracey & Gresser, 1988). We found that solutions of all amine buffers tried (Mops, HEPES, triethanolamine, Tricine) turned color upon irradiation in the presence of V_i (Cremo & Wolcott, 1990), suggesting that these buffers may also strongly inhibit V_i-dependent protein photocleavage reactions. As many of these buffers have been shown to complex with V_i (Crans & Shin, 1988), it is possible that they are involved as reactants in the V_i-dependent photoreactions.

To determine the reason for the Tris inhibition of cleavage at the V2 site, we examined the effect of irradiation upon the absorption spectra (Figure 5A) and the ^{51}V NMR spectra of Tris–V_i solutions (Figure 5B). Irradiation generated a species absorbing at 450 nm over the same time course as the loss of total signal in the ^{51}V NMR experiment (Figure 5B). These effects of irradiation were observed only in the presence of Tris, indicating that Tris was a reactant or a catalyst in a light-catalyzed reduction of vanadium(V) to vanadium(IV). It is known that chelated vanadyl ions are more slowly oxidized by molecular oxygen than the free VO^{2+} cation (Chasteen, 1981). Vanadyl cations interact more strongly with Tris than does V_i (Crans et al., 1989). Here, the vandium(IV) product(s) appeared to be reoxidized by oxygen much more slowly than would be expected for free vanadyl cations at this pH (Dean & Herringshaw, 1963; North & Post, 1984). These data taken together suggest that the role of Tris is to form a relatively stable complex with the vanadium(IV) that is generated by irradiation. This stabilization of the vanadium(IV) effectively lowered the concentration of vanadium(V) as the irradiation proceeded, explaining why preirradiated Tris–V_i solutions were less effective in cleaving S1 at the V2 site (Figure 4).

The fact that the reduced vanadium species was not competent to cleave the protein is not surprising, as it has been shown that the first step in S1 photocleavage at the V1 site is an oxidation of serine-180 to an aldehyde (Cremo et al., 1988). Thus photocleavage most likely involves a further oxidation by vanadium(V), the highest and most potent oxidation state of vanadium. Visible light has been previously shown to catalyze reductions of vanadium(V) to vanadium(IV) (Satapathy et al., 1963), oxidations of carbonyl compounds by vanadium(V) (Panwar & Gaur, 1967), and an uncharacterized decomposition of piperidine metavanadate ($C_5H_{11}N{\cdot}HVO_3$; Baudisch & Gates, 1934). Vanadium(V) promotes oxidative decarboxylation of the phenolate ligand *N*,*N*′-ethylenebis[(*o*-hydroxyphenyl)glycine] (Bonadies & Carrano, 1986). Recently, it has been shown that V_i, under conditions very similar to those reported here (20 mM Tris, pH 8.0), will spontaneously oxidize dihydroxyacetone, forming vanadium(IV) (Drueckhammer et al., 1989). Further studies of these oxidations may serve as model systems to examine the mechanisms of photocleavage of polypeptide chains by V_i.

By using ^{51}V NMR spectroscopy, we have shown that the oligomer tetravanadate preferentially bound to the S1–MgADP–V_i complex (Figure 6), whereas binding of monovanadate or divanadate was not detected. It was difficult to ascertain whether pentavanadate also bound, because the signal for this species was poorly resolved from the tetravanadate signal. In this experiment, the active site was blocked from interaction with free V_i; thus the observed binding was most likely at the V2 site. The binding of tetravanadate was accompanied by signal broadening. The broadening was not explained by a trivial increase in viscosity of the solution from the added protein, as the signals for mono- and divanadate did not broaden. We were not able to identify a signal corresponding to the protein-bound vanadium. Apparent equilibrium dissociation constants were calculated from the two data points, at 103 and 206 μM protein (Figure 6), for which a significant loss in the integrated free tetravanadate signal was detected (K_d's = 860 and 400 μM, respectively).

Although we have directly shown by ^{51}V NMR spectroscopy that tetravanadate binds to the V2 site, we have not demon-

strated that this species also binds to the V1 site. Conditions have not been established for selectively blocking V2 binding, so that V1 binding alone could be followed by ^{51}V NMR. We have shown, however, that the V1 site was cleaved under the same conditions as the V2 site. In an experiment identical with that in Figure 6 (data not shown), except that unmodified S1 was used to titrate the V_i, we found again that mono- and divanadate binding to the protein could not be detected, whereas tetravanadate binding was detected by signal broadening. Thus, it is not likely that either mono- or divanadate was responsible for cleavage at the V1 site in the absence of nucleotide. Thus, as tetravanadate was the only other species present in appreciable amounts, it was reasonable to conclude that tetravanadate bound to the V1 site in the absence of nucleotide to effect photocleavage.

The structure of tetravanadate is of interest here in that it appears to have a special ability to bind to the protein. Since it is assigned as a single resonance in the ^{51}V NMR spectrum, the environment of each vanadium nuclei is presumably identical. One structure consistent with this observation is the monocyclic tetrahedral tetramer $V_4O_{12}^{4-}$, whereas the linear tetramer $V_4O_{13}^{6-}$ would give two signals, one for the terminal and one for the central nuclei (Heath & Howarth, 1981). The structure of tetravanadate has recently been reinvestigated by ^{51}V NMR studies in liquid-crystalline solution (Tracey & Radley, 1985), and by estimation of the number of water molecules produced upon formation of tetravanadate from monovanadate (Tracey et al., 1988). Both studies are consistent with an adamantane-like structure, $H_4V_4O_{14}^{4-}$, where all four vanadium nuclei are pentacoordinate with nearly trigonal-bipyramidal geometry. It is possible that interactions with S1 stabilize a form of tetravanadate that is not stable in solution.

Why does tetravanadate bind specifically to the V1 and V2 sites on S1? As a polyanionic species, tetravanadate probably binds ionically to a polycationic portion of the nucleotide binding site (VI) which is specific for the triphosphate moiety of ATP. It may form an ester with the hydroxyl of serine-180, as it is known that this residue is oxidized by irradiation of the S1–MgADP–V_i complex (Cremo et al., 1989). It is thought that the stability of the S1–MgADP–V_i complex is due to the ability of V_i to adopt a stable trigonal-bipyramidal geometry (Goodno, 1982). In this way the complex would mimic the geometry of the transition state for the hydrolysis of ATP. If tetravanadate adopts the trigonal-bipyramidal adamantane-like structure discussed above, whereas both mono- and divanadate are tetrahedral species, this difference in coordination geometry may explain the specificity of tetravanadate for the V1 site.

The nature of the interaction of tetravanadate with the V2 site is less obvious, although it seems likely to also involve interaction with serine(s) and positively charged residues on the surface of the protein. Recently it has been shown that a V_i-mediated photocleavage of ribulose-1,5-bisphosphate carboxylase/oxygenase may also involve a prior oxidation of a serine residue (Mogel & McFadden, 1989). Even in the absence of light, V_i will spontaneously oxidize dihydroxyacetone by a mechanism that is thought to involve esterification of the V_i to a hydroxyl (Drueckhammer et al., 1989). The interaction of monovanadate with various aliphatic alcohols has been recently thoroughly investigated (Gresser & Tracey, 1985, 1986; Tracey et al., 1988; Tracey & Gresser, 1988). From these works, the equilibrium association binding constants for typical monoesters from monovanadate and alcohols have been found to be quite weak, on the order of 10 M^{-1}. Interaction with tyrosine is about 10-fold stronger (Tracey & Gresser, 1986). It seems unlikely that specific cleavage could result from simple esterification and subsequent oxidation of exposed serine residues. In model studies with di- and tripeptides, Redner and co-workers (Rehder et al., 1988) have shown that the hydroxyl functional group in peptides will esterify to V_i under conditions where serine alone shows no complexation. Thus, it seems reasonable to invoke some kind of protein-specific stabilization, allowing for specific serine interaction.

Although to exact amino acids involved at the V2 cleavage site have not been determined, it is likely that the site is at the most 20 amino acids NH_2 terminal to lysine-636, in agreement with the position estimated by Mocz (1989). The sequence in this region from residue 614 to residue 643, [KSMTLLAFLFSGAQAGEEGGGGKKGGKKKG] suggests that serine-615 or -624 (in boldface), or both, may be involved in the cleavage reaction, whereas the next serine is 34 residues away. The exposed lysine-rich portion of the sequence nearby may provide favorable charge interactions for the polyanionic tetravanadate. Recently, it has been shown by other methods that tetravanadate strongly inhibits 6-phosphogluconate dehydrogenases (association constant of 77 $\times 10^3$ M^{-1}), whereas mono, di and pentavanadate inhibition could not be detected (Crans et al., 1990). Thus, it is possible that the structural aspects of tetravanadate binding sites will be elucidated by studies with these and other similar enzymes.

The lysine-rich region of the S1 heavy chain that appears to be very close to the V2 cleavage site is thought to be an actin binding site (Chaussiepied & Morales, 1988; Yamamoto, 1989). Thus experiments are in progress to use V2 cleavage as a new, specific way to alter this region of the heavy chain and examine its effects upon ATPase activities and actin interactions (Cremo, unpublished data).

Finally, it is of interest to compare the V_i photocleavage reactions for myosin S1 to those for the ATPase dynein. V_i together with UV light cleaves dynein at two sites, termed V1 and V2, within both the α and β heavy chains. For both S1 and dynein, the V1 site is thought to involve the ATP binding site directly, as cleavage inactivates both proteins. Like S1, and V1 site of dynein can be cleaved by irradiation with UV light under two different sets of conditions: at relatively low V_i concentrations but in the presence of nucleotide where a ternary metal–nucleotide–V_i complex is formed at the active site or in the absence of nucleotides but at higher V_i concentrations where oligomers are formed in appreciable quantities. For dynein, at very low (nanomolar to micromolar) V_i concentrations, and in the presence of MgATP, the V1 site is specifically cleaved without cleavage at the V2 site (Gibbons et al., 1987). Monomeric V_i is probably responsible for the cleavage, as at these very low total V_i concentrations the concentration of oligomers is very low (Gibbons et al., 1987). Without nucleotide, and at higher V_i concentrations (50–200 μM) where oligomer concentrations become significant, the V1 site of dynein is cleaved along with the V2 site (Tang & Gibbons, 1987). The V1 site of S1 can be cleaved either by formation of the S1–MgADP–V_i complex at the active site or by irradiation in the presence of V_i alone at higher concentrations (cleaving the V2 site at the same time).

For both dynein and S1, and V2 site cleavage appears to involve V_i oligomers. For S1 we present evidence of tetravanadate binding. For dynein, evidence has been presented that trivanadate may be the oligomer involved (Tang & Gibbons, 1987), although direct binding of oligomers remains to be investigated for this enzyme. As observed for dynein

(Tang & Gibbons, 1987), V2 cleavage of S1 proceeds at relatively high concentrations of transition metals, whereas transition metals either slow or inhibit cleavage at the V1 site for both enzymes in the presence of nucleotides. For both enzymes, inhibition of cleavage by transition metals at the V1 site may reflect a tightly bound complex containing V_i and transition metal. In contrast, a similar V_i–transition metal interaction at the V2 site may be much less favorable. One interesting difference in the two enzymes is that the V2 cleavage of S1 appears to not be drastically inhibited if the active site is occupied with nucleotide [this work and Mocz (1989)], whereas very low concentrations of Mg–nucleotide completely inhibit V2 cleavage of dynein. This suggests that in dynein the V2 cleavage site may form part of the ATP binding site (Tang & Gibbons, 1987) whereas in S1 the sites may be structurally separate.

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Registry No. ADP, 58-64-0; Tris, 77-86-1; V_i, 37353-31-4.

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