Alteration of the ATP Hydrolysis and Actin Binding Properties of Thrombin-Cut Myosin Subfragment 1†

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ABSTRACT: We have characterized various structural and enzymatic properties of the (68K–30K)-S-1 derivative obtained by thombic cleavage [Chaussepied, P., Mornet, D., Audemard, E., Derancourt, J., & Kassab, R. (1986) *Biochemistry* (preceding paper in this issue)]. The far-ultraviolet CD spectra and thiol reactivity measurements indicated an unchanged overall polypeptide conformation of the enzyme whereas the CD spectra in the near-ultraviolet region suggested a local change in the environments of phenylalanine side chains; the latter finding was rationalized by considering the existence of about five of these amino acids in the vicinity of the cleavage sites. When the binding of Mg^{2+}-ATP and Mg^{2+}-ADP to the derivative was assessed by CD spectroscopy, distinct spectra were obtained with the two nucleotides as with native subfragment 1 (S-1), but some spectral features were unique to the nicked S-1. Stern–Volmer fluorescence quenching studies using acrylamide and the analogues 1,N^6-ethenoadenosine 5′-triphosphate and 1,N^6-ethenoadenosine 5′-diphosphate indicated that the complexes formed with the modified S-1 have a solute quencher accessibility close to that observed for the complexes with the normal S-1. However, in contrast to the parent enzyme, the thrombin-cut S-1 was unable to bind irreversibly Mg^{2+}-ATP, nor did it form a stable Mg^{2+}-ADP–sodium vanadate complex or achieve the entrapping of Mg^{2+}-ADP after cross-linking of SH_1 and SH_2 with *N,N′-p*-phenylenedimaleimide. Additionally, the amplitude of the P_i burst was very low, indicating that the inactivation of the proteolyzed S-1 was linked to the suppression of the hydrolysis step in the ATPase cycle. The binding constant of actin for the derivative was decreased at least 250-fold both in the rigor state and in the presence of Mg^{2+}-ADP, but it was unaffected in the presence of Mg^{2+}-ATP, which still dissociated the acto–(68K–30K)-S-1 complex. Only the COOH-terminal 30K fragment was cross-linked by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide to a single actin but not at all the NH_2-terminal 68K segment, which contains most of the 50K heavy chain moiety. Because the clip site at Lys^{560}-Ser^{561} is sequentially located in the proposed flexible loop within the S-1 heavy chain [Mornet, D., Ue, K., & Morales, M. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1658–1662], we conclude that the native folding of this region is essential both for the conformational transitions involved in the tight binding of Mg^{2+}-ATP and Mg^{2+}-ADP–P_i and for the firm attachment of actin to the myosin head heavy chain.

We have described the limited proteolysis of skeletal chymotryptic myosin S-1[1] by thrombin and chymotrypsin as a process made possible by the prior reversible blocking with 5,5′-dithiobis(2-nitrobenzoic acid) of certain surface thiols within the head molecule. The use of thrombin, the most specific protease as compared to chymotrypsin or trypsin, led to the production of a nicked S-1 form, referred to as (68K–30K)-S-1, whose heavy chain is only cut within the 50K segment at a primary site between Lys-560 and Ser-561 and to a limited extent at a secondary site between Lys-553 and Tyr-554 (Chaussepied et al., 1986). The cleaved –SH-free S-1 species exhibited very low ATPase activities. We suggested that the native folding of the polypeptide chain including the clip sites was somehow required for the maintenance of an active conformation of the ATPase site. Because the scission of the S-1 heavy chain was restricted to its 50K region, the nicked S-1 species appears valuable in elucidating structure–function relationships of the largest, central 50K heavy chain portion, which is known to be concerned with the binding of actin and nucleotides to S-1 (Mornet et al., 1981a; Sutoh, 1983; Mahmood & Yount, 1984). Previously, extensive studies on the trypsin-digested skeletal S-1 were quite useful in delineating the importance of the "connector segments" of the heavy chain in the enzyme activity (Mornet et al., 1979; Chaussepied et al., 1983).

In the present work, we investigated several functional and conformational properties of the isolated thrombin-cut (68K–30K)-S-1. The results show that while the overall polypeptide structure of S-1 was maintained in the derivative, the binding of Mg^{2+}-ATP is associated with the suppression of the hydrolytic step of the ATPase cycle and with the inability of the modified enzyme to undergo the transition toward the stable

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[1] Abbreviations: S-1, myosin chymotryptic subfragment 1; acto–S1, actomyosin–S-1; ATP, adenosine 5′-triphosphate; ϵ-ADP, 1,N^6-ethenoadenosine 5′-diphosphate; ϵ-ATP, 1,N^6-ethenoadenosine 5′-triphosphate; pPDM, *N,N′-p*-phenylenedimaleimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; IAEDANS, *N*-(iodoacetyl)-*N′*-(5-sulfo-1-naphthyl)-ethylenediamine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; Mops, 4-morpholinepropanesulfonic acid; V_i, sodium vanadate; DTE, dithioerythritol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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M–ADP–P_i state. Furthermore, the interaction of the cut S-1 with actin weakened in the rigor state and in the complex containing Mg^{2+}-ADP but was unchanged in the presence of Mg^{2+}-ATP. On the other hand, the cross-linking of actin to the 50K heavy chain segment in the acto–(68K–30K)–S-1 rigor complex was totally abolished. Together, the data suggested that the conformation of the region around the clip site is important for the tight binding and/or hydrolysis step of ATP and for the firm attachment of actin to the myosin head.

Materials and Methods

Materials. Thrombin from bovin plasma was from Serva; α-chymotrypsin (code C.D.S.) and trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl acetone were purchased from Worthington Biochemicals. ϵ-ATP and ϵ-ADP were obtained from Pharmacia (Uppsala). All other chemicals were of the highest analytical grade. Stock solutions of vanadate (V_2O_5) were prepared according to Goodno (1979).

Preparation of Proteins and S-1 Derivatives. Rabbit skeletal myosin was prepared according to Offer et. al. (1973). Chymotryptic S-1 was obtained as described by Wagner and Weeds (1977) and was labeled with 1,5-IAEDANS as reported by Mornet et al. (1981a). The (68K–30K)-S-1 derivative was prepared by thrombic digestion of DTNB-modified S-1 following the procedure described by Chaussepied et al. (1986). Trypsin-split (27K–50K–20K)-S-1 was isolated as specified by Mornet et al. (1981b); its modification with DTNB and subsequent conversion by thrombic digestion into (27K–40K–20K)-S-1 were conducted as for S-1 (Chaussepied et al., 1986). pPDM–S-1 was prepared according to Wells and Yount (1979). F-Actin was prepared as in Eisenberg and Kielly (1974) and was labeled by 1,5-IAEDANS as reported by Takashi et al. (1976).

Concentrations were estimated by using $A^{1\%}_{280nm} = 7.5$ (Wagner & Weeds, 1977) for the native S-1 and $A^{1\%}_{278nm} = 11$ (West et al., 1967) for F-actin. The concentration of thrombin-split S-1 were determined by the Bradford assay (Bradford, 1976) with S-1 as the standard. Calculations were based on molecular weights of 115 000 for S-1 (Mornet et al., 1981b) and 42 000 for actin (Collins & Elzinga, 1975).

Thiol Titrations. The reactivity of thiols in S-1 and (68K–30K)-S-1 was measured by monitoring at 412 nm the time course of the reaction of the proteins (4 mg/mL), with DTNB (30-fold excess over S-1) in 100 mM Hepes buffer, pH 8.0 at 4 °C.

Spectral Procedures. Fluorescence measurements were carried out with a Hitachi Perkin-Elmer Model MPF-4 spectrofluorometer thermostated at 15 °C. The pathway of the CD cell was 5 mm. The interaction of ϵ-ATP and ϵ-ADP with S-1 and (68K–30K)-S-1 was studied by the quenching of fluorescence of the free nucleotide with acrylamide as described by Ando et al. (1982) and by Rosenfeld and Taylor (1984). The Stern–Volmer constant (K_{sv}) was determined from the plot

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescence in the absence and in the presence of quencher, respectively, [Q] is the concentration of acrylamide, and K_{sv} is the constant of the collisional quenching, the static constant being negligible in this system. The range of acrylamide concentration (0–100 mM) led us to use eq 1 because we can consider that there is only one class of fluorophore (Rosenfeld & Taylor, 1984).

CD measurements were performed as outlined previously (Murphy, 1974) on a computerized Jobin et Yvon Model Mark V autodichrograph. The far-ultraviolet spectra were interpreted by the simplified method of Siegel et al. (1980).

Kinetic Analyses. Quenched-flow experiments were carried out in a quenched-flow apparatus that has already been described (Barman & Travers, 1985). ATP chase and P_i burst experiments were carried out as described previously (Biosca et al., 1984). In ATP chase experiments, one titrates ATPase active sites; P_i burst experiments give information about the chemical step in that the intermediate M**–ADP–P_i is measured (Trentham et al., 1976). The experimental conditions in these experiments are given in the legend to Figure 6.

The theory of ATP chase and P_i burst experiments are interpreted with reference to the first four steps of the Bagshaw–Trentham scheme (Scheme I) (Trentham et al., 1976). For a full description of the theoretical treatment of Scheme I, the reader is referred to Trentham et al. (1976) and Barman et al. (1983).

Scheme I

$$M + ATP \underset{K_1}{\rightleftharpoons} M\text{–}ATP \xrightarrow[k_2]{} M^*\text{–}ATP \underset{K_3}{\rightleftharpoons} M^{**}\text{–}ADP\text{–}P_i \xrightarrow[k_4]{} M + ADP + P_i$$

Association of acto–S1 and acto–(68K–30K)-S-1 in the absence and presence of nucleotides was measured by sedimentation in a Beckman airfuge essentially as described by Chalovich and Eisenberg (1982). The conditions were thrombin-cut S-1 (5 μM) in 40 mM Tris-HCl buffer, pH 7.5, 5 mM KCl, 2.5 mM $MgCl_2$, and 0.5 mM DTE (25 mM ionic strength) was mixed with varying free actin concentration (0–100 μM). The mixture was then centrifuged at 178000g for 35 min at 22 °C, and the fraction of S-1 bound was estimated as follows. For native S-1, the fraction of enzyme remaining in the supernatant was determined from its K^+-ATPase activity relative to that of an S-1 standard (Chalovich & Eisenberg, 1982) and by densitometric measurements of the 95K heavy chain band present on $NaDodSO_4$ electrophoretic gels. The two methods gave results in good agreement. For the inactive (68K–30K)-S-1, the latter procedure was employed with estimation of the 68K bands relative to a (68K–30K)-S-1 standard. The tryptic digestion of S-1 and thrombin-split S-1 in the presence of nucleotides was performed as reported by Mornet et al. (1985b).

Cross-Linking Experiments. Covalent cross-linking between F-actin and thrombin-split S-1 was performed essentially by the method of Mornet et al. (1981c).

Electrophoresis. Polyacrylamide gradient gel electrophoresis (5–18%) in the presence of sodium dodecyl sulfate ($NaDodSO_4$) was carried out as reported previously (Mornet et al., 1981b). The gels were scanned with a Shimadzu Model CS930 high-resolution gel scanner equipped with a computerized integrator.

Results

Conformational Properties of Thrombin-Split S-1. The investigations to be described concerning the conformation and enzymological properties of the thrombin-generated (68K–30K)-S-1 were performed in comparison with the native S-1 and the trypsin-cut (27K–50K–20K)-S-1 used as controls. Figure 1 shows a typical gel electrophoretic pattern of the three purified proteins employed throughout this work. Unlike the tryptic S-1, the thrombin-modified enzyme bears intact connector segments and an A1 light chain, but it has lost all its ATPase activities. To assess if the overall polypeptide conformation of the thrombin derivative was affected, we com-

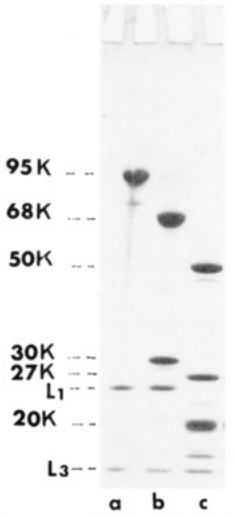

FIGURE 1: Polypeptide composition of thrombin and trypsin-fragmented S-1 preparations. Electrophoretic patterns on 0.1% Na-DodSO$_4$–polyacrylamide gels of thrombin-split (68K–30K)-S-1 (b) and trypsin-split (27K–50K–20K)-S-1 (c) prepared from native chymotryptic S-1 (A1 + A2) (a) as described under Materials and Methods.

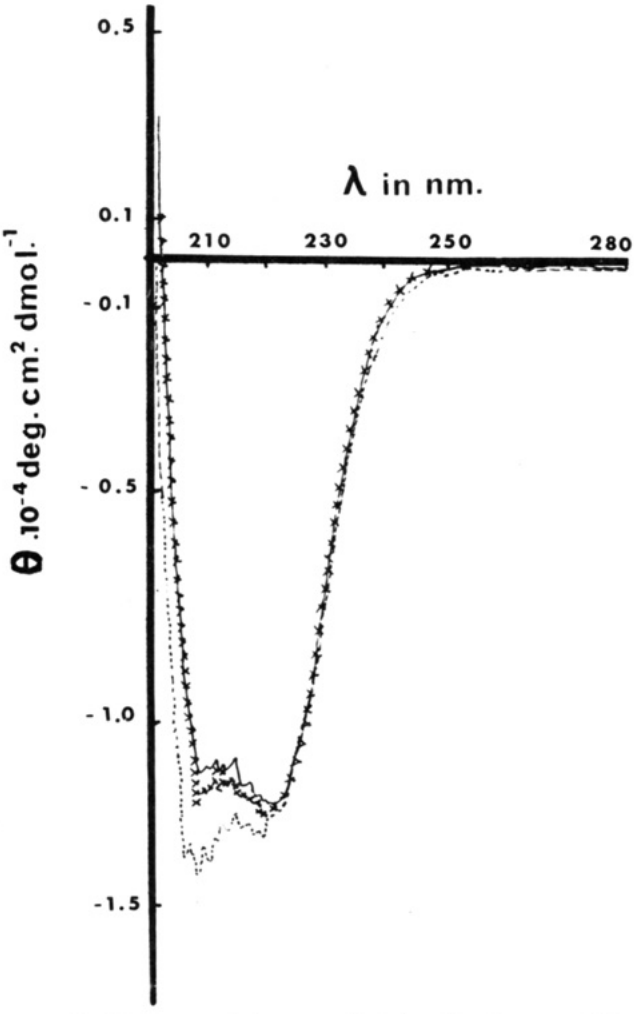

FIGURE 2: CD spectra of chymotryptic S-1 and its fragmented derivatives in the far-ultraviolet region. Comparative spectra for native S-1 (—), thrombin-cut S-1 (×), and trypsin-cut S-1 (– –). Conditions: 1 μM protein in 20 mM Tris-HCl buffer, pH 7.5 at 20 °C.

pared its far-ultraviolet CD spectrum with those obtained with native S-1 and (27K–50K–20K)-S-1. The results are illustrated in Figure 2. So far, only spectra for papain S-1 preparations have been reported (Murphy, 1974; Wu & Yang, 1976) but not for chymotryptic S-1. As shown in Figure 2, below 250 nm, both the native chymotryptic S-1 and the thrombic (68K–30K)-S-1 derivative exhibit the same minimum and intensities of elipticity near 210 nm. We determined an α-helix content according to the method of Siegel et al. (1980) of 40.8 and 40.5%, respectively. In contrast, the (27K–50K–20K)-S-1 showed reproducibly a relatively different spectrum with a significant increase of helical structure (44.8%). Thus, the S-1 secondary structure appears more sensitive to the tryptic cuts than to the selective cleavage of the heavy chain with thrombin.

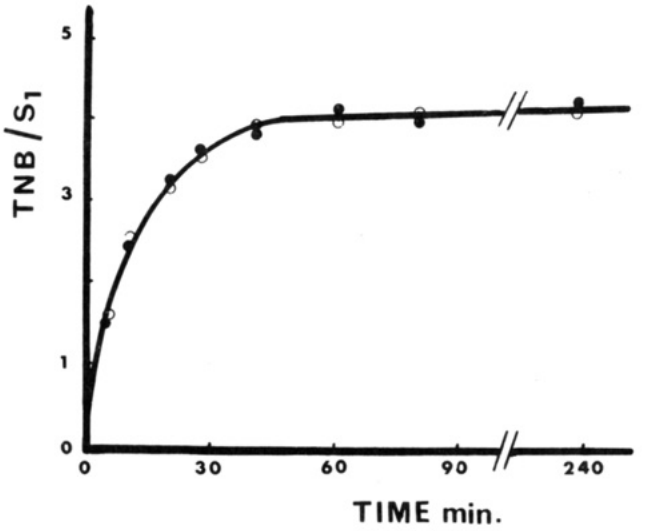

FIGURE 3: Comparative time courses of thiol titration with DTNB in native S-1 and (68K–30K)-S-1. The proteins (35 μM) were reacted with DTNB (20-fold excess) at 4 °C in 50 mM Hepes buffer, pH 8.0. The number of TNB groups released per mole of S-1 was monitored at 410 nm as described under Materials and Methods. Native chymotryptic S-1 (●); thrombin-cut (68K–30K)-S-1 (○).

These spectroscopic data of structure were further complemented by comparing the chemical reactivity of the thiols within S-1 and thrombin-split S-1 during their substitution with DTNB at pH 8.0, 4 °C. As shown in Figure 3, the amount of TNB groups released and the time course of the reaction were essentially the same for both proteins. This indicates that the proteolytic production and purification of the thrombin-cut S-1 did not apparently change the microenvironment or accessibility of the reacting thiols in spite of the fact that the proteolysis was initially mediated by the blocking of some of these thiols. Also, cross-linking experiments with dibromobimane led to the inter-thiol cross-linking between the 20K and 50K regions within the thrombin-cut S-1 (data not shown) as recently reported for native S-1 (Mornet et al., 1985a).

Interaction of Nucleotides with (68K–30K)-S-1. The critical properties of S-1 related to nucleotide binding and hydrolysis were explored with the thrombin S-1 derivative by several approaches. First, we investigated the near-ultraviolet CD spectrum of S-1, between 250 and 310 nm, which is sensitive to the interaction of ATP and ADP (Murphy, 1974). The spectrum of native chymotryptic S-1, shown in Figure 4A, exhibits features very similar to those present in the spectra previously established for papain-produced S-1 (Murphy, 1974; Burke et al., 1976; Wu & Yang, 1976). Also, as expected, the binding of Mg^{2+}-ATP and Mg^{2+}-ADP induced in each case specific changes in the spectrum, in particular near 258 and 282 nm. The spectrum recorded for (68K–30K)-S-1 is depicted in Figure 4B. Its overall shape was similar to that of the parent S-1 except for the 260-nm region, where the intensity of the peaks was significantly decreased. Moreover,

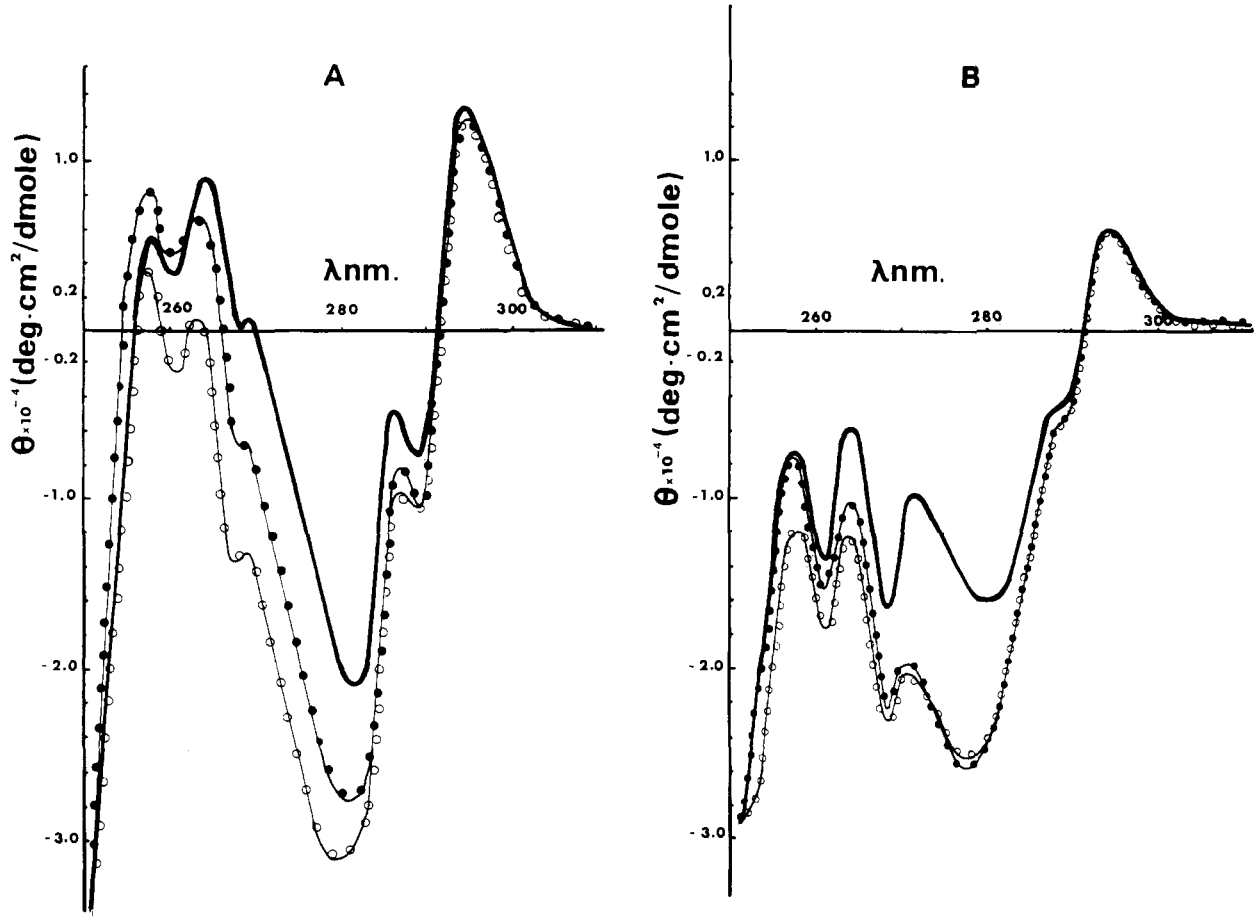

FIGURE 4: Comparative near-ultraviolet CD spectra of chymotryptic S-1 and (68K–30K)-S-1 in the absence and in the presence of nucleotides. The spectra for native S-1 (A) and thrombin-cut S-1 (B) were measured with 40 μM protein in 0.2 M KCl and 50 mM Mops buffer, pH 7.0 at 20 °C; CD measurements were performed in the absence (—) and in the presence of 0.5 mM Mg^{2+}-ATP (●) or 0.5 mM Mg^{2+}-ADP (○).

the addition of ATP and ADP led also to distinct spectra as observed with the native S-1. However, in contrast to the latter case, ATP did not increase the intensity of the peak at 258 nm, and the trough at 280 nm was similar for both nucleotides whereas it was deeper for ADP than for ATP in the case of the native S-1.

In a parallel study, we used the fluorescence acrylamide quenching method to analyze the accessibility of the fluorescent nucleotides Mg^{2+}-ε-ATP and Mg^{2+}-ε-ADP bound to (68K–30K)-S-1. The titration of the fluorescence enhancement upon addition of Mg^{2+}-ε-ATP to the derivative in comparison with native S-1 is shown in Figure 5A. The analogue was bound at the enzyme active site and not at a secondary site since the subsequent addition of increasing Mg^{2+}-ATP concentrations led to a concomitant decrease of the fluorescence enhancement (results not shown). Another set of experiments allowed us to determine the Stern–Volmer constant (K_{sv}) values of 5.8 M^{-1} for thrombin-cut S-1 and 7.4 M^{-1} for native enzyme (Table I). A similar study was also undertaken for the binding of Mg^{2+}-ε-ADP (Figure 5B) and Mg^{2+}-ε-ADP–V_i (Figure 5C). For native S-1, the addition of V_i led to a significant increase in the amplitude of the plateau of the saturation curve. This was interpreted as the burying of the nucleotide into the enzyme (Rosenfeld & Taylor, 1984). No such effect was observed with the thrombin-modified S-1. The same conclusion can be drawn from the K_{sv} values of 7.5 and 7.7 M^{-1} for the Mg^{2+}-ε-ADP complexes and 4.7 and 7.5 M^{-1} for the Mg^{2+}-ε-ADP–V_i complexes corresponding to S-1 and (68K–30K)-S-1, respectively (Table I).

A key property of native S-1 is that it binds ATP tightly in a two-step process that is followed by hydrolysis and release

Table I: Stern–Volmer Constants for Complexes of ε-ATP and ε-ADP with Native S-1 and (68K–30K)-S-1[a]

	K_{sv} (M^{-1})		
	Mg^{2+}–ε-ATP	Mg^{2+}-ε-ADP	Mg^{2+}-ε-ADP–V_i
nucleotide	58.1	60.4	61.0
nucleotide–S-1 complexes	7.4	7.7	4.5
nucleotide–(68K–30K)-S-1 complexes	5.8	7.5	7.5

[a] The Stern–Volmer constants (K_{sv}) for the different etheno-containing nucleotides with or without added enzymes were obtained as described under Materials and Methods. Conditions were 40 mM Tris-HCl, 2 mM $MgCl_2$, and 5 mM KCl, pH 7.5 at 15 °C. The concentrations of the nucleotides and S-1 or (68K–30K)-S-1 were 4 and 5 μM, respectively. Acrylamide concentration was varied between 0 and 100 mM. When present, V_i was employed at 20 μM.

of products (Trentham et al., 1976). A way of studying the binding process is by the ATP chase technique: in addition to giving information on the kinetic constants involved, this method titrates S-1 ATPase sites [Barman et al. (1983) and references cited therein]. The key feature of this technique is that it only measures sites that bind tightly and then hydrolyze ATP.

As shown in Figure 6, the S-1 preparation used in our experiments gave a titration value of 0.91 mol of $[^{32}P]P_i$/mol of S-1. The steady-state value was 0.033 s^{-1}, and if one assumes that the enzyme was 91% active, k_{cat} = 0.036 s^{-1}. This is in good agreement with previous work under the same experimental conditions (Barman et al., 1983).

We now carried out an ATP chase experiment on (68K–30K)-S-1. The chase period was increased from 2 min (for

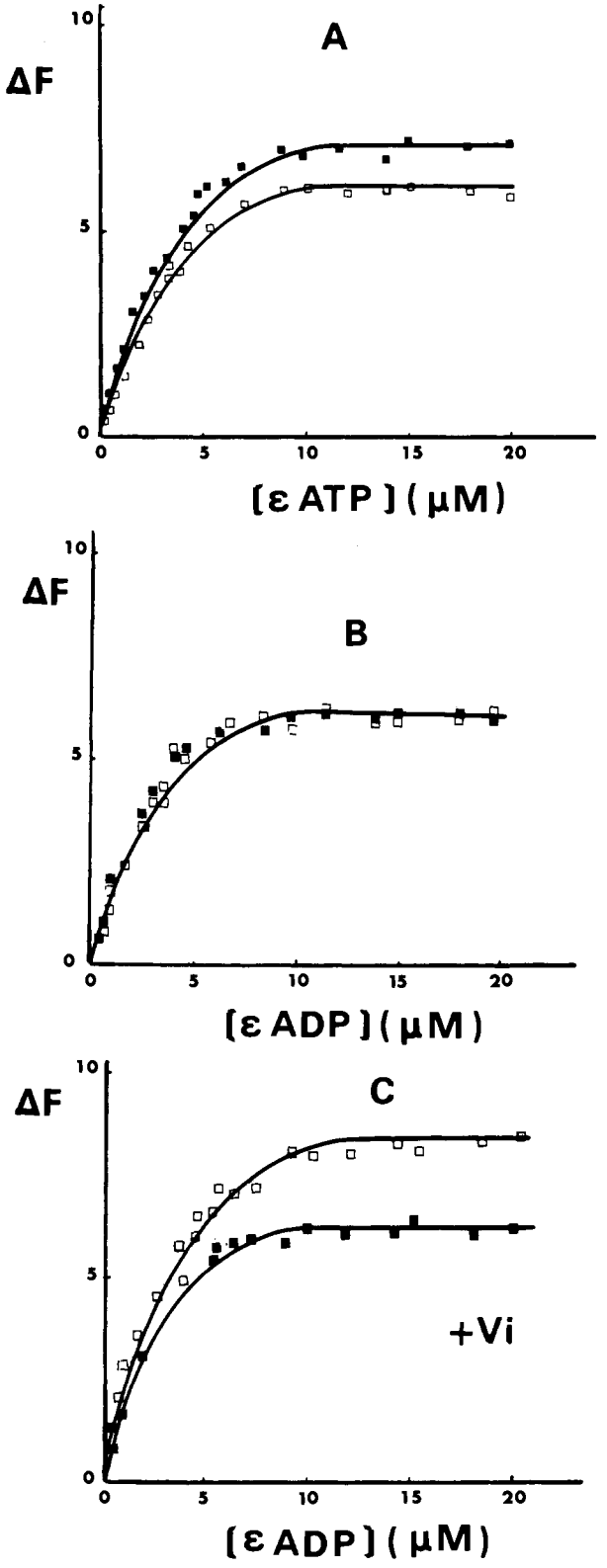

FIGURE 5: Titration of the binding of fluorescent nucleotide analogues to native S-1 and (68K–30K)-S-1. Increasing concentrations of ϵ-ATP (A), ϵ-ADP (B), and ϵ-ADP–V_i (C) at 0–20 μM were added to a solution containing 4 μM S-1 (□) or (68K–30K)-S-1 (■), 100 mM acrylamide, 40 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 5 mM KCl at 15 °C. The nucleotide fluorescence was measured at 410 nm with excitation at 320 nm.

S-1) to 30 min to take account of the low turnover of the preparation [for a discussion, see Barman & Travers (1985)]. The titration value obtained (curve not shown) corresponds to the amount of unreacted S-1 remaining (as determined by SDS gel electrophoresis). This result shows that (68K–30K)-S-1 does not bind ATP in the essentially irreversible manner of S-1 or, alternatively, that it does not hydrolyze ATP.

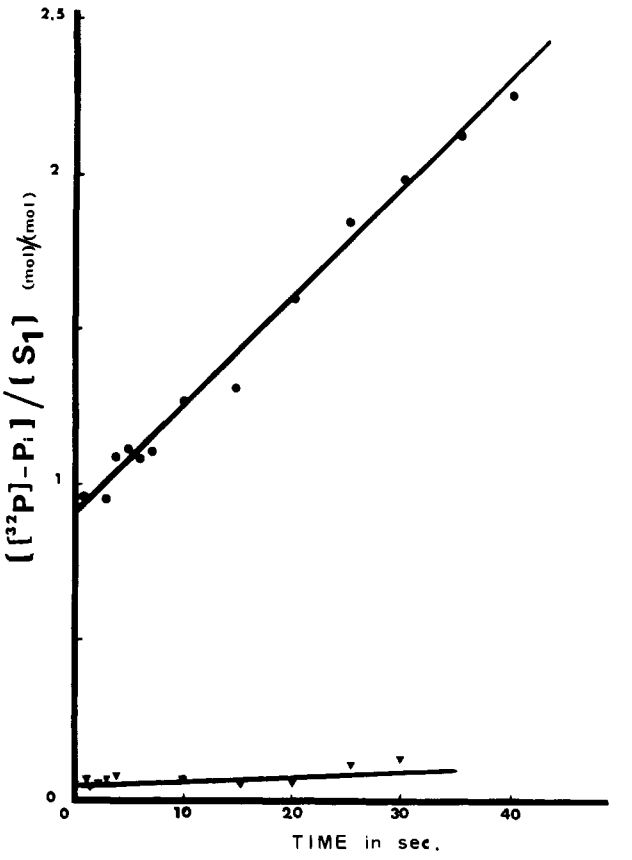

FIGURE 6: Time course for P_i burst of (68K–30K)-S-1 and titration of the ATPase site of unmodified S-1 at 15 °C. In P_i burst experiments, the reaction mixtures were 8.7 μM (68K–30K)-S-1 and 24 μM [γ-^{32}P]ATP (▼). The reaction mixtures were quenched in 22% trichloroacetic acid containing 1 mM NaH_2PO_4 at the times shown, and the [^{32}P]P_i was determined by the method of Reimann and Umfleet (1978). In titration experiments, the reaction mixtures were 2 μM S-1 plus 8 μM [γ-^{32}P]ATP (●). The reaction mixtures were quenched in 12 mM unlabeled ATP at the times shown and treated as described by Barman and Travers (1985). The buffer was 50 mM Tris base, 5 mM KCl, 2 mM magnesium acetate, and 0.1 mM DTE adjusted to pH 8.0 with acetic acid.

We next investigated if (68K–30K)-S-1 was able to hydrolyze ATP. The result of a P_i burst experiment, carried out on (68K–30K)-S-1 prepared from an S-1 titrating at 91% active, is illustrated in Figure 6. The amplitude was 0.033 (±0.005) mol of [^{32}P]P_i/mol of protein; this compares with 0.7 for S-1 (curve not shown). The steady-state value was 0.0025 (±0.00035) s^{-1}. It was important to check that this reduction in ability to hydrolyze ATP was due to the cleavage of the 68K–30K band rather than to the chemical treatment involved in the preparation of the modified protein. Thus, S-1 was treated with DTNB. The TNB groups incorporated were then removed with DTE (see Materials and Methods). When the regenerated enzyme was titrated by the ATP chase method, an amplitude of 0.73 equiv of active sites/mol of S-1 was obtained. This shows that the chemical treatment of S-1 leads to a loss of about 20% of its sites. We conclude that the more than 90% loss of the active sites of (68K–30K)-S-1 is due to the cleavage of the heavy chain.

In addition, the data shown in Table II indicate that the (68K–30K)-S-1 derivative is unable to trap magnesium nucleotides at the ATPase site either in the presence of V_i or after cross-linking of SH_1 and SH_2 with pPDM. Under both conditions, maximally only about 0.2 mol of nucleotide/mol of protein remains associated with the nicked S-1.

Table II: Comparative Trapping of ^{14}C-Labeled Nucleotides by Native S-1 and (68K–30K)-S-1[a]

	amount of trapped nucleotide[b]			controls[b]	
	Mg^{2+}-ADP–V_i	Mg^{2+}-ATP (pPDM)	Mg^{2+}-ADP (pPDM)	Mg^{2+}-ATP	Mg^{2+}-ADP
S-1	0.91	0.70	0.85	0.06	0.05
(68K–30K)-S-1	0.20	0.23	0.19	0.09	0.06

[a] In trapping assays with V_i, S-1 and (68K–30K)-S-1 were mixed with 2 mM [^{14}C]ADP-Mg^{2+} and 3 mM sodium vanadate in 50 mM Hepes buffer, pH 8.0. Trapping experiments with pPDM were performed as reported under Materials and Methods. The cross-link was assessed by the loss of the K^+- and Ca^{2+}-ATPase activities for S-1 and by thiol titration with DTNB (20-fold excess, 12 h, 4 °C) before and after the pPDM reaction for both S-1 and (68K–30K)-S-1. Controls contained the proteins mixed with 2 mM [^{14}C]ATP-Mg^{2+} or [^{14}C]ADP-Mg^{2+}. The nucleotide–enzyme complexes were isolated over a Sephadex G-50 column (20 × 1.5 cm) eluted with 50 mM Hepes, 200 mM KCl, and 0.3% NaN_3, pH 7.0. [b] The results are expressed as moles of nucleotide bound per mole of S-1 or (68K–30K)-S-1 with a standard deviation of 0.05.

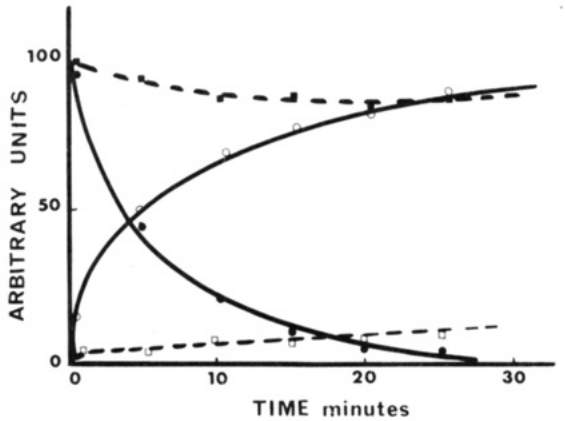

FIGURE 7: Nucleotide-induced tryptic degradation of the NH_2-terminal 27K heavy chain fragment in native and (68K–30K)-S-1. Chymotryptic S-1 (circle symbols) and thrombin-cut S-1 (square symbols) were digested at a protein concentration of 17 μM in 50 mM Tris-HCl, pH 8.0, with a trypsin to substrate weight ratio of 1:25, in the presence of 2.5 mM Mg^{2+}-ATP at 25 °C. The nucleotide was added 2 min after the start of the proteolytic reaction; during this initial 2-min period, a total release of intact 27K fragment was achieved in both protein samples. At the indicated times and following the introduction of ATP, aliquots were analyzed by polyacrylamide gel electrophoresis. The amount of 27K peptide (●, ■) and its 22K breakdown product (○, □) was estimated by densitometry of corresponding bands on the gel as specified under Materials and Methods.

Finally, to further assess the mode of nucleotide binding to the thrombin-cut S-1, we employed a proteolytic approach based on the determination of the kinetics of the nucleotide-promoted tryptic conversion of the NH_2-terminal 27K heavy chain fragment into a 22K product (Hozumi, 1983). The rate and amplitude of the process are dependent on the nature of the bound nucleotide (Mornet et al., 1985b). The densitometric diagram shown in Figure 7 clearly indicates that the nicked S-1 behaves quite differently to the intact enzyme. Its digestion in the presence of Mg^{2+}-ATP (5 mM) did not yield a significant amount of 22K peptide, and the concentration of the parent 27K fragment was only little decreased (about 10%) under conditions where the 27K → 22K conversion for native S-1 was quantitative.

Interaction of Actin with (68K–30K)-S-1. F-Actin associates reversibly with the thrombin-modified S-1. We performed the measurement of its binding by the sedimentation method, in the absence and in the presence of Mg^{2+}-ATP and Mg^{2+}-ADP. The values of the binding constants were compared to those determined for native S-1 under similar conditions (Table III). The affinity of the proteolytic derivative for actin in the absence of nucleotide and in the presence of Mg^{2+}-ADP was reduced 250-fold and 200-fold, respectively. In contrast, the binding constant measured in the presence of Mg^{2+}-ATP was unaffected.

In order to examine if 20K and 50K heavy chain regions were still operant in the interaction between the thrombin-split S-1 and F-actin, we studied the cross-linking of the derivative

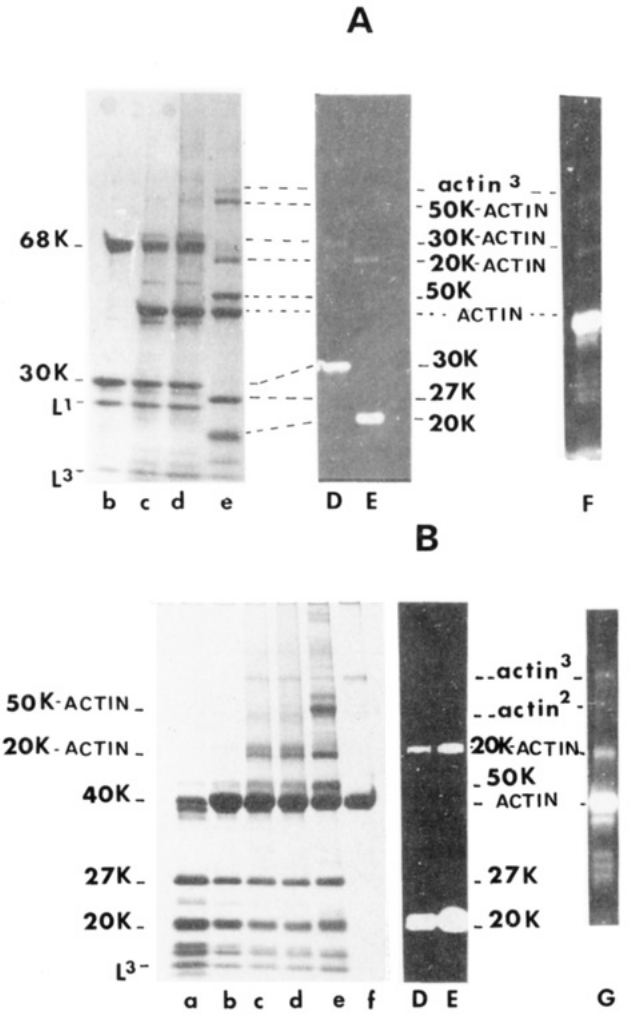

FIGURE 8: Time course of the covalent cross-linking between actin and thrombin-split S-1's. (A) Cross-linking process between F-actin and fluorescent 1,5-IAEDANS-labeled (68K–30K)-S-1. After reaction of the derivative (b) with EDC-activated F-actin as indicated under Materials and Methods, the protein samples were analyzed by polyacrylamide gel electrophoresis after 10 (c) and 20 min (d and D) of cross-linking. Trypsin-split AEDANS–(27K–50K–20K)-S-1 (e and E) cross-linked to EDC–actin by a 20-min condensation reaction is run as control. The gel was stained with Coomassie blue (b–e) and viewed under ultraviolet light (D–F). In lane F, AEDANS–F-actin was cross-linked to (68K–30K)-S-1 for 20 min. (B) Cross-linking of F-actin to (27K–40K–20K)-S-1. The 1,5-IAEDANS-labeled S-1 derivative was condensed to EDC–actin as in (A), and the gel pattern of the protein mixture was established at the interval times of 0 (a), 5 (b), 10 (c), and 15 min (d and D) of the cross-linking process. (e and E) Control complex between F-actin and AEDANS–(27K–50K–20K)-S-1 covalently cross-linked after 20-min reaction. (f) Actin after 15 min of EDC treatment. The gel was stained with Coomassie blue (a–f) and viewed under ultraviolet light (D, E, and G). In lane G, the AEDANS–F-actin was used as previously in (A), and the condensation time corresponded to 20 min.

to actin in the presence of EDC. The results are illustrated in Figure 8A in comparison with those obtained with the

Table III: Comparative Binding Constants for Complexes between Native S-1 or Thrombin-Cut S-1 and Actin in the Absence and Presence of Nucleotides[a]

	K_a (M^{-1})		
	alone	ATP-Mg^{2+}	ADP-Mg^{2+}
S-1	7.2×10^7	2.0×10^4	6.2×10^6
(68K–30K)-S-1	3.0×10^5	1.7×10^4	4.5×10^4

[a] The values were obtained from the slopes of the Scatchard plots obtained from binding experiments described under Materials and Methods. The correlation coefficient of the slope was greater than 0.85.

tryptic (27K–50K–20K)-S-1 used as control. The reaction generated a new species with a mass of 72K, which incorporated the fluorescence of AEDANS–(68K–30K)-S-1 as well as the fluorescence of AEDANS–actin (Figure 8A, lane F). This product, which migrates with a lower mobility than the authentic actin–20K entity with a mass of 62K (Mornet et al., 1981a) produced by trypsin-cut S-1, is obviously the adduct of actin with the COOH-terminal 30K peptide. In contrast, no cross-linked product between actin and the NH_2-terminal 68K heavy chain segment could be detected. Such a product with a mass near 110K would have been formed if actin was cross-linked to the 50K moiety of the segment. The band intensity at the 110K–120K position of the electrophoretic gel was accounted for only by the actin trimer present also in the controls. Further cross-linking experiments were carried out with the thrombin derivative (27K–40K–20K)-S-1 prepared from DTNB-modified (27K–50K–20K)-S-1. Its cross-linking to actin yielded the actin–20K band but not any product corresponding to actin–40K (Figure 8B). Finally, although all the proteolytic derivatives employed in a set of cross-linking experiments were issued from the same S-1 preparation, we observed reproducibly striking differences in the amplitude of the cross-linking of actin to the COOH-terminal segment of the heavy chain within the different derivatives. Thus, the intensity of the actin–30K band released from (68K–30K)-S-1 (Figure 8A) was noticeably lower relative to the actin–20K band derived from (27K–40K–20K)-S-1 (Figure 8B) and even much lower relative to the actin–20K band produced by the tryptic (27K–50K–20K)-S-1.

Discussion

The results presented here extend those described in the accompanying paper (Chaussepied et al., 1986) concerning the production of the thrombic-cut S-1. They constitute the initial characterization of the thrombin-modified S-1, which may represent a useful protein species for examining specific relationships between the structure of the heavy chain and the function of the enzyme. The limited proteolytic scission of the skeletal heavy chain at the connector regions flanking the 50K segment or at other internal sites within the 27K and 50K fragments (Labbé et al., 1984; Mornet et al., 1984) did not alter the intrinsic steady-state ATPase activities of the split S-1. On the contrary, abolition of these ATPases always accompanied the modification of the S-1 heavy chain at Lys^{560}-Ser^{561} by thrombin. Consequently, to understand the possible mechanisms involved with S-1 inactivation by thrombin, we have undertaken the present study, which compares various functional and structural properties of native S-1 to those exhibited by the thrombin-split enzyme. Because the cleavage area is located within the 50K segment, which is thought to contain portions of the actin binding site as well as of the ATPase active site (Mornet et al., 1981c; Mahmood & Yount, 1984), we thoroughly investigated the mode of interaction of the S-1 derivative with nucleotide and actin.

To ascertain that the enzymological behavior of the nicked S-1 was not due to a significant conformational change in the overall protein structure, we first analyzed the derivative by CD spectroscopy. As judged by the far-ultraviolet spectra, the conformation of the inactive thrombin-split S-1 was more alike that of the native S-1 than the conformation of the conventional active tryptic (27K–50K–20K)-S-1. The scission of the connector segments within the latter derivative produced approximately at 12% increase of the helical content of the protein. Changes in the conformation of (27K–50K–20K)-S-1 were also observed in recent studied using ^1H nuclear magnetic resonance spectroscopy (Goodearl et al., 1985). On the other hand, CD spectra in the near-ultraviolet region of thrombin-split S-1 and native S-1 were not similar. The main difference lies near 260 nm, where the spectrum usually reflects the environments of phenylalanine residues (Burke et al., 1976). In this part of the spectrum, native S-1 exhibited a pair of peaks and a shoulder having a higher intensity than the triplet produced by the thrombin derivative. This finding suggests a change in the environment of the phenylalanine side chain in the cleaved S-1. Another explanation could be that the DTNB treatment remained in memory inside the heavy chain after DTE reversion. However, such process was not detected by the thrombin pattern of such a reversed S-1 [see Chaussepied et al. (1986)]. Examination of the amino acid sequence around Lys^{560}-Ser^{561} shows that 5 of the 45 phenylalanine residues residing in the S-1 heavy chain are located near the clip site within the 50K sequence. Thus, it is conceivable that the thrombin cut has generated a local conformational change affecting the peptide stretch including these aromatic residues. Nevertheless, such a possible conformational change did not influence the CD spectrum near 285–300 nm corresponding to tryptophan side chains; also, it did not alter the chemical reactivity of the thiols accessible to titration with DTNB compared with the native enzyme.

The tight binding and hydrolysis of Mg^{2+}-ATP are important functional properties of the intact native S-1. Using near-ultraviolet CD spectroscopy, we found that the differential effect of Mg^{2+}-ATP and Mg^{2+}-ADP observed with S-1 was maintained in the thrombin-modified enzyme. Fluorescence acrylamide quenching studies of the emission of ϵ-ATP and ϵ-ADP indicated no noticeable environmental changes around the base moiety of both analogues. These are bound at the original ATPase site within the thrombin-split S-1 as suggested by competition experiments between Mg^{2+}-ATP and the fluorescent analogues. From these results, it appears that (68K–30K)-S-1 is able to bind ATP as confirmed by the ability of the nucleotide substrate to dissociate the complex between actin and this derivative. However, this binding does not have the essentially irreversible character observed with S-1. This was suggested by our failure to isolate a stable protein–Mg^{2+}-ATP complex on mixing the inactive split S-1 with the nucleotide and to trap Mg^{2+}-ADP by cross-linking between SH_1 and SH_2 thiols present within the enzyme derivative (Table I). Further, the ATP bound to (68K–30K)-S-1 is not hydrolyzed to ADP–P_i. The low activity remaining in the modified preparations (Figure 6) can be explained by the presence of unmodified S-1 (at least as judged by $NaDodSO_4$ gel electrophoresis). Nevertheless, we cannot totally exclude the possibility that the (68K–30K)-S-1 has a low ATPase activity. The finding that the split S-1 cannot form the ADP–V_i complex, a stable analogue of the ADP–P_i intermediate, strongly suggests that the proteolytic cut has triggered an S-1 structure unable to move toward the ADP–P_i conformation. Because V_i binds tightly in the presence of Mg^{2+}-ADP

at the ATPase site as an analogue of the γ-phosphate of ATP, it is possible that the binding of ADP to (68K–30K)-S-1 did not induce a conformational change that favors tight binding of V_i or the polyphosphate chain to the active site. Indeed, a lack of such a nucleotide-promoted conformational change is suggested by the disappearance of the tryptic conversion of the NH_2-terminal 27K fragment into a 22K peptide, which occurs with normal S-1 but not with (68K–30K)-S-1. Recently, we showed that this conversion is associated with the positioning of the polyphosphate chain on S-1 and its magnitude is dependent on the nature of the phosphorylated ligand bound at the ATPase site (Mornet et al., 1985b).

Another crucial feature of S-1 is that binding of nucleotides should be transmitted to the actin site. The peptide bond cleaved in the S-1 heavy chain by thrombin has not impaired such intersite communication as indicated by the results of binding studies of the derivative to actin in the absence and presence of nucleotides (Table III). The affinity for F-actin was similar to that of unmodified S-1 in the presence of ATP. In contrast, the binding of ADP decreased only slightly the weak affinity observed in the rigor state. This indicates that specific ternary complexes are formed between acto–(68K–30K)-S-1 and Mg^{2+}-ATP or Mg^{2+}-ADP. On the other hand, the proteolytic cut seems to affect the binding of S-1 at the rigor state but not at the active state. Assuming that the binding constant of Mg^{2+}-ATP to (68K–30K)-S-1 was not modified, this could explain that the affinity of actin to the Mg^{2+}-ATP–S-1 derivative complex was unaffected. The lower affinity for actin in the rigor complex can be related to the changes observed in the EDC-promoted cross-linking between F-actin and the modified S-1. The cross-linking of an actin monomer and the 50K segment within the (68K–30K)-S-1 was abolished, or it might be so weak that we were unable to detect it. Considering that the actin site on the 50K peptide is localized on its COOH-terminal moiety (Sutoh, 1983) where the thrombin cleavage has occurred, an alteration of the conformation of this site can be anticipated. Moreover the additional observation that, under optimal experimental conditions, the extent of actin cross-linking to the 20K segment included in the 30K fragment was also decreased suggests that the conformational change has been propagated to the second class of actin sites on the 20K region.

Thus, the structural integrity of the 50K portion proteolyzed by thrombin appears to be required not only for the catalytic activity of S-1 but also for its firm binding to actin. As the sites cut by thrombin lie within the primary loop segment thought to be present in S-1 linking portions of the 50K and 20K fragments (Mornet et al., 1985a), we propose that the structural freedom of this region may be critical for conformational transitions occurring during ATP hydrolysis and actin interaction. When the present work was terminated, we became aware of a brief report describing the natural proteolytic cleavage of the scallop S-1 heavy chain into 63K and 31K peptides with parallel inhibition of the K^+-ATPase activity (Szentkiralyi, 1985).

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Registry No. Mg^{2+}-ATP, 1476-84-2; Mg^{2+}-ADP, 7384-99-8; ATPase, 9000-83-3; thrombin, 9002-04-4.

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Inhibition of Myofibrillar and Actomyosin Subfragment 1 Adenosinetriphosphatase by Adenosine 5′-Diphosphate, Pyrophosphate, and Adenyl-5′-yl Imidodiphosphate

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ABSTRACT: Adenosine 5′-diphosphate (ADP), inorganic pyrophosphate (PP_i), and adenyl-5′-yl imidodiphosphate (AMPPNP) act as competitive inhibitors of the ATPase of myofibrils and actomyosin subfragment 1 (acto-S1). At I = 0.2 M, pH 7, and 15 °C, the inhibition constants for rabbit myofibrils are 0.17, 3, and 5 mM, respectively; the values for frog myofibrils at 0 °C are very similar, being 0.22, 1.5, and 2.5 mM. The inhibition constant of AMPPNP is about 2 orders of magnitude larger than the reported dissociation constant for fibers [Marston, S. B., Rodger, C. D., & Tregear, R. T. (1976) *J. Mol. Biol. 104*, 263–276]. A possible reason for this difference is that AMPPNP binding results in the dissociation of one head of each myosin molecule. The inhibition constants for rabbit acto-S1 cross-linked with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide measured under the same conditions were 0.12, 2.6, and 3.5 mM for ADP, PP_i, and AMPPNP, respectively. The inhibition of cross-linked and native acto-S1 was compared at low ionic strength and was found to be similar. The value for ADP is very similar to reported values of the dissociation constant whereas the inhibition constants for AMPPNP and PP_i are an order of magnitude weaker [Greene, L. E., & Eisenberg, E. (1980) *J. Biol. Chem. 255*, 543–548].

Knowledge of the steady-state ATPase properties of a muscle fiber is one of the requirements for understanding the relation between the unconstrained acto–myosin ATPase cycle in solution and the cycle coupled to work production in a muscle. As an extension of our work on the ATP dependence of the ATPase of rabbit psoas muscle fibers and myofibrils (Glyn & Sleep, 1985), we report in this paper an investigation of the inhibitory properties of products and substrate analogues. Each of the ligands chosen for study, adenosine 5′-diphosphate (ADP), inorganic pyrophosphate (PP_i), and adenyl-5′-yl imidodiphosphate (AMPPNP), tends to dissociate actin from subfragment 1 (S1); the binding constants of actin to S1 are reduced by factors of 20, 300, and 600, respectively (Greene & Eisenberg, 1980), values which are to be compared with the factor of 3000 resulting from ATP binding. AMPPNP binding has been reported to reduce the tension of a muscle fiber in rigor to about half, necessitating a stretch of about 2 nm per half-sarcomere to regain the original tension (Kuhn, 1973; Marston et al., 1976). ADP binding has a relatively small effect.

In simple models of the cross-bridge cycle [e.g., see Eisenberg & Hill (1985)], there are generally two attached states, the one before the power stroke (often being labeled 90°) and the one after the power stroke (45°). On this basis, the observed chemomechanical effect would be accounted for in terms of AMPPNP binding, tending to favor population of the 90° state rather than the rigor 45° state. However, from studies on the effect of AMPPNP on the structure of insect fibers, Reedy et al. (1983) suggested that AMPPNP binding may lead to one head of each myosin molecule dissociating. Data from several other sources, for example, digestion patterns of myofibrils (Chen & Reisler, 1984), orientation of spin-label (Thomas & Cooke, 1980), and fluorescence polarization (Yanagida, 1981), suggest that AMPPNP and PP_i binding results in the partial dissociation of myosin heads. Thus, the observed chemomechanical effect could also be accounted for in terms of AMPPNP reducing the binding constant of myosin heads for actin to the extent where strained heads become dissociated whereas unstrained heads remain bound. Dissociation of the strained head necessitates stretching of the muscle to regain the original tension. Comparison of the inhibition constants of myofibrillar ATPase with the dissociation constant of myofibrils and fibers offers a useful approach to clarifying this issue. If AMPPNP binding tends to dissociate one head from actin, then the AMPPNP dissociation constant of this head will be small and thus easily measurable. The behavior of this easily dissociated head will tend to dominate the binding behavior of fibers. In the case of inhibition studies, the opposite effect is operative. Heads which bind to actin have a much higher ATPase, and thus the ATPase measurements which give the inhibition constant report primarily on these active heads which display a high inhibition constant. Our observation that the inhibition constants for AMPPNP and PP_i were not equal to the reported values of the dissociation constants is thus consistent, at least in part, with this model in which AMPPNP and PP_i binding results in the dissociation of one head. The model accounts for the chemomechanical response of AMPPNP binding in terms of tension reduction because on each myosin the head that is under the greater strain becomes dissociated and not because of a shift in the population *of heads* from 45° to 90° states.

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