Effect of limited trypsin digestion on the biochemical kinetics of skeletal myosin subfragment-1

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ABSTRACT We have investigated the effect of limited trypsin digestion of chymotryptic myosin Subfragment-1 (S-1) on its kinetic properties. We find that V_{max} (i.e., the extrapolated maximal ATPase activity at infinite actin) remains approximately constant, independent of the period of digestion. We also find that the apparent actin activation constant, K_{ATPase}, and the apparent dissociation constant, $K_{binding}$, are both significantly weakened by trypsin digestion of S-1, and that these kinetic parameters change in concert.

In addition, we investigated the effect of limited trypsin digestion on the initial phosphate burst. We find that trypsin digestion has no effect on the rate of the tryptophan fluorescence enhancement that occurs after ATP binds to digested S-1, but that the magnitude of the fluorescence enhancement falls ~40% with digestion. Digested S-1 also showed anomalous behavior in that the fluorescence magnitude increased and the fluorescence rate dropped in the presence of actin. Trypsin digestion also decreased the magnitude of the chemically measured Pi burst ~35%, but this magnitude was essentially unaffected by actin. A possible explanation for this behavior is discussed.

INTRODUCTION

Muscle contracts as a result of the sliding of actin filaments relative to the myosin filaments (1, 2). According to the cross-bridge theory of muscle contraction (3), it is the globular head of the myosin molecule that interacts with the actin filament and moves the actin filament relative to the myosin filament. The energy source used to perform work in this system is the free energy of hydrolysis of ATP (4). To study contraction from a molecular viewpoint, biochemists have turned to the actin activated myosin ATPase activity, which is the in vitro correlate to muscle contraction in vivo. These studies have led to the proposal of several models for actin activation, one of which is shown in Fig. 1 (5–11). Central to this model is the assumption that the binding of actin to the myosin-nucleotide intermediates is directly responsible for the activation of the ATPase activity. When modeling with the six-state model in Fig. 1, it is also assumed that the apparent dissociation constant in the presence of ATP, $K_{binding}$, represents only the physiologically relevant binding (i.e., binding which is directly involved in the ATPase activation). When modeling the ATPase mechanism, $K_{binding}$ is a weighted "average" of the individual dissociation constants K_3, K_{13}, and K_{14} seen in Fig. 1.

Since the proposal of the model in Fig. 1, it has been reported that trypsin digestion of myosin S-1 leads to a ternary complex of fragments that remain associated at low ionic strength (12–14). These fragments consist of a 25-Kd fragment important to nucleotide binding, a 20-Kd fragment involved in regulation and the binding of light chains and a 50-Kd fragment that is largely responsible for actin binding and ATPase activity (15–18). It has also been reported that digested S-1 binds to actin with an affinity similar to undigested S-1, yet possesses an actin activation that is much weaker than that of the undigested subfragment (19, 20). This differential effect of digestion on binding and activation may suggest that the majority of the measured binding is not physiologically relevant (i.e., not directly related to the activation process), or that different fragments of the ternary complex are responsible for binding and activation.

A weakness of the previous studies is that the binding measurements were made either in the absence of ATP (19) or under conditions where very little binding (~1%) was observed (20), and caution should be exercised in drawing conclusions from these data. In the current work we have investigated the actin activation kinetics of the ternary complex formed by digestion of S-1 with trypsin. We find that the apparent activation constant, K_{ATPase}, becomes appreciably weaker after trypsin digestion. Significantly, however, we find that $K_{binding}$ becomes weaker to about the same extent. These studies imply that actin binding to S-1 during hydrolysis of ATP is specific to the activation process, and that it is likely that the domains responsible for actin binding to myosin in the presence of ATP and for actin activation are the same.

We have also investigated the effect of limited trypsin digestion of S-1 on the rate and magnitude of the fluorescence burst and the magnitude of the chemically mea-

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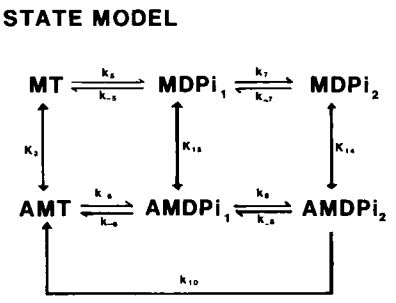

FIGURE 1 The six state model. In this model: A = actin, M = Subfragment-1, T = ATP, D = ADP, Pi = inorganic phosphate. The dissociation constants K_3, K_{13}, K_{14} are assumed to represent rapid equilibrium processes.

sured phosphate burst. When ATP is mixed with S-1 two fluorescence enhancements can be observed, and these are generally believed to arise from the M + ATP → M*ATP transition and the M*ATP → M**ADPPi$_1$ transition (see Fig. 1). At saturating ATP concentrations (i.e., >0.5 mM), the first fluorescence transient is very fast and only the second transition known as the fluorescence burst is observed. To observe the faster transient, very low ATP concentrations must be used. Another way of measuring a burst for S-1 is to use chemical quench techniques. When ATP is added to S-1 and the reaction is quenched at short time points (i.e., 10–200 ms), ADP and Pi bound to S-1 in the state M**ADPPi$_1$ are released back into the medium. If the concentration of released Pi is followed as a function of time, it rises rapidly at first and then reaches a plateau. The plateau is, therefore, the magnitude of a Pi burst, and it represents the concentration of M**ADPPi$_1$ at steady state. Biochemists generally regard the "burst" as arising from a conformational change in the S-1 molecule that occurs during the initial hydrolysis of ATP. Because digested S-1 consists of three fragments which would presumably possess increased degrees of freedom in comparison to undigested S-1, it was interesting to determine how trypsin digestion affects the magnitude and rate of the burst.

MATERIALS AND METHODS

Proteins

Actin, skeletal myosin, and subfragment-1 were prepared as described previously (5). Standard SDS polyacrylamide gels were performed to monitor the digestion of S-1 and the purity of the proteins, and to follow the proportions of the 95, 75, 50, 25, and 20 kDalton fragments present after digestion. Before digestion, S-1 at 10 mg/ml was dialyzed in 0.1 M NaCl, 25 mM $NaHPO_4$, pH 7.0. Trypsin (T8642; Sigma Chemical Co., St. Louis, MO) was then added to a final concentration of 0.1 mg/ml, and the protein was digested at 10°C for the required time. The reaction was then quenched with trypsin inhibitor (T9003; Sigma Chemical Co.), and the digested protein immediately used in ATPase and binding assays without further purification. For burst experiments the digested S-1 was dialyzed to 10 mM Imidazole, pH 7.0, 1 mM DTT for 7 h before use. In general, when experiments were performed, the digested S-1 was compared with unmanipulated S-1 only. Additional experiments were run to determine the effect of the required additional dialyses on undigested S-1. These control experiments (Fig. 6 *a*) were performed by first dialysing the undigested S-1 in the high salt digestion conditions for 24 h and then redialyzing for 24 h to low salt conditions. Further proof that the manipulation of the protein does not account for the entire drop in burst magnitude seen after trypsin digestion came from controls in which the burst magnitude in the absence of actin was measured immediately following digestion and compared with undigested protein otherwise treated the same way, but at higher ionic strength (Fig. 6 *b*).

Binding assay

The binding assay used was similar to the airfuge technique used previously (21, 22), except that the pelleting of bound species was carried out in a temperature controlled preparative ultracentrifuge using a Dupont Sorvall rotor TFT80, modified to take 0.4-ml tubes. Pelleting of the actoS-1 complexes was performed at 55,000 RPM (=150,000 g) for 25 min at 10°C.

ATPase assays

The ATPase assays were carried out as described previously using (gamma-^{32}P)ATP and extracting the hydrolyzed radioactive inorganic phosphate (9, 22) as the phospho-molybdate complex.

Quench flow and stopped flow assays

These experiments were carried out as described previously using machines designed by Dr. Ken Johnson at Penn State University (22). Stopped flow measurements were performed by mixing S-1 in one syringe with actin + ATP in the second syringe, which allows for the best possible mixing. Quench flow measurements were performed by mixing ATP in one syringe with Actin + S-1 in the other. This type of mixing is required because actin does hydrolyze ATP at a rate sufficient to significantly increase the ^{32}Pi blank during the course of a single quench flow experiment (1–2 h). In the case of fluorescence measurements, no correction for the scattering and fluorescence of actin were made as these corrections tend to be small under the conditions used. In general the magnitude of the recorded fluoresence transient was ~20% of the background tryptophan fluorescence. Chemical chase measurements were performed as described previously (6). In these experiments, actoS-1 in one syringe of the quench flow machine was mixed with ATP in the other, and at a predetermined time (300–400 ms) the mixture is "chased" with 50–100-fold excess cold ATP. In stopped flow studies the ATP concentrations used were 1 mM when saturation of ATP was desired to observe only the fluorescence change associated with the initial Pi burst, and 15–20 μM ATP when both fluorescence transients were to be observed. On the other hand, in quench flow experiments, the concentrations of (gamma-^{32}P)ATP that can be used are limited by the 1–2% blank of ^{32}P that exists. In these experiments 0.1–0.2 mM ATP were used.

Data analysis

The binding and ATPase data were analyzed using a hyperbolic fitting procedure based on Nelder and Mead (22, 23). The stopped flow data

were analyzed by first averaging five to ten traces and then determining the rate and magnitude using a least squares technique (22).

RESULTS

Effect of tryptic digestion on actin activation and actin binding

In our initial studies we digested S-1 at 25°C, and we found that both the apparent activation constant, K_{ATPase}, and the apparent dissociation constant, $K_{binding}$, became too "weak" to measure accurately. For this reason we decided to carry out the digestion at 10°C, and to measure the kinetic parameters as a function of the digestion time. In this way, we anticipated that a pattern of changes in the parameters would emerge.

In Fig. 2, polyacrylamide gels show the effect of limited tryptic digestion of chymotryptic skeletal S-1 for up to 100 min at 10°C. At times greater than 5 min none of the original heavy chain (95 kD) remains, and the major fragments that appear are the 75- and 20-kD fragments. Further digestion reduces the 75-kD fragment with increased appearance of the 50- and 25-kD fragments, and the 75-kD fragment vanishes before 40 min. Note that at short digestion times the S-1 is not homogeneous but consists of a mixture of molecules at different stages of digestion. For this reason the kinetic constants measured will be essentially a weighted average of individual populations. At longer digestion times the proteins become more homogeneous as the ternary complex of 50-, 25-, and 20-kD fragments.

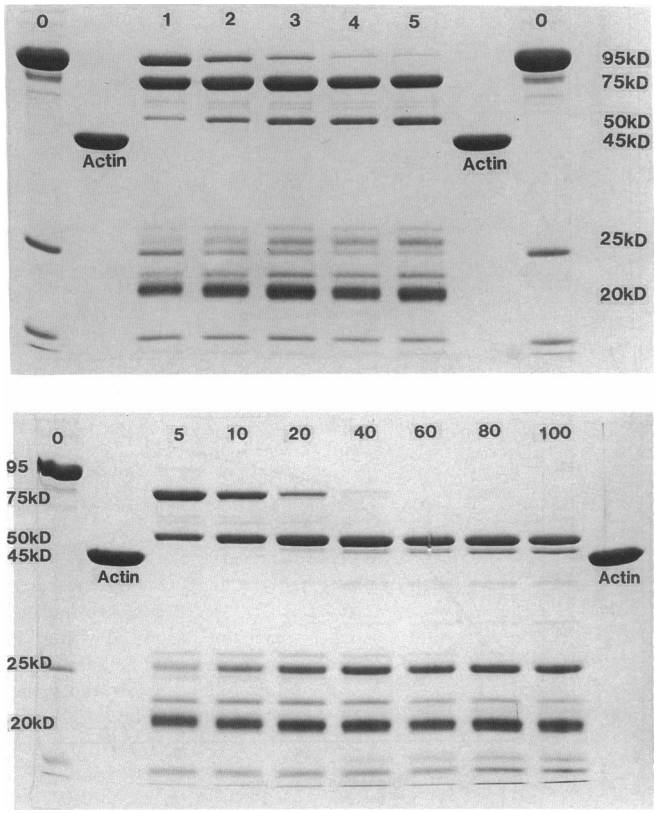

FIGURE 2 Polyacrylamide gels of tryptically digested S-1. In 2 *a* the rows from left to right are: rows 1 and 9, undigested S-1; rows 2 and 8, actin; rows 3–7, are 1, 2, 3, 4, and 5 min of tryptic digestion at 10°C. In 2 *b* row 1 is undigested S-1; rows 2 and 10 are actin, and rows 3–9 are for 5, 10, 20, 40, 60, 80, and 100 min of tryptic digestion at 10°C. The approximate molecular weights are shown on the figure.

Fig. 3 *a* shows double reciprocal plots of the ATPase activation and the actin binding of undigested chymotryptic S-1 during steady-state hydrolysis of ATP. These data show that the apparent activation constant, K_{ATPase}, is about seven-fold stronger than the apparent dissociation constant, $K_{binding}$. Figs. 3, *b* and *c* show the same data for S-1 digested 3 and 20 min, respectively. The double reciprocal plots appear linear despite the fact that the partially digested proteins consist of mixtures of S-1 molecules at different stages of digestion (see Fig. 2). The linearity observed in this case is not unexpected because the sum of two hyperbolic equations with very different $K_{apparent}$'s can appear nearly linear when plotted in double reciprocal form (6). Note that the K_{ATPase} and $K_{binding}$ have weakened significantly with digestion (the scales on the ordinate and abscissa have been altered to allow comparison of the different plots). However, the ratio of $K_{binding}$ to K_{ATPase} remains approximately equal to that observed in Fig. 3 *a*.

In Fig. 4, the V_{max}, K_{ATPase}, and $K_{binding}$ are plotted vs. the time of tryptic digestion, and all three kinetic constants are seen to vary as a function of digestion. In Fig. 4 *a*, V_{max} changes by only a factor of two between 0 and 60 min of digestion, whereas K_{ATPase} and $K_{binding}$ change much more dramatically. After 20 min of digestion, K_{ATPase} increases ~10–12-fold and $K_{binding}$ ~15-fold, and the ratio of these constants remains approximately constant independent of digestion. It is clear that actin binding and actin activation parallel each other closely, indicating that actin activation of S-1 and the actin binding to S-1 during steady-state hydrolysis of ATP are closely related.

Effect of tryptic digestion on the presteady state kinetics

When skeletal S-1 is mixed with ATP two enhancements of tryptophan fluorescence have been observed to occur and are generally believed to represent conformational changes in the S-1 molecule. The first fluorescence enhancement is generally believed to be associated with the binding of ATP: M + ATP → M*ATP, is very rapid, and can only be observed if very low ATP concentrations are used (11, 24–26). The second fluorescence enhance-

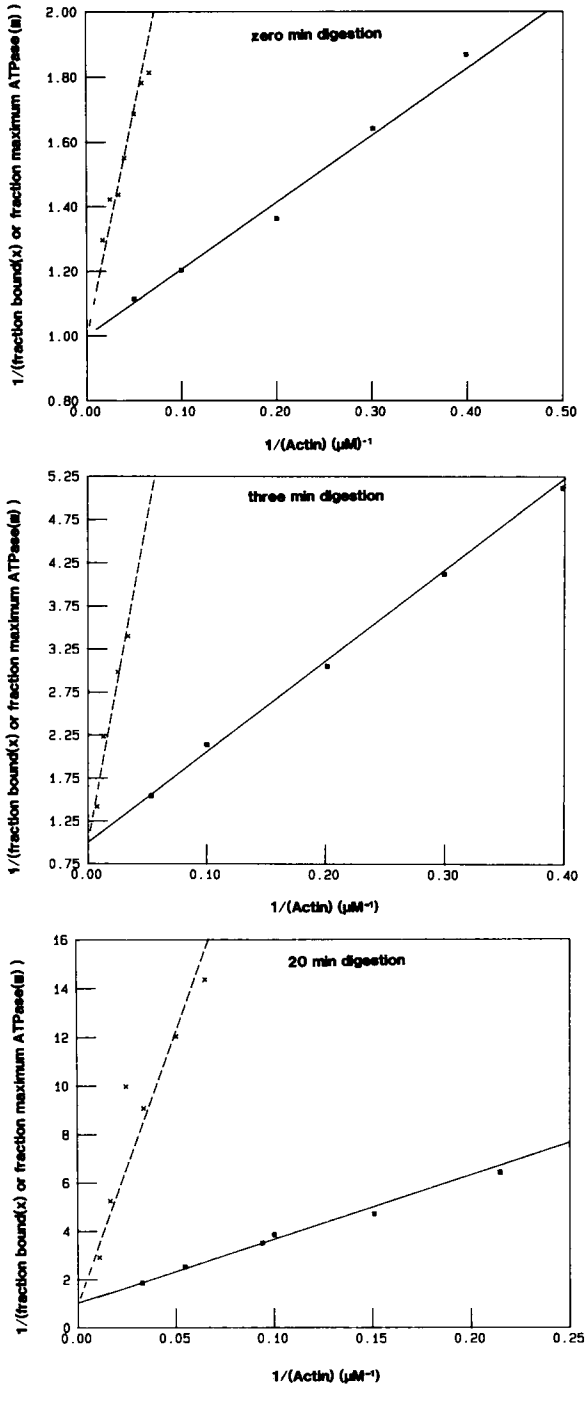

FIGURE 3 Comparison of actin activation of the S-1 ATPase activity and actin binding to S-1 during steady-state hydrolysis of ATP. In all three parts of the figure the ATPase activity has been normalized to the extrapolated V_{max} so that both the activation and binding plots can be extrapolated through 1.0 for comparison. In 3 *a* undigested S-1 has been used, and there is a seven-fold difference between K_{ATPase} and $K_{binding}$. In 3, *b* and *c*, 3 and 20 min of digestion have been used with ratios of $K_{binding}$ to K_{ATPase} of 6.6 and 9.6, respectively. Conditions: 10° C; 10 mM Imidazole, pH 7.0; 1 mM $MgCl_2$; 1 mM MgATP; 1 mM DTT; ATPase experiments were carried out at 0.5 μM total S-1, and binding experiments were performed at 0.2–0.4 μM S-1. The actin concentration was varied as shown.

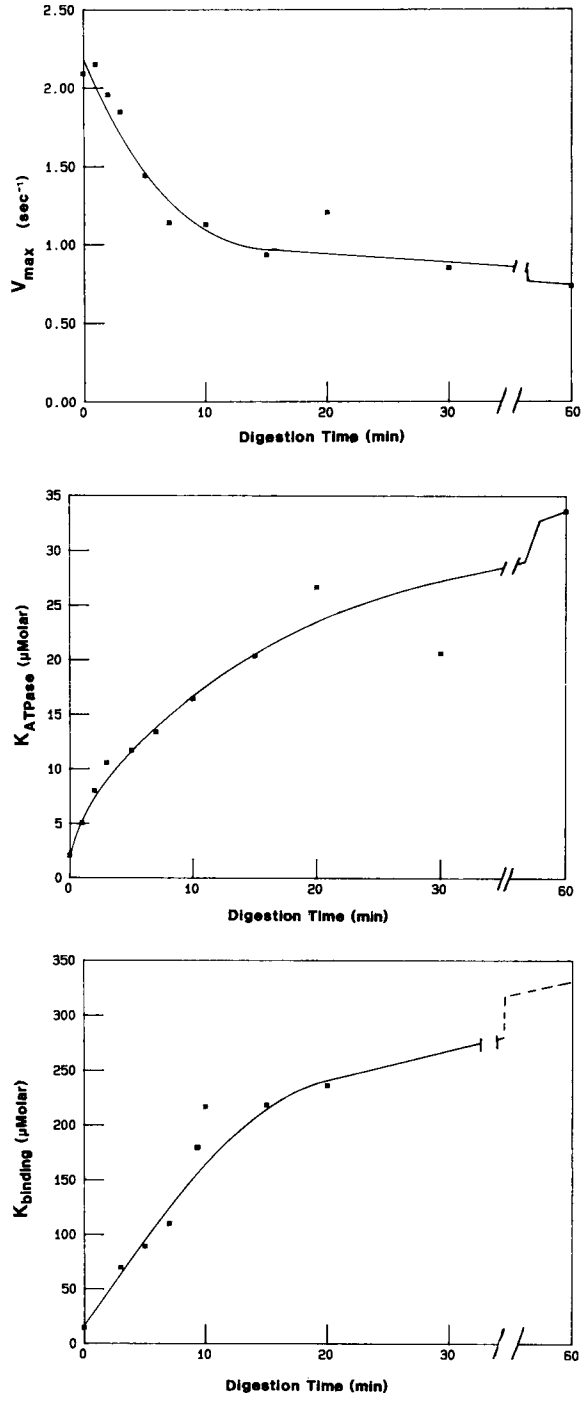

FIGURE 4 The dependence of K_{ATPase}, V_{max}, and $K_{binding}$ on the period of tryptic digestion. (4 *a*) The dependence of V_{max} on the period of digestion. (4 *b*) Dependence of K_{ATPase} on the period of tryptic digestion. (4 *c*) Dependence of $K_{binding}$ on the period of tryptic digestion. Conditions: as in Fig. 3.

ment is generally believed to be associated with the initial hydrolysis of ATP on the S-1 surface, M*ATP → M**ADPPi$_1$, and is referred to here as the "fluorescence burst" (24–26). Limited tryptic digestion of the S-1 molecule splits it into three fragments, and, therefore, digestion may either eliminate or otherwise alter the conformational changes that occur.

Presteady state fluorescence measurements

Fig. 5 shows the ATP-induced enhancement of tryptophan fluorescence before and after limited trypsin digestion of chymotryptic S-1 at 1 mM ATP. Because 1 mM ATP should be kinetically saturating, it is assumed here that the fluorescence transition observed in Fig. 5 is the one associated with the initial hydrolysis of ATP. Clearly the digested S-1 shows a fluorescence burst, and the lines drawn through the data represent single exponential fits to the data. As shown in Table 1, column *A*, the rate constants for the fluorescence enhancement are essentially identical for undigested S-1 and for digested S-1 up to 60 min of tryptic digestion at 10°C (i.e., the rates are in the range of 16.6–17.5 s). However, there is a difference between the fluorescence magnitudes of digested and undigested proteins, with the digested proteins having a significantly lower magnitude.

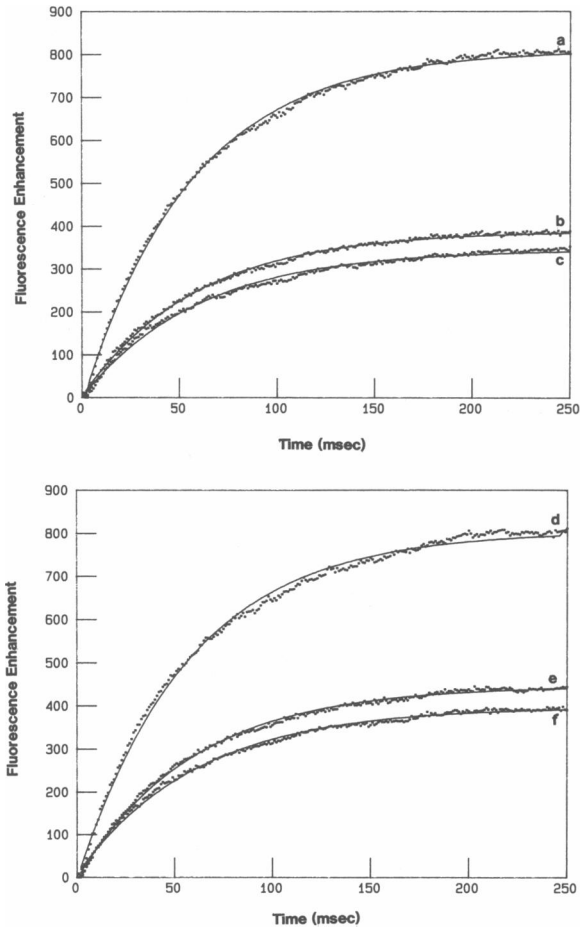

FIGURE 5 Effect of limited tryptic digestion on the rate and magnitude of the tryptophan fluorescence enhancement when S-1 or digested S-1 are added to ATP in the stopped flow machine. (5 *a*) The traces are (*a*) undigested S-1; (*b*) 10 min of digestion; (*c*) 60 min of digestion. The dots are the experimental points. The line drawn through the points is a least squares single exponential fit. The rate constants for the fit are: 17.5 s^{-1}, 17.1 s^{-1}, and 16.8 s^{-1}, respectively. (5 *b*) The traces are (*d*) undigested S-1; (*e*) 20 min of digestion; (*f*) 40 min of digestion. As in 5 *a*, the rate constants for the least squares fit are: 17.5 s^{-1}, 17.1 s^{-1}, and 16.6 s^{-1}, respectively. Figs. 5, *a* and *b*, came from two different protein preparations. Conditions: as in Fig. 3. In all runs the final S-1 concentration was 10 μM, and the ATP concentration was 1 mM.

In Fig. 5 the undigested proteins used were not treated identically to the digested proteins, and it was important to determine if the reduction in burst magnitude observed was caused by the additional dialyses before and after digestion rather than the actual tryptic digestion. For this reason we performed separate experiments to determine the effect of the two dialyses alone on undigested S-1. In Fig. 6 *a* the effect of the two additional dialyses on the fluorescence magnitude is shown to be a 12% reduction in magnitude, although reductions as high as 20% were occasionally observed. This drop in magnitude cannot account for the much larger drop in burst magnitude seen in Fig. 5. To directly demonstrate that digestion can lower the magnitude, we performed the control in Fig. 6 *b*, in which digested and undigested S-1 were treated identically. S-1 was initially dialyzed to high salt digestion conditions, and then half of the protein was digested for 40 min with trypsin while the other half was not. Both proteins were then used immediately for a determination of the fluorescence magnitude. Clearly the burst magnitude of the digested S-1 is 20–25% lower than the undigested S-1, and therefore digestion directly lowers the magnitude of the fluorescence burst.

In the case of undigested S-1, the fluorescence enhancement measured in Fig. 5 represents only the enhancement associated with the initial hydrolysis of ATP. Digested proteins, however, could conceivably differ from undigested S-1 by having a different proportion of the total fluorescence enhancement associated with each of the two transitions. Table 2 compares the magnitude of the fluorescence enhancement at 1 mM ATP with the enhancement at 20 μM ATP. The fluorescence enhancement measured near stoichiometry of S-1 and ATP measures both the M + ATP → M*ATP transition as well as the M*ATP → M**ADPPi$_1$ transition, and, therefore, represents the total enhancement of fluorescence. The table shows that digested S-1 and undigested S-1 have different total fluorescence magnitudes, but that fractional distribution in each of the two transitions appears the same. The fluorescence burst is approxi-

TABLE 1 Summary of data

	A Fluorescence rate and magnitude (zero actin)		*B* Fluorescence rate and magnitude (10 μM actin)		*C* CHASE	*D* Quench burst magnitude (zero actin)		*E* Quench burst magnitude (10 μM actin)	
	Rel. Mag.	Rate	Rel. Mag.	Rate	Magnitude	Raw. Mag.	Cor. Mag.	Raw. Mag.	Cor. Mag.
		s^{-1}		s^{-1}					
Undig. S-1	1.0	17.5	0.70	21.4	0.79	0.60	(0.76)	0.50	(0.63)
5 min dig.	0.70*	17.4*	0.81*	18.3*	—	—	—	—	—
10 min dig.	0.53*	16.9*	0.74*	18.1*	—	—	—	—	—
10 min dig.	0.49	17.1	0.68	16.2	0.56	0.37	(0.66)	0.33	(0.59)
20 min dig.	0.56	17.1	0.77	15.8	0.58	0.38	(0.66)	0.38	(0.66)
40 min dig.	0.49	16.6	0.74	14.6	0.62	0.37	(0.60)	0.34	(0.55)
60 min dig.	0.45	16.8	0.62	14.3	0.50	0.35	(0.70)	0.34	(0.69)

The quench flow magnitude in the absence of actin and the irreversible binding magnitude appear in the table only. In columns *A* and *B*, Rel. Mag. means relative to unmanipulated S-1 taken as 1.0. In columns *D* and *E*, Cor. Mag. is the raw magnitude divided by the chase magnitude given in column *C* in the appropriate row. The values given in Columns *C*, *D*, *E* are averages from three different experiments. Conditions: Columns *A* and *B* as Fig. 3; Columns *C*, *D*, and *E* as Fig. 8.

*Data were obtained from a different protein preparation. The relative values of the fluorescence magnitudes were determined by normalizing the magnitude of the undigested S-1 from the two different preparations.

mately two-thirds of the total fluorescence enhancement both for undigested and digested S-1 (see Discussion).

Fig. 7 shows the effect of 10 μM actin on the rate and magnitude of the fluorescence transient. In each part of Fig. 7, *a* was in the absence of actin and *b* was in the presence of actin. In Fig. 7 *a*, actin caused the rate to increase from 17.5 to 21.4 s^{-1}, but the magnitude fell ~25%. In the case of digested protein the rate constant decreased slightly from 17.1 s^{-1} in the absence of actin to 16.2 s^{-1} in the presence of actin at 20 min of tryptic digestion (Fig. 7 *b*), and similarly from 16.6 to 14.6 s^{-1} at 40 min of digestion (Fig. 7 *c*). While these differences are very small, Table 1, columns *A* and *B*, shows a consistent decrease in the fluorescence rates in the presence of actin. In addition, the fluorescence magnitude *increases* with actin in Figures 7, *b* and *c* (i.e., curve *b* is higher than *a*). Based on the model in Fig. 1, it is difficult to explain the fall in fluorescence rate and the increase in magnitude in the presence of actin, observed with the digested proteins.

Chemical measurements of the magnitude of the Pi burst

One possible explanation for the anamolous effects of actin on the fluorescence of digested S-1 is that the fluorescence enhancement may not be identical to the chemical Pi burst magnitude in this case (24–26). Hence we decided to compare the fluorescence burst with direct measurements of the Pi burst using quench flow chemical techniques.

Fig. 8 shows the phosphate production, as a function of time, in the presence of actin, for S-1 after 0, 10, and 60 min of trypsin digestion. The protein concentrations used in this experiment were 15 μM S-1 and 10 μM actin, to approximate the conditions of the fluorescence burst experiments. The apparent burst magnitude (i.e., the extrapolated intercept at $t = 0$) is highest for undigested S-1, and is approximately 0.56 μmol Pi/μmol S-1. It should be mentioned that in chemical burst measurements when the steady-state phosphate production rate is high, the extrapolated magnitude obtained is an underestimation of the concentration of myosin-product states, a simple back extrapolation to zero time does not give the right correction (11). But this small error should not affect the subsequent discussion. As expected from Fig. 4, the steady-state ATPase rate drops with increasing trypsin digestion.

Fig. 8 also shows that the magnitude of the Pi burst in the presence of actin is *lower* for digested S-1 than for the undigested S-1, although the magnitude appears to be independent of the digestion time after 10 min. One possible explanation for the decreased magnitude is that the concentration of active protein has been reduced. In the case of undigested S-1, it has been found that active S-1 irreversibly binds ATP, and the fraction of active sites can be determined using chemical chase techniques (5, 24) in which (gamma-^{32}P)ATP is mixed with S-1 long enough for S-1 to bind ATP and establish an equilibrium between myosin-ATP and myosin-product states. The mixture is then chased with 100-fold excess cold ATP to prevent further radioactive ATP binding and to allow the irreversibly bound radioactive ATP to be hydrolyzed. The amount of radiolabeled Pi released in this experiment is a measure of the total number of active sites. In column *C* of Table 1 it can be seen that the chase magnitude falls

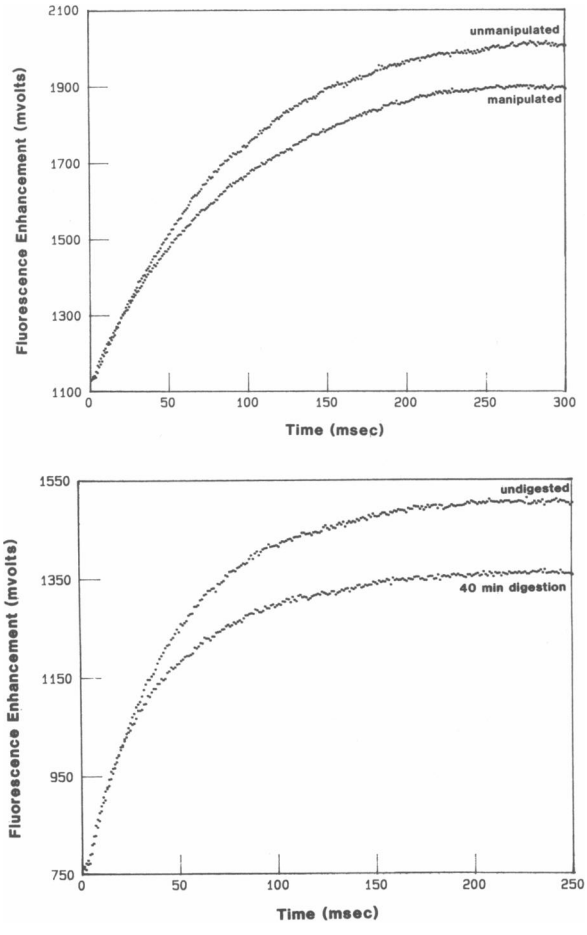

FIGURE 6 Effect of dialysis on the magnitude of the fluorescence transient. (6 *a*) In this experiment undigested S-1 was dialyzed for 24 h into high salt conditions normally used for digestion, and dialyzed back to our usual low salt conditions for 24 h. Dialyzed S-1 is compared with unmanipulated S-1 from the same preparation. Conditions as Fig. 5. (6 *b*) In this experiment S-1 was dialyzed into the high salt conditions usually used for digestion. Half of the S-1 was then digested for 40 min according to protocol, followed by the immediate measurement of the fluorescence magnitude without further manipulation. Conditions: as in Fig. 5 with the addition of 15 mM NaCl.

TABLE 2 Comparison of fluorescence magnitudes

	A Fluorescence 1 mM ATP		*B* Fluorescence 20 μM ATP	
	Rel. Mag.	Rate	Rel. Mag.	Rate
Undig. S-1	1.0	18 s^{-1}	1.52	11 s^{-1}
20 min dig.	0.46	18 s^{-1}	0.64	15.7 s^{-1}
40 min dig.	0.43	16 s^{-1}	0.67	15.7 s^{-1}

Conditions as Fig. 5, except that column *B* performed at 20 μM ATP. Rel. Mag. means relative to unmanipulated S-1 taken as 1.0.

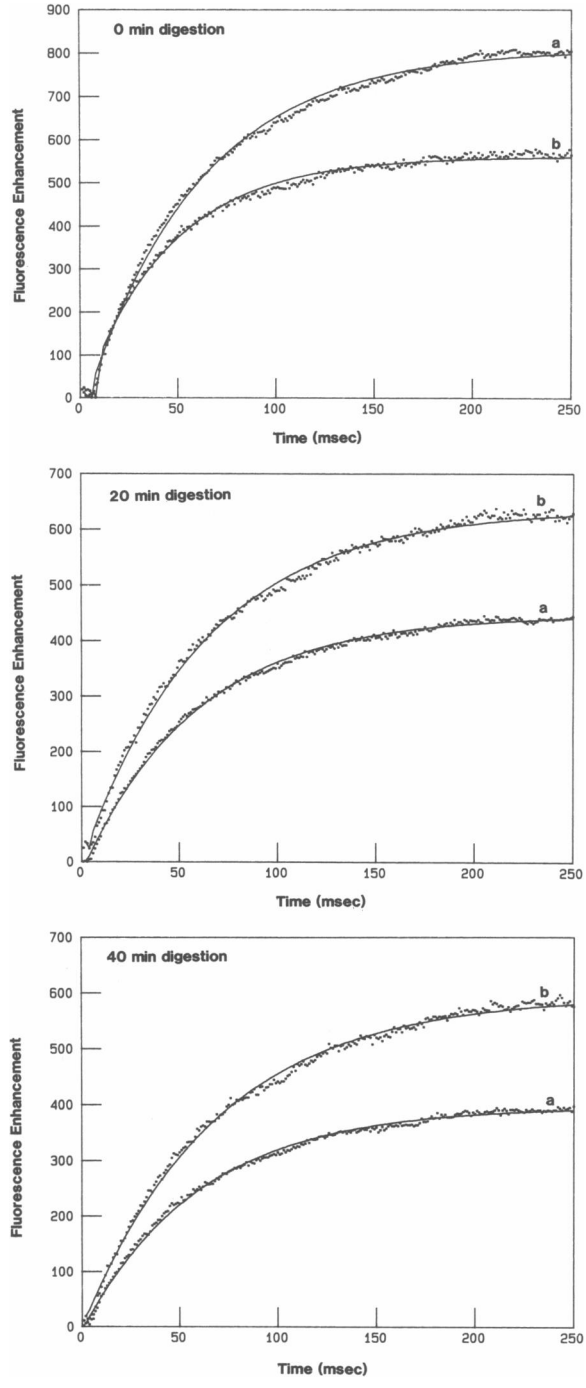

FIGURE 7 Effect of actin on the fluorescence enhancement rate and magnitude of undigested and tryptically digested S-1. (7 *a*) Undigested protein; the fluorescence magnitude in the absence of actin *a* is larger than the magnitude in the presence of actin *b*. The data are the points. The smooth line through the data is a least squares single exponential fit. Rate of the processes are: (*a*) 17.5 s^{-1}; (*b*) 21.4 s^{-1}. (7 *b*) S-1 tryptically digested for 20 min. Magnitude in the presence of actin, *b*, larger than in its absence, *a*. Rate constants are: (*a*) 17.1 s^{-1}; (*b*) 15.8 s^{-1}. (7 *c*) As 7 *b*, but after 40 min of digestion. Rate constants: (*a*) 16.6 s^{-1}; (*b*) 14.6 s^{-1}. Conditions: as in Fig. 3, except that the plots marked *a* are in the absence of actin, and plots marked *b* have 10 μM total actin present. The S-1 concentration used was 10 μM.

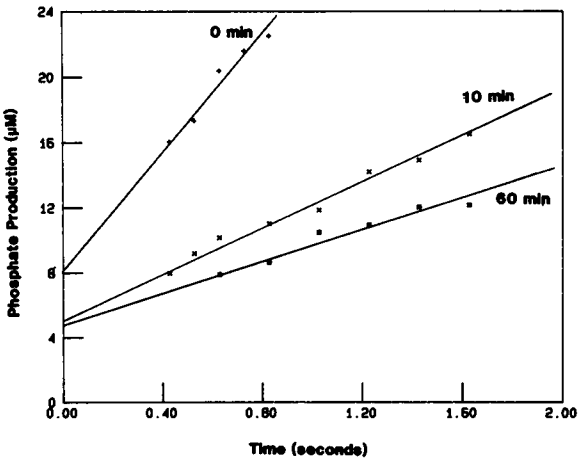

FIGURE 8 Effect of limited tryptic digestion on the quench flow magnitude in the presence of actin. In this figure the extrapolated magnitude falls as a function of the period of digestion. The steady-state rates for undigested S-1 and for S-1 after 10 and 60 min of tryptic digestion (i.e., slope of the plots) also fall as a function of digestion. Conditions: 10° C; 10 mM Imidazole, pH 7.0; 0.15 mM MgATP; 1 mM $MgCl_2$; 1 mM DTT; 4.25 mM KCl. The KCl is added to keep the ionic strength equal to Fig. 3. Also present is 10 μM total actin, 12–15 μM S-1.

with digestion, and in columns *D* it can be seen that the burst per active site (i.e., ratio of Pi burst to the chase magnitude) remains approximately constant. This implies that the fall in Pi burst observed arises from a fall in the concentration of active sites.

A comparison of columns *D* and *E* of Table 1 shows that the magnitude of the chemical Pi burst for digested S-1 either does not change significantly in the presence of actin, or falls slightly, which is in contrast with the increase in the fluorescence magnitude with actin in columns *A* and *B*. The fact that the chemical burst magnitudes for digested S-1 did not change significantly in the presence of actin is to be expected because the ATPase activity is low for these digested proteins at 10 μM actin. The magnitude of the burst for undigested S-1 decreased in the presence of actin, both in the fluorescence and chemical quench burst measurements, due presumably to the ATPase activation, and the redistribution of kinetic intermediates that result. Again it is important to determine that the drop in Pi burst is not due to the additional dialyses required for digestion. The controls carried out in this case were identical to the ones in Fig. 6, but in this case using quench flow measurements. The results are essentially the same with approximately a 15% reduction in the burst and chase magnitudes (see Methods) due to dialysis alone, and a 20–25% drop in the chase magnitude due to digestion (data not shown).

DISCUSSION

The data presented here show that during trypsin digestion of chymotryptic S-1, both $K_{binding}$ and K_{ATPase} increase (i.e., become weaker). Specifically, during the trypsin digestion of S-1 from the original 95-K dalton fragment to the three fragments of molecular weight 50, 25, and 20 K daltons (approximately complete after 30 min at 10°C), K_{ATPase} increases ~10–12-fold and $K_{binding}$ changes ~15-fold. Thus, the ratio of $K_{binding}/K_{ATPase}$ remains approximately constant (within a factor of two) independent of the degree of digestion by trypsin, supporting the hypothesis that the binding of actin to the myosin nucleotide complexes is directly responsible for activation as depicted in the current models for activation (see Fig. 1).

The stopped flow measurements are remarkable for showing that in fact a significant burst is observed with digested S-1, and the rate of the burst in the absence of actin is unaltered by digestion. A comment should be made about the actin concentration used in the stopped flow experiments reported here. In prior work with undigested S-1 (5–7, 9, 10), a special attempt was made to achieve an actin concentration equal to $K_{binding}$ so that half the S-1 was actin bound during the measurement of the fluorescence transient. However, in the case of trypsin-digested S-1 this is not feasible because digestion significantly increases $K_{binding}$. Generally, at final actin concentrations >20 μM, difficulties in mixing cause noisy exponentials, requiring the averaging of many traces. We tried to avoid this problem by working at a moderate actin concentration where mixing is satisfactory, and where significant activation of the ATPase activity is achieved. The weakest K_{ATPase} measured was ~30 μM, and, therefore, at 10 μM actin at least 25% V_{max} was achieved in all experiments. Because the quench flow experiments were performed for comparison with the stopped flow measurements, the same actin concentrations were used in quench experiments as well.

The stopped flow fluorescence data also show that the magnitude of the fluorescence signal rises in the presence of actin. In the case of undigested S-1 it is observed that the magnitude drops in the presence of actin, and this is generally accepted to be due to the actin activation of the ATPase activity, leading to a shift in the relative distribution of the M*ATP and M**ADPPi states. Because activation is lower for the digested species it should be expected that either the magnitude remain constant or fall slightly in the presence of actin. One possible explanation for the rise in fluorescence magnitude observed in the presence of actin is that actin may have an effect on the rate of the AM + ATP $\rightarrow$ M*ATP + A transition. As has been demonstrated before (24–26), the tryptophan fluorescence enhancement of S-1 at saturating ATP

consists of two transitions: A fast fluorescence change presumably due to the M + ATP to M*ATP transition (which tends to be over within the dead time of the apparatus at 1 mM ATP), and a slower fluorescence change generally considered equivalent to the initial Pi burst (M*ATP → M**ADPPi). Table 2 shows the comparison of fluorescence magnitudes at 1 mM and 20 μM ATP. At 20 μM ATP, the rate of ATP binding to S-1 decreases, allowing the first fluorescence transient to be observed. In Table 2, the magnitude at 20 μM ATP is ~50% larger both for undigested and trypsin digested S-1. The interesting fact is that the fluorescence magnitude of digested S-1 in the presence of actin (see Table 1) is approximately equal to the fluorescence magnitude at 20 μM ATP in the absence of actin, and the corresponding fluorescence rates are approximately equal as well. Hence, actin may significantly slow the rate of the first fluorescence transient for digested S-1, although how it does so remains unclear.

The data in Table 1 show that the "chase" magnitude (column *C*) and the quench magnitude (column *D*) fall about the same percentage with digestion. We have not investigated whether the chase measurement of digested S-1 is actually a measure of the fraction of active sites, but it is interesting that the burst magnitude is approximately correlated with the chase magnitude. This may suggest that the drop in the burst magnitude is due to a drop in the number of active sites rather than a change in the burst of active molecules. If the chemical burst magnitudes are divided by the chase magnitudes (column *D*), then the "corrected" Quench Burst magnitude for digested proteins is seen to be almost unchanged from the value for undigested proteins. This may imply that digestion simply denatures some of the active sites, whereas others remain active.

In the presence of actin, digested S-1 shows either that the burst magnitude is unchanged or falls slightly (Table 1, column *E*) as compared with the burst in the absence of actin, and therefore the rise in fluorescence magnitude (column *B*) is not due to an increase in population of the myosin-product states. The magnitudes of the chemical burst and the fluorescence burst of undigested S-1 fell 20 and 30%, respectively, at 10 μM actin, and this decrease is generally attributed to the increasing ATPase rate which results in a shift toward the M*T state lowering the magnitude of the burst.

In summary then, tryptically digested S-1 appears to obey the same kind of kinetic scheme for actin activation as does undigested S-1. We have found that the activation constant correlates with the binding affinity, and that V_{max} remains approximately constant. In addition, we have shown that the tryptophan fluorescence enhancement rate remains approximately constant with tryptic digestion, and although the magnitude of the burst falls with digestion, it remains a significant fraction of the burst magnitude of undigested S-1.

The only anomalous behavior observed was that the fluorescence magnitude of digested S-1 increased in the presence of actin, and this may be an actin effect on the rate of ATP binding to the AM complex.

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