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Effect of Actin, ATP, Phosphates, and pH on Vanadate-Induced Photocleavage of Myosin Subfragment 1†

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ABSTRACT: Near-UV irradiation in the presence of vanadate cleaves the heavy chain of myosin subfragment 1 at three specific sites located at 23, 31, and 74 kDa from the N-terminus. Increasing the pH from 6.0 to 8.5, gradually, reduces the efficiency of the cleavage and comletely eliminates the 31-kDa cut. Actin specifically inhibits the photocleavage at the sites located 31 and 74 kDa from the N-terminus. ATP strongly protects from cleavage at the 23- and 31-kDa sites and less strongly from the cut at the 74-kDa site. ADP and pyrophosphate have similar, but less pronounced, effects as ATP. Orthophosphate inhibits the photocleavage at the 23- and 74-kDa sites with a similar efficiency. In the ternary actin-S-1-ATP complex, the photocleavage is inhibited at all sites, and the effects of actin and ATP are additive. Photocleavages affect the K^+(EDTA)-, Ca^{2+}-, and actin-activated ATPase activity of subfragment 1. Loss of all three ATPases is caused by cleavage at the 23-kDa site, while the cut at the 74-kDa site only leads to the loss of actin-activated ATPase activity. It is concluded that subfragment 1 contains at least two distinct phosphate binding sites, the first being part of the "consensus" ATP binding site wherein the 23-kDa photocleavage site is located. This site is responsible for the binding and hydrolysis of ATP. It is possible that the 31-kDa cleavage site is also associated with the "consensus" site through a loop. The 74-kDa cleavage site is a part of another phosphate binding site which may play a role in the regulation of the myosin-actin interaction.

Myosin is a ubiquitous protein in eukaryotes, responsible for biological motility and muscle contraction by coupling the actin interaction with the hydrolysis of ATP. Distinct ATP and actin binding sites reside on the head segment of myosin, named subfragment 1 (S-1).[1] Because of the biological significance of the myosin-ATP and myosin-actin interactions, it is essential to characterize the respective binding sites of ATP and actin on S-1. The finding that the S-1 heavy chain can be cleaved into three trypsin-resistant fragments [27, 50, and 20 kDa, aligned in this order from the N-terminus (Balint et al., 1978; Mornet et al., 1979)] provided a convenient framework for the localization of the binding sites.

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[1] Abbreviations: S-1, subfragment 1 of myosin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTE, dithioerythritol; IAA, iodoacetamide; HC, heavy chain; LC, light chain; LB, line-broadening parameter for the exponential multiplication of free induction decay (FID).

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The location of an actin binding site on S-1 was first attempted by covalently cross-linking F-actin to S-1 in the presence of the zero-length cross-linker EDC (Mornet et al., 1981; Sutoh, 1983). By this method, actin was shown to bind to a region of the 50-kDa fragment located within 5 kDa from the C-terminus of the fragment and to the N-terminal region of the 20-kDa fragment. Chaussepied and Morales (1988) have shown, using an "anti-peptide" directed against the connector region between the 50- and 20-kDa fragments, the involvement of this latter region in actin binding. Suzuki et al. (1987) reported, by using a synthetic heptapeptide corresponding to the sequence around the SH_1 thiol, that this sequence may also participate in actin binding. Finally, we have recently shown that the positively charged Arg-143 to Arg-147 sequence on the 27-kDa fragment also may be an actin binding site (Muhlrad, 1989; Dan-Goor & Muhlrad, 1990).

An early study of the binding of an ATP photoaffinity analogue (Szilagyi et al., 1979) first suggested that the substrate binding site of S-1 resides on the 27-kDa fragment but the more precise location of the site was deduced by Walker et al. (1982) from a sequence homology analysis noting that a characteristic GXXXXGK corresponds to 178–185. The deduction of Walker et al. has recently been supported by the studies of Cremo et al. (1989), who found that the "phosphate analog", vanadate (coupled with UV irradiation), promotes cleavage at Ser-180. It is interesting that sites other than Walker's "A" (or consensus) sequence have been implicated as either forming or being near to the substrate binding site. For example, Mahmood and Yount (1984) found that an ATP photoaffinity analogue with azido attached to the ribose moiety actually attaches to the 50-kDa fragment. Walker et al. (1982) themselves pointed out a "B" ATP-associated sequence which Burke et al. (1990) have now found near the reactive thiol (Cys-707).

Recently, vanadate, which is a good structural analogue of phosphate (Lindquist et al., 1973), has been introduced to characterize the phosphate binding site of S-1 and the myosin-catalyzed ATP hydrolysis. The substitution of phosphate by vanadate has been shown to greatly stabilize the predominant intermediate of ATP hydrolysis (Goodno, 1979). The S-1 heavy chain has been shown to be cleaved by irradiation with near-UV light in the presence of vanadate at 23 and 74 kDa from the N-terminus (Mocz, 1989; Cremo et al., 1988a), and it has been assumed that the sites of the vanadate-induced cleavage actually assign the location of the phosphate binding sites. We have studied the vanadate binding of myosin and S-1 by ^{51}V NMR spectroscopy and found that the three vanadate species present in the solution at pH 7.0 all bind to the proteins; the presence ATP reduces the binding of the oligomeric species while increasing the binding of monomeric vanadate, and photocleavage occurs only when tetrameric vanadate binds to S-1 (Ringel et al., 1990). This last finding supports the conclusions reported earlier by Cremo et al. (1988a) and Cremo and Wilcott (1990). Because of the importance of vanadate in simulating phosphate binding, we decided to thoroughly characterize the vanadate-induced cleavage sites in the S-1 heavy chain, describe the effect of various cuts on S-1 function, and find out how actin, ATP, and other phosphates affect the vanadate-induced photocleavage.

Materials and Methods

Reagents. ATP, ADP, sodium pyrophosphate, sodium orthovanadate, deuterium oxide, chymotrypsin, DTE, IAA, and HEPES were best-grade products of Sigma. SDS electrophoresis and immunoblot reagents were purchased from Bio-Rad. Alkaline phosphatase conjugated antibodies were purchased from KPL Laboratories. All other chemicals were of reagent grade. A stock solution of vanadate (100 mM) was prepared according to the method of Goodno (1979).

Proteins. Myosin and actin were prepared from back and leg muscles of rabbit, according to Tonomura et al. (1966) and Spudich and Watt (1971), respectively. S-1 was prepared by digesting myosin filaments with chymotrypsin (Weeds & Taylor, 1975). S-1 and actin concentrations were estimated by assuming an $A(1\%)$ at 280 nm of 7.5 and an $A(1\%)$ at 290 nm of 6.4, respectively. Molecular masses of S-1 and actin were assumed to be 115 and 42 kDa, respectively.

Irradiation of S-1. This was carried out in a medium containing 5–8 μM S-1, 2 mM $MgCl_2$, 30 mM HEPES, pH 7.0, and 0.5–1.0 mM Vi in a 0.2–1.0-mL volume, if not stated otherwise. The samples were irradiated on ice by an UV transilluminator (U.V.P. Inc.) with a near-ultraviolet light (peak 365 nm) for 2–30 min.

SDS–PAGE. Electrophoretic analysis of the samples was carried out on 7–18% polyacrylamide gradient gels. The peptide bands were visualized by staining with Coomassie blue. Molecular masses of the peptide bands were estimated by comparing their electrophoretic mobilities with those of authentic markers. After electrophoretic separation, a gel slice containing a Coomassie blue stained band of the fragment of interest was extracted with 20% v/v pyridine in water for 24 h at room temperature, and the fragment concentration was taken to be proportional to the absorbance of the eluted dye at 605 nm.

Immunoblot. Following electrophoresis, peptide bands were transferred from the gel by a Western blot procedure to a nitrocellulose membrane. Part of the nitrocellulose was stained by the general protein stain amido black, while other parts were immunostained with mAb.25 (anti-50-kDa N-terminus) monoclonal, N3.36 (anti-S-1 N-terminus) monoclonal, and anti-20-kDa polyclonal antibodies. The monoclonal antibodies were recently characterized in our laboratory (Dan-Goor et al., 1990), and the polyclonal antibody was raised in rabbits against the 20-kDa fragment isolated from chicken skeletal S-1 (Muhlrad and Groschel-Stewart, unpublished results). The second antibodies were alkaline phosphatase conjugates of anti-mouse IgG and anti-rabbit IgG, used for the monoclonal and polyclonal antibodies, respectively. The reacting protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium by their reaction with alkaline phosphatase.

NMR Measurements. NMR measurements of ^{51}V were carried out on a Varian VXR 300s spectrometer, at 78.86 MHz, interfaced with a Sun-3 computer and equipped with a 5-mm multinuclear probe. T_1 measurements of mono-, di-, and tetravanadate reveal values of $<10^{-2}$ s, thus allowing a total accumulation time of 0.05 s per spectrum for a 90° pulse without relaxation delay. The rapid relaxation times assure the accuracy of signal integration measurements. Typically, 0.8-mL samples were used, 5000–10 000 transients were accumulated (5–10 min), the spectral width was 8 kHz, and the data memory size was 16K. A 10-Hz exponential line broadening was used before Fourier transformation was performed. The sample buffer contained 10% D_2O for locking and shimming, and the sample temperature was 18 °C. Assignments of the various vanadate signals are according to Csermely et al. (1985). The chemical shifts reported are relative to the external reference standard $VOCl_3$ (0 ppm).

Alkylation of the SH_1 Thiol of S-1 by Iodoacetamide. S-1, 45 μm in 30 mM NaCl and 10 mM HEPES, pH 7.0, was

reacted with a 2-fold molar excess of IAA at 0 °C overnight, essentially as described by Takashi et al. (1976). At the end of the incubation period, the K^+(EDTA)-activated ATPase activity of S-1 decreased to about 10% of the native S-1, while the Ca^{2+}-activated ATPase activity increased 4-fold, indicating the specific alkylation of the SH_1 thiol.

ATPase Assay. Following irradiation, vanadate was removed from the S-1 samples by fractionation with ammonium sulfate and by dialysis. S-1 was precipitated by 2.5 volumes of saturated ammonium sulfate, redissolved in 100 mM KCl and 30 mM Tris-HCl, pH 7.8, and dialyzed against 100 volumes of the same buffer from 48 h (one change) and then against 100 volumes of 30 mM Tris HCl, pH 7.8, for an additional 48 h (one change). For ATPase assays on these samples, the reaction was carried out at 25 °C in 1-mL times aliquots containing 0.1 μM S-1 and 2 mM ATP in 600 mM KCl, 50 mM Tris-HCl, pH 8.0, and either 6 mM EDTA [for K^+(EDTA)-activated ATPase] or 6 mM $CaCl_2$ (for Ca^{+2}-activated ATPase). Actin-activated S-1 ATPase was measured at 25 °C in samples containing 0.1 μM S-1, 1 μM F-actin, 2 mM ATP, 2 mM $MgCl_2$, and 20 mM imidazole hydrochloride buffer, pH 7.0. ATPase activities (micromoles of P_i per milligram of S-1 per minute) were calculated from the production of inorganic phosphate (P_i), using the Fiske–Subbarow method. During the incubation time, less than 15% of the ATP added was hydrolyzed.

Measurement of the Binding of S-1 to Actin by Sedimentation. The binding of S-1 to actin was measured by sedimenting acto–S-1 solutions under the same conditions used for the UV irradiation. The samples were centrifuged at 240000*g* at 0 °C for 20 min (Chalovich & Eisenberg, 1982). The supernatant was analyzed by SDS–PAGE. The amount of S-1 in the supernatant was estimated from the intensity of its Coomassie blue stained band. Appropriate S-1 controls were also run and were taken into account when calculating the fraction of actin bound S-1.

Results

Peptide Products of Vanadate-Induced Photocleavage of S-1. S-1 was irradiated by near-UV light in the presence of 0.5 mM Vi on ice for various time intervals, and the peptide products of the photocleavage were analyzed by SDS–PAGE (Figure 1, top). A considerable cleavage was observed even after 2 min of irradiation, and after 15 min, almost all of the S-1 heavy chain (95-kDa band) was cleaved while the LC1 and LC3 myosin light chain bands only slightly decreased in intensity. No cleavage was observed when S-1 was irradiated in the absence of Vi. The main products of cleavage of the S-1 heavy chain in order to decreasing molecular mass were an intermediate 74-kDa peptide, then bands of 51, 46, 31, 23, and 21 kDa. All of the products, except the 46- and the 31-kDa bands, have previously been reported by Mocz (1989) and Cremo et al. (1988a). In order to characterize the two newly found bands, the irradiated S-1 was analyzed by immunoblot (Figure 1, bottom). The blot was stained with three antibodies, which reacted with the N-terminus of the 50-kDa tryptic fragment, with the N-terminus of the heavy chain, and with the 20-kDa tryptic fragment, respectively. The 31-kDa band reacted with the anti-heavy-chain N-terminus and the anti-50-kDa N-terminus antibodies, indicating that this band contained a 31-kDa stretch of heavy chain starting from the N-terminus. The time course of the appearance of the various photocleavage products of the S-1 heavy chain was recorded (Figure 2); the cleavage scheme deduced is outlined in Figure 3. In the beginning, the concentration of the 74-kDa band was high, but after 2 min of irradiation, its molar fraction started to decrease, implying that this band is an intermediate product of the photocleavage. Accompanying the 74-kDa band

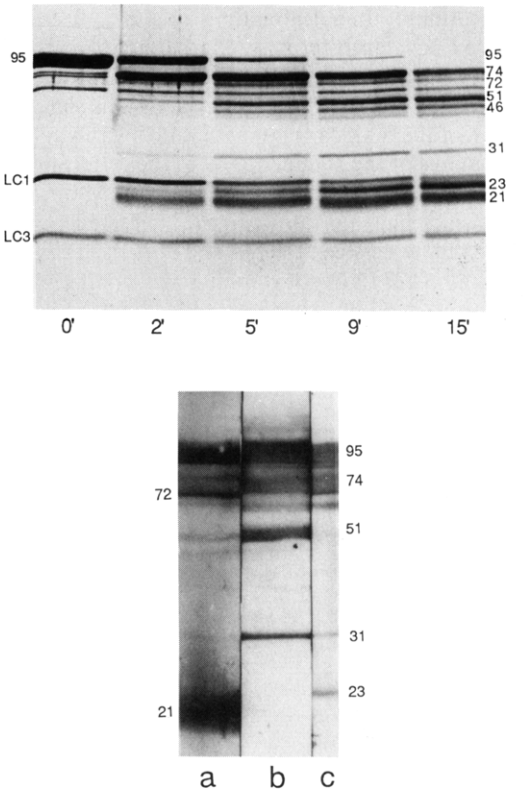

FIGURE 1: SDS gel electrophoretogram and immunoblot of S-1 following vanadate-induced photocleavage. 8 μM S-1 was irradiated in 0.5 mM Vi, 2 mM $MgCl_2$, and 30 mM HEPES, pH 7.0, and analyzed by SDS–PAGE (top) as described under Materials and Methods. Vertical numbers, molecular masses in kilodaltons: LC1 and LC3, light chain 1 and 3, respectively; horizontal numbers, time of irradiation in minutes. A sample taken after 5 min of irradiation was transferred from the gel to nitrocellulose and analyzed by immunoblot (bottom) as described under Materials and Methods. The nitrocellulose was immunostained by anti-20-kDa (a), anti-50-kDa N-terminus (b), and anti-S-1 N-terminus (c) antibodies.

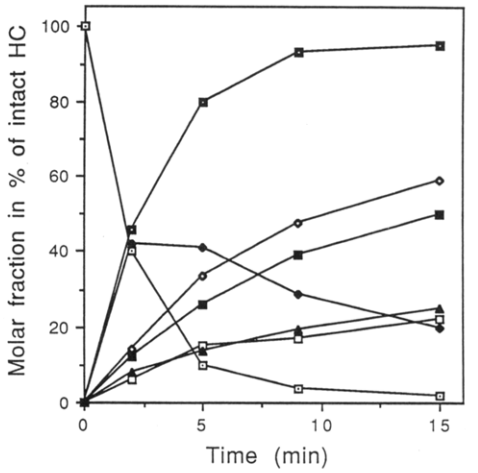

FIGURE 2: Time course of the formation of the cleavage products of the S-1 heavy chain following near-UV irradiation. The Coomassie blue stained bands of the S-1 heavy-chain fragments were cut off from the gel shown in Figure 1 and extracted by 20% pyridine as described under Materials and Methods. The concentrations of the fragments were taken to be proportional to the absorbance of the eluted dye and expressed as the molar fraction (in percent) of the intact S-1 heavy chain (HC) by taking into account the molecular mass of each fragment (ordinate). The concentration of the intact heavy chain before the irradiation was normalized to 100. Irradiation time is given on the abscissa. Symbols: 95-kDa heavy chain (⊡); 74- (♦), 51- (■), 46- (□), 31- (▲), 23- (◊), and 21-kDa (◘) fragments.

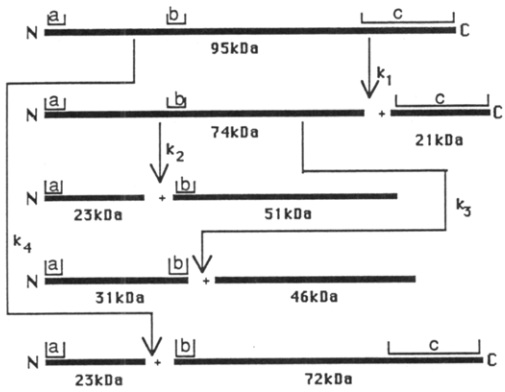

FIGURE 3: Scheme of vanadate-induced photocleavage of the S-1 heavy chain. Fragments are labeled by thick lines and the reactions (k_1–k_4) by thin lines. The top thick line is the 95-kDa intact heavy chain. Above the thick lines, the letters and the inverted bracket assign the epitopes of the anti-S-1 N-terminus (a), anti-50 kDa N-terminus (b), and anti-20-kDa (c) antibodies.

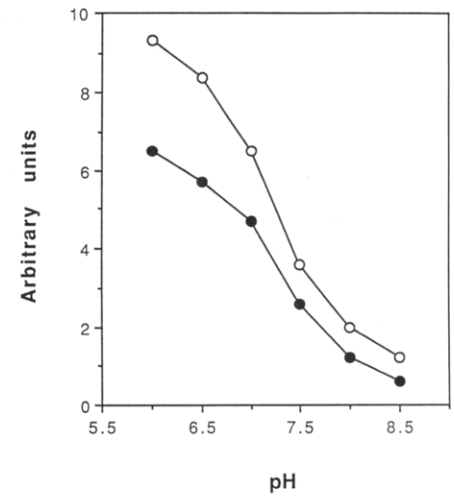

FIGURE 4: Effect of pH on the appearance of 31- (●) and 46-kDa (○) fragments during vanadate-induced photocleavage of S-1. S-1 (8 μM) was irradiated in 0.5 mM vanadate, 2 mM $MgCl_2$, and 30 mM HEPES buffer at each pH and analyzed by SDS–PAGE as described under Materials and Methods. Concentrations are presented in arbitrary units normalized to the concentration of intact heavy chain before photocleavage (100 arbitrary units).

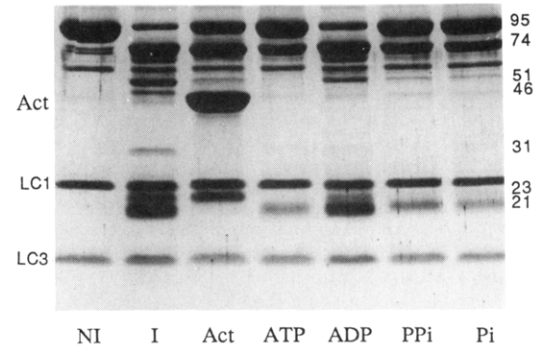

FIGURE 5: SDS gel electrophoretogram of S-1 following irradiation in the presence of actin, ATP, ADP, PP_i, or P_i. S-1 (8 μM) was irradiated for 9 min in 0.5 mM Vi, 2 mM $MgCl_2$, and 30 mM HEPES, pH 7.0. 20 μM F-actin (Act), 2 mM ATP, 2 mM ADP, 2 mM PP_i, and 40 mM P_i were also added when indicated at the bottom of the figure. Nonirradiated (NI) and irradiated S-1 without addition of the ligands (I). Vertical numbers are molecular masses in kilodaltons; Act, actin; LC1 and LC3, light chain 1 and 3, respectively.

there emerged the 21-kDa band, which according to the results of the immunoblot contained the C-terminal region of the S-1 heavy chain [see also Mocz (1989)]. This band is also produced by photocleavage of the S-1 heavy chain, 74 kDa from the N-terminus (k_1 reaction). The 23-kDa band (k_2) appeared at a lower rate; it contained the 1–180 sequence of the S-1 heavy chain (Cremo et al., 1989); accordingly, it reacted only with the anti-S-1 N-terminus antibody (Figure 1, bottom). The 51-kDa band was generated mainly from the 74-kDa intermediate fragment, following cleavage of its 23-kDa N-terminal region. The 46- and 31-kDa bands (k_3) emerged together at the slowest rate, and, therefore, they appeared as relatively minor bands. Their parallel appearance shows that they derive from a common precursor. Again, this precursor is the 74-kDa fragment, cleaved in this case at 31 kDa from the N-terminus. In addition to the main route of the cleavage (k_1, k_2, k_3 reactions), there also was a minor route. In this route, the heavy chain was first cleaved at the 23-kDa junction (k_4). This became the major route in the presence of actin when the cleavage at the 74-kDa/21-kDa junction was inhibited. The 72-kDa fragment can be further cleaved at 21 kDa from the C-terminus, producing 51- and 21-kDa fragments.

Effect of pH on Vanadate-Induced Photocleavage. The Vi-induced photocleavage of S-1 was studied in the pH range 6.0–8.5, using 0.5 pH unit increments in HEPES buffer. The extent of the photocleavage decreased slightly with increasing pH as judged by quantitative evaluation of the SDS–PAGE (data not shown). This phenomenon is probably caused by the decrease of the fraction of tetravanadate species with increasing pH [see also Cremo and Wilcott (1990)]. The effect was most conspicuous in the case of the 46- and 31-kDa bands (Figure 4). These bands were relatively intense at pH 6.0 and were essentially eliminated at pH 8.5. The pH dependencies of the production of the two bands are parallel and resemble an ionization curve with a pK of 6.8.

Effect of ATP, ADP, PP_i, P_i, and Actin on Vanadate-Induced Photocleavage of S-1. Actin, ATP, and other phosphate compounds significantly influenced the photocleavage of S-1 (Figure 5). The addition of any of the ligands practically eliminates the 31-kDa and the 46-kDa bands. These bands disappear upon addition of PP_i and P_i at low concentrations, 0.2 and 1.0 mM, respectively (data not shown). ATP also strongly inhibited the cleavage at the 23-kDa/72-kDa junction and mildly inhibits at the 74-kDa/21-kDa junction. PP_i and ADP had a similar but weaker effect, while P_i moderately inhibited the photocleavage at both locations. The effect of actin, which itself did not suffer photocleavage (results not shown), is qualitatively different since it strongly protects the 74-kDa/21-kDa junction but it does not affect the cleavage at 23 kDa from the N-terminus. These qualitative findings were supported by quantitative measurements of the 23- and 21-kDa fragments during photocleavage in the presence of various ligands (Figure 6).

Since ATP and actin affected the photocleavage of S-1 differently, it was of interest to see how the cleavage proceeded when S-1 forms a ternary complex with ATP and actin. Since the affinity between S-1 and actin was strongly reduced by ATP, it was necessary to carry out the photocleavage in the presence of a large molar excess of actin over S-1 and then measure the amount of S-1 which is in ternary complex by using the sedimentation assay (see Materials and Methods). In this way, we were able to compare the photocleavage of the ternary complex to cleavage of the binary complexes actin–S-1 and S-1–ATP and to that of free S-1 (Table I). We found that in the ternary complex, the photocleavage of S-1 at both the 23-kDa/72-kDa and 74-kDa/21-kDa junctions was inhibited. Thus, the effect of ATP and actin on the photocleavage is additive.

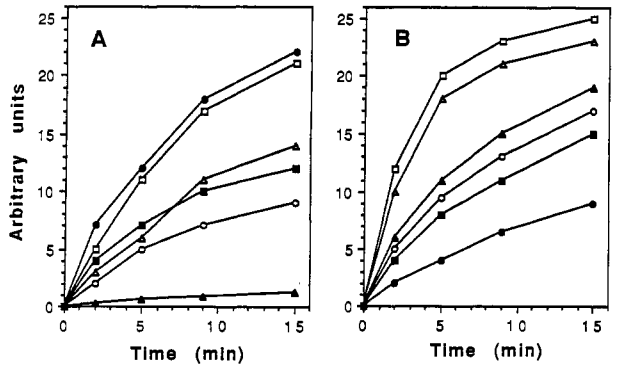

FIGURE 6: Effect of actin, ATP, ADP, PP_i, and P_i on the production of 23- (A) and 21-kDa (B) fragments during vanadate-induced photocleavage of S-1. S-1 (8 μM) was irradiated for time periods indicated on the abscissa in the presence of 0.5 mM Vi, 2 mM $MgCl_2$, and 30 mM HEPES, pH 7.0. Other additions: None (□); 20 μM F-actin (●); 2 mM ATP (▲); 2 mM ADP (△); 2 mM PP_i (○); 40 mM P_i (■). The production of the 23- and 21-kDa fragments was analyzed by extracting their Coomassie blue stained bands as described under Materials and Methods. The concentration of the fragments was expressed in arbitrary units which were normalized to that of the intact heavy chain before the irradiation (100 arbitrary units).

Table I: Vanadate-Induced Photocleavage of Free S-1, S-1–ATP, Actin–S-1, and Actin–S-1–ATP Complexes[a]

	UV cleavage products (arbitrary units[b])			
	95 kDa	31 kDa	23 kDa	21 kDa
(a) free S-1	6.9	4.6	14.2	22.4
(b) S-1–ATP	18.2		2.3	20.8
(c) actin–S-1	19.7		16.8	4.5
(d) actin–S-1–ATP	55.3		3.6	9.6

[a] S-1, 3 μM, was irradiated for 5 min in the presence of 0.5 mM Vi, 2 mM $MgCl_2$, 30 mM HEPES, pH 7.0, and 2 mM ATP to (b) and (d); 90 μM F-actin was also added to (c) and (d). The cleavage products were analyzed as described under Materials and Methods. According to the sedimentation assay (see Materials and Methods), 70% of S-1 in (d) is part of the ternary actin–S-1–ATP complex while the rest is not bound to actin. [b] Intact S-1 heavy chain (95 kDa) before the irradiation was normalized to 100 arbitrary units.

The inhibitory affect of ATP and other phosphate compounds may indicate that there is a competition between the phosphate compounds and vanadate for the phosphate binding sites on S-1. However, another possibility is that the inhibitory effect is caused by a direct reaction between the phosphate compounds and the vanadate in solution. This alternative does not seem to be valid for ATP, since the ^{51}V NMR studies indicated no interaction between ATP and vanadate in HEPES buffer, pH 7.0 (Ringel et al., 1990). However, the alternative cannot be excluded for phosphate and pyrophosphate since Gresser et al. (1986) showed by ^{51}V NMR that these compounds complex with vanadate at neutral pH and that complexation decreases with increasing pH. To study the inhibitory effect of PP_i and P_i on the photocleavage, S-1 was irradiated in the presence of 1 mM PP_i or 5 mM P_i and of 0.5 mM Vi at pH 7.0 and 8.5 (Table II). PP_i and P_i inhibited the photocleavage, and to about the same extent at both pHs. The effect of PP_i and P_i on the ^{51}V NMR spectrum was also studied under the same conditions, in the absence of S-1, (Figure 7). Addition of both PP_i and P_i significantly altered the ^{51}V NMR spectrum at pH 7.0; this alteration is mainly in the broadening and chemical shift of the monomeric vanadate signal and in a decrease in the concentration of the tetrameric vanadate. At pH 8.5, the addition of PP_i and P_i caused much less of a change in the ^{51}V NMR spectrum.

Effect of Vanadate-Induced Photocleavage on the ATPase Activity of S-1. The K^+(EDTA)-, Ca^{2+}-, and actin-activated

Table II: Effect of PP_i and P_i on Vanadate-Induced Photocleavage of S-1[a]

			UV cleavage products (arbitrary units[b])			
pH	PP_i, 1 mM	P_i, 5 mM	95 kDa	31 kDa	23 kDa	21 kDa
7.0	–	–	9.5	3.3	14.1	18.0
7.0	+	–	21.0		7.2	11.9
7.0	–	+	21.6		8.5	10.6
8.5	–	–	14.6	0.5	12.8	16.5
8.5	+	–	32.5		5.1	12.0
8.5	–	+	29.7		7.0	9.8

[a] S-1, 8 μM, was irradiated for 5 min in the presence of 0.5 mM Vi, 2 mM $MgCl_2$, and 30 mM HEPES, pH 7.0 or 8.5, and the cleavage products were analyzed as described under Materials and Methods. [b] Intact S-1 heavy chain (95 kDa) before irradiation was normalized to 100 arbitrary units.

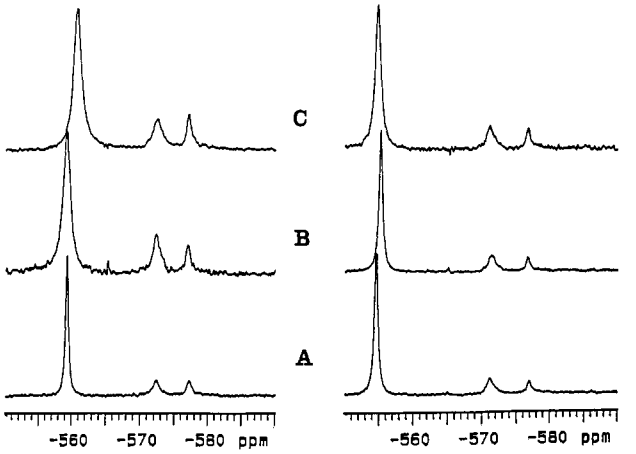

FIGURE 7: ^{51}V NMR spectra of vanadate in S-1 buffer at pH 7.0 and 8.5, in the absence or presence of P_i and PP_i. ^{51}V NMR spectra of 0.5 mM Vi samples at pH 7.0 (left) and pH 8.5 (right). (A) S-1 buffer alone (2 mM $MgCl_2$/30 mM HEPES); (B) S-1 buffer + 1 mM PP_i; (C) S-1 buffer + 5 mM P_i.

ATPase activities of S-1 were measured simultaneously with the cleavages at the 23-kDa/72-kDa and 74-kDa/21-kDa junctions (Figure 8A). The K^+(EDTA)- and even more the actin-activated ATPase activities sharply decreased, while the Ca^{2+}-activated ATPase activity first increased and then decreased following the irradiation. After 30 min, all of the activities neared zero. The rate of loss in actin- or K^+-(EDTA)-activated ATPase was faster than the rate of cleavage at both sites. When the photocleavage was carried out in the presence of ATP, the loss in K^+(EDTA)-ATPase and the cleavage at 23 kDa from the N-terminus became very slow and proceeded hand in hand (Figure 8B). This suggested that the loss in K^+(EDTA)-activated ATPase is caused by cleavage at the 23-kDa/72-kDa junction. Similarly, the loss in actin-activated ATPase activity of S-1 and the cleavage at 21 kDa from the C-terminus proceeded hand in hand, suggesting that this latter cleavage causes the loss in actin-activated ATPase. Actin was found to decrease significantly the rate of photocleavage at the 74-kDa/21-kDa junction (Figure 8C). This did not affect the time course of the loss in the K^+(EDTA)- and Ca^{2+}-activated ATPase activities. Only the rate of loss of the actin-activated ATPase of S-1 became slower. This indicates that the 74-kDa/21-kDa junction plays a role in actin binding in the presence of ATP. The observed simultaneous activation of the Ca^{2+}-activated ATPase and the inhibition of K^+(EDTA)-activated ATPase is a characteristic response to the modification of the SH_1 thiol of S-1 (Sekine & Kielley, 1964). Since this modification may take place during the irradiation of S-1, we tested how the protection of the SH_1

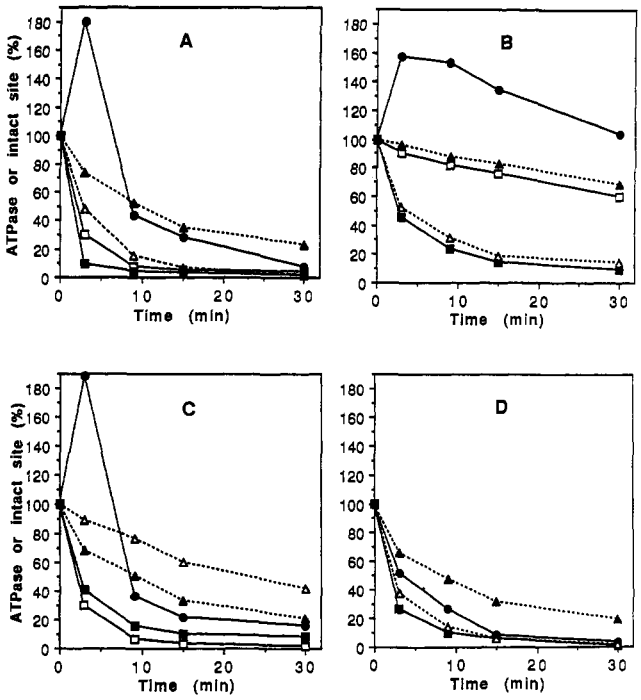

FIGURE 8: Effect of vanadate-induced photocleavage on the ATPase activity of S-1. S-1 (5 μM) was irradiated in 0.5 mM Vi, 2 mM $MgCl_2$, and 30 mM HEPES, pH 7.0, for the periods of time indicated on the abscissa. Other additions: none (A and D); 2 mM ATP (B); 20 μM F-actin (C). IAA-S-1 was irradiated in (D). After the photocleavage, Vi was removed from the solution. Photocleavage was followed by extracting the stained bands of the heavy-chain fragments, and the percent of intact, uncleaved, sites at 23 (▲) and 74 kDa (△) from the N-terminus was assessed from the production of the 23- and 21-kDa fragments, respectively. Actin- (■); K^+(EDTA)- (□), and Ca^{2+}-activated (●) ATPase activities of S-1 were expressed as a percent of the nonirradiated control. For details of all procedures, see Materials and Methods.

thiol affects the changes in the ATPase activities. After the photocleavage, we added 1 mM DTE to the reaction mixture in order to regenerate thiols which may have been oxidized during the irradiation and then assayed the ATPase activities. The effect of photocleavage on the ATPase activities was not influenced by the addition of DTE (data not shown). However, one cannot exclude the possibility that the SH_1 thiol oxidized irreversibly during the photocleavage and, therefore, the addition of DTE after the photocleavage did not affect the ATPase activity. In order to avoid the oxidation, we alkylated the SH_1 thiol with IAA, before the irradiation (Takashi et al., 1976). This significantly increased the Ca^{2+}-activated ATPase activity and reduced the K^+(EDTA)-activated ATPase activity. Any further modification of other S-1 functionalities is known to decrease the elevated Ca^{2+}-activated ATPase activity (Sekine & Kielley, 1964; Takashi et al., 1982). The vanadate-induced photocleavage of IAA-modified S-1, both at the 23-kDa/72-kDa and at the 74-kDa/21-kDa sites, was found to proceed similarly to the cleavage of the unmodified, native S-1, and the actin- and Ca^{2+}-activated ATPases decreased sharply after the onset of irradiation (Figure 8D). These latter findings support the notion that the change in the ATPase activities is not due to the modification of the SH_1 thiol, since it was protected in IAA-S-1, but to the photocleavage taking place at specific sites in the molecule.

Discussion

The principal aim of this paper was to characterize the phosphate binding sites of S-1 by taking advantage of the finding that near-UV irradiation, in the presence of vanadate, cleaves S-1 at specific sites. Since vanadate is a good structural analogue of phosphate (Lindquist et al., 1973), it is assumed that the cleavage sites participate in forming the phosphate binding sites of S-1. This assumption is supported by the observation that the cleavage at all three sites is suppressed by orthophosphate or polyphosphate. It seems that there are only two independent cleavage sites on S-1 located at 23 and 74 kDa from the N-terminus, whose cleavage is specifically inhibited by ATP and actin, respectively. This indicates two potential distinct phosphate binding sites within the S-1 heavy chain. The cleavage at the 31-kDa site took place independently of the 74-kDa/21-kDa junction, when 100 μM S-1 was irradiated in the presence of 200 μM vanadate (Ringel et al., 1990). However, it seems that the cleavage at 31 kDa depends on the cut at 23 kDa since we did not find conditions wherein the cut at 31 kDa occurred without a cut at 23 kDa from the N-terminus, or when the 23-kDa cut was suppressed without also suppressing the cut at the 31-kDa site. So we cannot exclude the possibility that the 23- and 31-kDa cleavage sites are parts of the same phosphate binding site, i.e., and that a loop exists between the two sites in the S-1 structure. Actually, the existence of a loop in this region was predicted by the model of Botts et al. (1989). Independent evidence exists which shows that the 23-kDa site is part of a phosphate binding site of S-1 since the cleavage at this site is assigned to Ser-180 (Cremo et al., 1989), i.e., a site which is located inside the "consensus" ATP binding site (residues 178–185) of S-1 (Walker et al., 1982). However, this is the first time that a site at 31 kDa from the N-terminus has been implicated in phosphate binding. By taking into account the size of the 27-kDa fragment whose C-terminus is at Lys-204 (Tong & Elzinga, 1990), the 31-kDa cleavage site should be somewhere around residue 239. This site is located in a highly conserved region of the S-1 heavy chain (Mornet et al., 1989), which suggests a possible functional significance. Former studies on vanadate-induced photocleavage of S-1 (Mocz, 1989; Cremo et al., 1988a) did not observe a change at 31 kDa from the N-terminus. The apparent lack of this cut can be explained by the higher ionic strength and/or pH used in these studies which according to our experience reduces the photocleavage at the 31-kDa site. We tried to assign the cleavage site by sequencing the 46-kDa fragment which is also generated by this cut, but found that the N-terminus of the fragment was blocked. Cleavage at the 31-kDa site is pH sensitive, and the pH dependence has the shape of an ionization curve with a pK of 6.8. The pK for deprotonation of imidazolium groups in proteins is between 6.0 and 7.0. It is plausible to assume that the photocleavage at the 31-kDa site only takes place when the imidazolium side chain of a specific histidine is unprotonated. The nearest histidine to the assumed cleavage site is His-253 (Tong & Elzinga, 1990) which is about 25 residues downstream in the sequence. This means that there is no histidine in the immediate neighborhood of the cut, but it does not exclude the proximity of a histidine to this place, in the three-dimensional structure of S-1.

Phosphate-containing compounds were found to inhibit the vanadate-induced photocleavage at all three sites. According to ^{51}V NMR measurements, ATP and ADP do not react with vanadate, therefore, it is assumed that their inhibitory effect was caused by competition between the nucleotides and the vanadate for the binding sites on S-1. P_i and PP_i do react with vanadate (Figure 7); however, the inhibitory effect of the two phosphate compounds was about the same at pH 8.5 as at pH 7.0 despite the fact that the direct interaction between the phosphates and vanadates was much less at a higher pH;

therefore, we assume that also in this case the inhibition is caused by competition between the compounds and vanadate. These results support the assumption that the sites of vanadate-induced photocleavage are parts of the phosphate binding sites of S-1. The polyphosphates, especially ATP, had a much stronger inhibitory effect on the photocleavage at the 23- and 31-kDa sites than that on the cut at 74 kDa from the N-terminus. This finding may be due to the higher affinity of polyphosphates to the 23- and 31-kDa sites (which are probably parts of a single phosphate binding site) than to the 74-kDa site. This assumption is supported by our earlier findings (Ringel et al., 1990) which showed that when 0.1 mM S-1 is irradiated in the presence of a low Vi concentration, i.e., 0.2 mM, only the 23- and 31-kDa sites are cleaved due to the competition between these sites and the 74-kDa site for the tetrameric vanadate. The significant difference between the polyphosphate affinities for the two phosphate binding sites can explain the results of earlier studies (Schliesefeld & Barany, 1968; Nauss et al., 1969) which concluded that there is only one polyphosphate binding site per S-1 (or two per myosin).

Actin was found to inhibit specifically the photocleavage at 74 kDa from the N-terminus. This site was implicated in actin binding in a number of studies, including inhibition of its trypsinolysis by actin (Mornet et al., 1979; Botts et al., 1982), covalent cross-linking of S-1 to actin (Mornet et al., 1981; Sutoh, 1983), and the binding of an "antipeptide" to this region (Chaussepied & Morales, 1988). The fact that both actin and phosphates bind to the 74-kDa/21-kDa junction raises the question about the role of this site in the regulation of the actin–myosin interaction in spite of the finding that this site is unimportant in the ATPase activity of S-1 (Chaussepied & Morales, 1988).

The photocleavage of the actin–S-1–ATP ternary complex was also studied. In this complex, the cleavage at all three sites of S-1 was highly suppressed, and the effect of actin and ATP was additive. ATP was found to inhibit the cleavage at 23 kDa from the N-terminus with the same efficiency, in both free S-1 and acto–S-1 in spite of the structural differences between the two forms. The similarity of the ATP effect is probably due to the direct competition between ATP and vanadate for the 23-kDa site, which constitutes a part of the "consensus" ATP binding site, both in free S-1 and in acto–S-1.

We observed, in agreement with Grammer et al. (1988) and Mocz (1989), that vanadate-induced photocleavage significantly affects the ATPase activity of S-1. The K^+(EDTA)- and actin-activated ATPase activities of S-1 sharply decreased upon photocleavage, and the rate of their decrease was higher than the rate of cleavage at either 74 or 23 kDa from the N-terminus. The Ca^{2+}-activated ATPase first increased and then dropped during the course of the irradiation. By regenerating reactive thiols with DTE after the photocleavage and by irradiating IAA–S-1 in the presence of vanadate, we excluded the posibility that the observed changes in the ATPase activity of S-1 were due to the modification of the SH_1 thiol. When the photocleavage was performed in the presence of ATP, the rate of loss in the K^+(EDTA)-activated ATPase activity and the rate of cleavage at 23 kDa from the N-terminus were considerably reduced, and the two curves run parallel to each other. This suggests that the loss in K^+-(EDTA)-activated ATPase is caused by the 23-kDa cut. However, this does not explain why the Ca^{2+}-activated ATPase first increases before it starts to decrease and why the rate of loss in K^+(EDTA)-activated ATPase is higher than the rate of cleavage at the 23-kDa site. One can interpret these results on the basis of the findings of Grammer et al. (1988) and Cremo et al. (1988b), who showed that the photocleavage of the S-1–MgADP–Vi complex at 23 kDa from the N-terminus proceeds in two steps. The first step is the photomodification of a serine residue (Ser-180), which is accompanied by both an increase in the Ca^{2+}-activated and a decrease in the K^+-(EDTA)-activated ATPase activities. The second step is the actual photocleavage accompanied by the loss of both ATPase activities. Accordingly, we assume that after 3 min of irradiation, a large fraction of the S-1 molecules is already photomodified but only a small fraction is cleaved at the 23-kDa site and, therefore, there are an increased Ca^{2+}- and a decreased K^+(EDTA)-activated ATPase activities. As the irradiation continues, more and more S-1 molecules get cleaved at the 23-kDa site, which leads to the drop in the Ca^{2+}-activated ATPase activity. The finding that not only the photocleavage but also the decrease in K^+(EDTA)-activated ATPase activity is strongly inhibited by ATP indicates that both the photomodification of the Ser-180 and the photocleavage are suppressed by ATP. The fast drop in actin-activated ATPase activity of S-1 during the course of irradiation is due both to the damage in the "consensus" ATP site at 23 kDa and to the cleavage at 74 kDa from the N-terminus. The effect of the cleavage at the 74-kDa/21-kDa site can be easily seen when the photocleavage of S-1 is carried out in the presence of ATP (Figure 8B). In this case, both the photomodification and the cleavage at the 23-kDa site are suppressed, and the decrease in actin-activated ATPase runs parallel with the cut at the 74-kDa site. The loss of actin-activated ATPase upon cleaving S-1 in this region is well-known and was first reported by Mornet et al. (1979) by trypsinolysis at 75 kDa from the N-terminus. The reason for the loss in actin-activated ATPase upon this cut is the decreased affinity between actin and split S-1 in the presence of ATP (Botts et al., 1982). These results support the notion [also expressed in the recent preliminary report of Cremo and Wilcott (1990)] that the photocleavage at 74 kDa from the N-terminus ruptures the normal interactions between S-1 and actin in the presence of ATP.

In conclusion, we found that near-UV irradiation in the presence of vanadate cleaves S-1 at three specific sites. The cleavage at 23 kDa from the N-terminus is protected by ATP and other phosphates, and the cut leads to the total loss of all three ATPase activities. The second cut is at 31 kDa from the N-terminus, and it can be eliminated by increasing the pH above 8.0, by actin, and by low concentration of phosphates. It is possible that the 23- and 31-kDa sites are parts of the same phosphate binding site. The third cleavage site is located at 74 kDa from the N-terminus. The photocleavage at this site is suppressed by actin and phosphates, and the cut leads to the loss of actin-activated ATPase. This site probably participates in the formation of a second independent phosphate binding site whose existence was first indicated by Chaussepied et al. (1986), and it may play a role in the regulation of the myosin–actin interaction.

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Registry No. ATP, 56-65-5; ADP, 58-64-0; ATPase, 9000-83-3; vanadate, 14333-18-7; pyrophosphate, 14000-31-8; orthophosphate, 14265-44-2.

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