Effects of Tryptic Digestion on Myosin Subfragment 1 and Its Actin-Activated Adenosinetriphosphatase†

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ABSTRACT: Myosin subfragment 1 (S-1) was fluorescently labeled at its rapidly reacting thiol ("SH_1"). Short exposure to trypsin cuts the S-1 heavy chain into three still-associated fragments (20K, 50K, and 27K) [Balint, M., Wolf, L., Tarcsafalvi, A., Gergely, J., & Sreter, F. A. (1978) *Arch. Biochem. Biophys. 190*, 793–799] which bind F-actin to the same extent as does the uncut labeled S-1, as indicated by time-resolved fluorescence anisotropy decay (at 4 °C, pH 7, in 0.15 M KCl and 5 mM $MgCl_2$, ±1 mM ADP). These results are thus in agreement with turbidity measurements on similar systems as reported by Mornet et al. [Mornet, D., Pantel, P., Audemard, E., & Kassab, R. (1979) *Biochem. Biophys. Res. Commun. 89*, 925–932]. The excited-state lifetime of the fluorescent label on cut S-1 is indistinguishable from that on normal S-1 (±ADP, ±F-actin). F-Actin activation of MgATPase of cut S-1 is lower than that for normal S-1 at moderate concentrations of F-actin, as reported by Mornet et al. (1979). But as the F-actin concentration is increased, the MgATPase activities for cut S-1 approach those for uncut S-1. In terms of an eight-species steady-state kinetics scheme involving actin binding to free S-1, S-1·ATP, S-1·ADP·P, and S-1·ADP, actin affinity for the species S-1·ADP·P was found to be 13.4 times greater for uncut S-1 than for cut S-1 [at 24 °C, pH 7.0, in 3 mM KCl, 1 mM ATP, 1 mM $MgCl_2$, and 20 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid].

As established by Balint et al. (1978), the heavy chain of myosin subfragment 1 (S-1),[1] during a short exposure to trypsin, can be cut in two places, thereby generating three heavy chain fragments: 20K, 50K, and 27K. Mornet et al. (1979) reported that these cuts do not seem to affect the gross physical or enzymatic properties of S-1 itself but essentially eliminate the F-actin activation of its MgATPase. Since communication between the actin-binding and nucleotide-binding (ATPase) sites of S-1 is of great importance in understanding energy transduction in muscle, we have examined further some of the physical properties of cut S-1 as well as the reduced ability of F-actin to activate the ATPase. The physical studies largely confirm the findings of Mornet et al. (1979) that the tryptically cut S-1 closely resembles the original S-1. However, we find that at sufficiently high F-actin concentrations MgATPase activation is almost fully restored.

Experimental Procedures

S-1 was prepared by digestion of rabbit muscle myosin [prepared according to Tonomura et al. (1966)] with α-chymotrypsin in the presence of EDTA (Weeds & Taylor, 1975) and was purified by gel filtration through a Sephacryl 200 column equilibrated with 0.15 M KCl–10 mM Tes, pH 7.0. The purified S-1 was concentrated to about 10 mg/mL with an Amicon MX-50 at 0 °C and was dialyzed against 50 mM ammonium acetate and 1 mM DTT, pH 7.0, 4 °C. Solid sucrose to 0.2 M was dissolved in the S-1 solution, and the resulting solution was lyophilized in a dry ice–2-propanol bath. The S-1 ATPase properties before and after this treatment are the same. Limited tryptic digestion of S-1 (30 min at 25 °C in 100 mM NaCl–25 mM sodium phosphate, pH 7.0) was carried out according to Yamamoto & Sekine (1979a). F-Actin was prepared from the acetone powder of rabbit skeletal muscles by the method of Spudich & Watt (1971). For fluorescence measurements, S-1 was labeled, prior to digestion, with 1,5-IAEDANS at the SH_1 position. It has been shown previously that such labeling does not affect the pattern of tryptic cutting (Hozumi & Muhlrad, 1981). The cut and uncut S-1's were then compared through measurement of time-resolved fluorescence anisotropy decay (TRFAD) giving the excited-state lifetime (τ), the rotational correlation time (Φ), and the equilibrium constant (K) for association of S-1 with F-actin (at 4 °C, pH 7.0, in 0.15 M KCl, 5 mM $MgCl_2$, and 20 mM TES). The procedure for making these measurements and analyzing the results was similar to that described previously (Highsmith et al., 1976; Wadzinski et al., 1979). ATPase activities of 0.1 μM unlabeled S-1 (at 24 °C, pH 7.0, in 3 mM KCl, 1 mM ATP, 1 mM $MgCl_2$, and 20 mM Tes) were measured by a modified Fiske–SubbaRow method (Fiske & SubbaRow, 1925) over a range of F-actin concentrations (0.05–6.4 μM in terms of actin monomer). $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out in 15% acrylamide according to Laemmli (1970).

Results

In agreement with reports by others [e.g., Balint et al. (1978)], limited tryptic digestion of S-1 was found to produce essentially three heavy chain fragments (20K, 50K, and 27K) which remain associated in low KCl solution but are separable by polyacrylamide gel electrophoresis. As shown in Figure 1, the heavy chain is almost completely degraded into the three fragments.

TRFAD measurements reveal no significant difference in the Φ values for digested and undigested labeled S-1, confirming that the fragments remain associated (Table I). Interaction of labeled S-1 (0.25 μM) with F-actin (1.0 μM) increased these two Φ values to about the same degree, indicating an equivalent extent of actin binding to cut and uncut

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[1] Abbreviations: 1,5-IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonate; DTT, dithiothreitol; S-1, myosin subfragment 1; SH_1, most reactive thiol of S-1; $NaDodSO_4$, sodium dodecyl sulfate; Tes, N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TRFAD, time-resolved fluorescence anisotropy decay; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-6903$01.25/0

Table I: TRFAD Measurements on Normal and Digested S-1's[a]

S-1 (0.25 μM)	[ADP] (mM)	Φ (ns) (no actin)	Φ (ns) (1 μM actin)	K (μM^{-1})	τ (ns) (no actin)	τ (ns) (1 μM actin)
control	0	155 ± 2 (3)	309 ± 2 (4)	0.819	20.25 ± 0.03 (4)	20.46 ± 0.04 (5)
digested	0	156 ± 5 (5)	308 ± 33 (4)	0.806	20.29 ± 0.04 (5)	20.38 ± 0.05 (5)
control	1.0	165 ± 3 (5)	191 ± 11 (5)	0.121	20.59 ± 0.03 (5)	20.67 ± 0.03 (5)
digested	1.0	155 ± 3 (4)	180 ± 10 (5)	0.121	20.54 ± 0.04 (5)	20.61 ± 0.07 (5)

[a] Conditions: 4 °C, pH 7.0, 0.15 M KCl, 5 mM $MgCl_2$, 20 mM Tes; S-1 from two different preparations; Φ and τ values based on data between 28 and 72 ns following excitation. Standard deviations are indicated following each value; the number of measurements is indicated in parentheses. In the calculation of K, the Φ value for actin–S-1 is taken to be 10^5 ns for both control and digested S-1's.

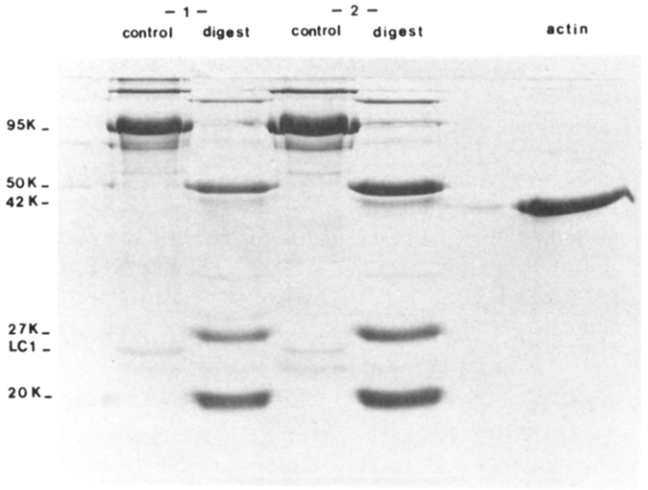


FIGURE 1: Polyacrylamide gel electrophoresis of normal and trypsin-digested S-1 for two different S-1 preparations. Conditions: 15% acrylamide, 22 °C.

S-1 (corresponding to about 45% of the S-1 bound). ADP reduced that binding (to about 10%) in both cases (Table I). These results thus support the findings of Mornet et al. (1979), who used unlabeled S-1 and F-actin in the presence and absence of MgATP and reported that progressive digestion of S-1 did not alter the extent of F-actin binding as indicated by turbidity measurements.

Small increases in the fluorescence lifetime (τ) of the label on S-1 in the presence of F-actin and/or ADP also reveal no significant differences between digested and undigested S-1 (Table I). Thus, the digestion process appears to cause no detectable change in the immediate environment of the fluorescent label at SH_1 under the conditions studied.

The present work confirms reports (Mornet et al., 1979; Yamamoto & Sekine, 1979b) that the ATPase activity of S-1 alone is little affected by trypsin digestion whereas the actin-activated ATPase at moderate F-actin concentrations is substantially diminished. However, Figure 2 shows that higher actin concentrations can largely overcome the inhibition. In all the kinetic measurements, the substrate (S) was in excess. For higher F-actin concentrations ([A]), the plots of $1/v_{[S]\to\infty}$ vs. $1/[A]$ for both digested and normal S-1's approach linearity and a common intercept, $1/v_{[S]\to\infty,[A]\to\infty}$, as $1/[A] \to 0$ (Figure 2).

Mornet et al. (1979), comparing ATPase activities of digested and normal S-1's at a single F-actin concentration, suggested that, since actin binding to S-1 appeared to be unimpaired following digestion, suppression of F-actin activation of ATPase might be due to inhibition of P_i release from the actin–S-1·ADP·P complex. The present work suggests that the P_i release mechanism is normal, but actin binding to some form of the S-1 may be inhibited. [Yamamoto & Sekine (1979b) also attributed lowered activation to a reduced affinity of S-1 for actin.]

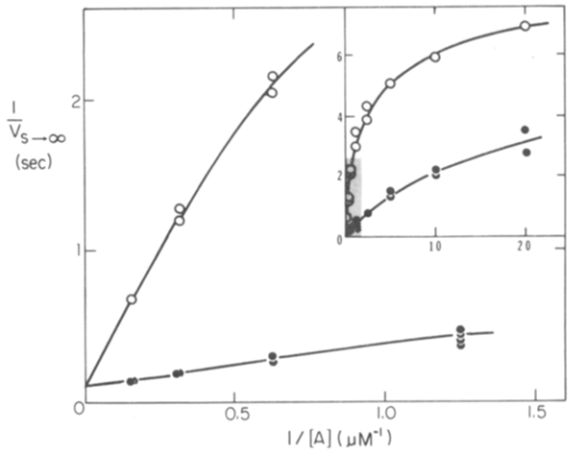
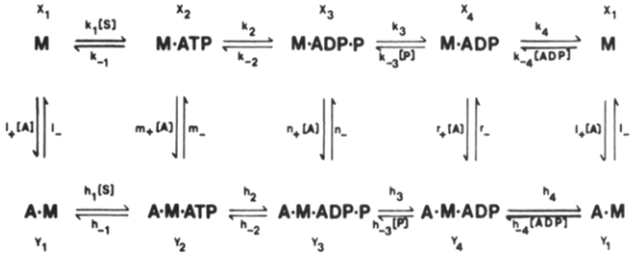


FIGURE 2: Double-reciprocal plot of ATPase activity in the presence of excess substrate ($v_{[S]\to\infty}$ in moles of P_i per moles of S-1 per second) vs. F-actin concentration, [A], in micromolar of actin momomer, for normal (●) and digested (○) S-1. Conditions: 0.1 μM S-1, 1.0 mM ATP, 1.0 mM $MgCl_2$, and 3 mM KCl, pH 7.0, 24 °C. [Assumed molecular mass: S-1, 115000 daltons; actin monomer, 42000 daltons.) Inset: Same axes; stippling designates area shown with expanded scale in the primary plot. Scaling difference: abscissa, 0.3/10; ordinate, 1/5.

Scheme I



Analysis

The difficulties in devising kinetic models applicable to an actomyosin or acto-S-1 ATPase system and in interpreting data in terms of such a model have been dealt with extensively elsewhere [e.g., see Taylor (1979)]. Scheme I is among the simpler models which might accommodate the present data. For convenience, the X's and Y's are used to denote the various species as indicated. In this scheme, inorganic phosphate, P_i, is assumed to be released from both A·M·ADP·P and M·ADP·P. In the steady state, the fraction of P_i released from A·M·ADP·P would be

$$\frac{h_3[Y_3]}{h_3[Y_3] + k_3[X_3]} = \frac{h_3[Y_3]}{v} \qquad (1)$$

where v is the total steady-state rate of P_i release for a given F-actin concentration, [A]. Steady-state analysis of Scheme I, utilizing 8-order determinants together with the simplifying assumptions that $[P_i]$ and [ADP] are negligible and substrate concentration ([S]) is in excess, shows that

$$\frac{h_3[Y_3]_{[S]\to\infty}}{v_{[S]\to\infty}} = 1 - [k_3/(h_3N)](1/[A]) - \text{higher order terms in } 1/[A] \quad (2)$$

where N is the association constant for the binding of actin to M·ADP·P. The coefficients of the higher order terms in $1/[A]$ are complicated expressions involving rate constants for various reactions in Scheme I.

With excess substrate in the absence of actin, the steady-state enzymatic rate under Scheme I is

$$v_{[S]\to\infty,[A]=0} = k_3[X_3]_{[S]\to\infty,[A]=0} \quad (3)$$

Experimentally, this rate was found to have a value of 0.111 s^{-1} for both normal and digested S-1. For high concentrations of actin, Scheme I gives

$$v_{[S]\to\infty,[A]\to\infty} = h_3[Y_3]_{[S]\to\infty,[A]\to\infty} \quad (4)$$

In Figure 2, it is seen that the two curves for $1/v_{[S]\to\infty}$ vs. $1/[A]$ for normal and digested S-1 approach the same intercept, corresponding to $v_{[S]\to\infty,[A]\to\infty} = 9.58\ s^{-1}$. It is assumed that these equivalent rates for normal and digested S-1 reflect the same k_3 and the same h_3 for the two systems.

In terms of Scheme I, for equal S-1 concentrations, let $[A]_n$ be some actin concentration in the normal S-1 system and $[A]_d$ be a corresponding concentration in the digested system such that the steady-state enzymatic activities of the two systems are equal. Since the activities of the two systems are equal at both extremes ($[A] = 0$ and $[A] \to \infty$), it is reasonable to assume that, for any intermediate common activity, the fraction of P_i released from the Y_3 species in each system is the same. The right-hand side of eq 2 for normal S-1, with actin concentration $[A]_n$ and actin affinity constant N_n, can then be equated to the corresponding expression for digested S-1, with $[A]_d$ and N_d. For sufficiently high actin concentrations, the higher order terms in $1/[A]$ become negligible, in which case the equation reduces to

$$N_n/N_d = [A]_d/[A]_n \quad (5)$$

This is equivalent to saying that N_n/N_d can be measured as the reciprocal of the ratio of the slopes for normal and digested S-1 in Figure 2, i.e., $N_n/N_d = 13.4$. (However, it can be seen from eq 2 that, if either the k_3's or the h_3's were not the same for the two systems, this simple relationship between slopes and affinity constants would not hold.)

It might also be noted that as [A] becomes large, $[Y_3]_{[S]\to\infty}/[X_3]_{[S]\to\infty}$ approaches its equilibrium value for the given [A]. The left-hand side of eq 2, with $(k_3[X_3]_{[S]\to\infty})/(h_3[Y_3]_{[S]\to\infty}) \equiv q$, can be expressed as $1/(1+q)$, which approaches $1 - q$ as [A] becomes large and q becomes correspondingly small. So, for sufficiently large [A], eq 2 becomes

$$1 - q = 1 - (k_3/h_3)[1/(N[A])] \quad (6)$$

or

$$N = \frac{[Y_3]_{[S]\to\infty,[A]\text{ large}}}{[A][X_3]_{[S]\to\infty,[A]\text{ large}}} \quad (7)$$

where eq 7 is the standard form for an affinity constant expressed in terms of equilibrium concentrations.

If the steady-state system is in equilibrium for all values of [A], the right-hand side of eq 2 can be expressed as $[1 + k_3/(h_3N[A])]^{-1}$. Equation 5 would then be valid without additional stipulation of large [A] values. Thus (within the limitations set forth above) if the reactions in Scheme I are in equilibrium, either generally or as a result of large [A] values, eq 5 will hold.

Discussion

Kinetic analysis of MgATPase data indicates that the affinity of F-actin for the phosphate-releasing species of S-1 is considerably larger in normal than in digested S-1. The TRFAD method of measuring S-1–actin affinity constants showed no difference between normal and digested S-1 in either the presence or the absence of MgADP. Previous TRFAD work on the F-actin affinity for normal S-1 in the presence of MgATP, its analogue MgAMP-P(NH)P, or $MgPP_i$ had given S-1–actin affinity constants at least an order of magnitude lower than that with MgADP (S. Highsmith, unpublished results). It is therefore likely that the concentrations of the acto-S-1 complex in the presence of MgATP are so low, in both the normal and digested S-1 systems, that neither the fluorescence nor the turbidity method would distinguish a difference in actin–S-1 affinities. Calculations show that this would be the case for a typical set of concentrations when the affinity constant is $10^4\ M^{-1}$ or less. There is thus no obvious inconsistency between kinetic results, which indicate a difference in the actin affinities of normal and digested S-1, and the physical measurements, which detect no difference.

In this work, S-1 digestion seemed to affect actin affinity (during ATP hydrolysis) but not the signals (Φ, τ) issuing from a fluorophore located on SH_1 in the 20K region (Balint et al., 1978) near the site of the proteolytic cut. A possible explanation is that the structure in this portion of the actin-binding site is held together quite well by extensive interchain bonds but that the actin affinity under stressful conditions (the ADP·P state) is lower because under such conditions the contact with the 50K region is impaired by the proteolysis. Recent work by Mornet et al. (1981) indicating actin-binding sites in both the 20K and 50K regions of S-1 may bear on this point.

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