Bovine Cardiac Myosin Subfragment 1

TRANSIENT KINETICS OF ATP HYDROLYSIS

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The kinetics of binding and hydrolysis of ATP by bovine cardiac myosin subfragment 1 has been reinvestigated. More than 90% of the total fluorescence amplitude associated with ATP hydrolysis occurs with an apparent second-order rate constant of $8.1 \times 10^7 \text{m}^{-1} \text{s}^{-1}$ and a limiting rate constant of $\sim 140 \text{s}^{-1}$ (100 mM KCl, 50 mM 1,3-bis-(tris(hydroxymethyl)methylamino)-propane, 10 mM MgCl₂, pH 7.0, 20°C); the remaining 10% occurs more slowly ($\sim 1 \text{s}^{-1}$). The observed rate constants are independent of subfragment 1 concentration under pseudo first-order conditions for ATP with respect to protein. The fraction of protein which hydrolyzes ATP rapidly is not a function of the nucleotide or protein concentration and appears to be constant irrespective of ionic strength or temperature within the range studied (50–100 mM KCl, pH 7.0, 15–20°C). These data are compared to that obtained previously using subfragment 1 prepared by a different method which showed ATP-dependent aggregation of two protein species.

Previous studies have shown that cardiac myosin subfragment 1 is kinetically heterogeneous when reacted with ATP and ADP (1–3). This heterogeneity complicates kinetic analysis, and hence the transient kinetics of ATP hydrolysis by cardiac S1† are less well understood than the corresponding processes for skeletal S1. This is particularly unfortunate, since comparison of the properties of cardiac S1 and skeletal S1 could extend our knowledge of the relationship between the enzymatic properties of myosin in solution and the physiological properties of the corresponding muscle. Furthermore, such detailed information is necessary to understand the molecular basis of changes in cardiac contractility induced by adaptive or disease states, e.g. overload hypertrophy and thyrotoxicosis (3–7). Recently, Flamig and Cusanovich (2, 3) demonstrated that bovine cardiac S1 could be prepared which, although kinetically heterogeneous, was resolvable into two components: a monomeric component which hydrolyzed ATP rapidly and was interconvertible with a slower reacting species of higher molecular weight. Nevertheless, the system remained complex and was not ideal for more detailed analysis.

We report here that cardiac S1 prepared by a different method does not show such aggregation-linked kinetic heterogeneity. This greatly simplifies the analysis of kinetic data and provides a material which is tractable in kinetic studies. The kinetic properties of this protein will be shown to be essentially identical to those of the monomeric species of the cardiac S1 previously isolated.

MATERIALS AND METHODS

Bovine cardiac myosin was prepared from left ventricles as described by Siemankowski and White (8). This procedure differed from our previous preparation in that the crude myosin was extracted from myofibrils rather than minced muscle. Crude myosin was precipitated by dialysis to low ionic strength, and any remaining actomyosin was dissociated by ATP (20 mM); actin was removed by high-speed centrifugation, and myosin was obtained by ammonium sulfate fractionation of the resulting supernatant (37–45% saturation). S1 was prepared by chymotryptic digestion as described previously (2, 3) except that the buffer contained 0.6 M NaCl and 1 mM EDTA and the DEAE-cellulose chromatography step was omitted as this results in S1 which has a significantly lower actin-activated ATPase activity.

Material having maximum actin-activated ATPase activity precipitated between 50 and 55% saturation with ammonium sulfate. S1 was used immediately after dialysis into the appropriate buffer or was lyopholized (at approximately 50 mg/ml) in 10 mM MOPS, 50 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.02% azide, pH 7.0, in the presence of 5 mg of sucrose/mg of protein. Lyopholization did not alter the concentration of active sites/mole protein or the actin-activated ATPase activity (see below).

The concentration of cardiac myosin was determined using $A_{280} = 5.53 \text{cm}^{-1}$, a light-scattering correction of $1.4 \times A_{280}$, and a molecular weight of 468,000. The concentration of cardiac S1 was determined using $A_{280} = 6.4 \text{cm}^{-1}$ (9) and a molecular weight of 115,000.

The number of active sites/mole of S1 was estimated essentially by the method of Hackney and Clark (10). The reaction mixture contained 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 5 mM A₅₅, 44 units of lactate dehydrogenase, 30 units of pyruvate kinase, and approximately 10 nmol of S1 in 1 ml of 0.1 M KCl, 50 mM BTP, 10 mM MgCl₂, 0.1 mM dithiothreitol, pH 7.0, at 22°C. The coupling enzymes were dialyzed into the buffer before the assay to remove ammonium sulfate. Aliquots of ATP (1 or 2 nmol) were added until the rate of decrease of $A_{550}$ did not change on further addition of nucleotide. The steady state Mg-ATPase activity of S1 was measured at 22°C using the same assay system, initiating the reaction by addition of ATP to give a final concentration of 80 μM to 2 mM (1). The actin-activated ATPase activity of S1 was followed in a pH stat at pH 7.0 and 25°C (11). The assay mixture contained 1 mg of S1 in 10 mM KCl, 0.1 mM dithiothreitol, 2 mM MgCl₂, and 4–20 mM actin. Cardiac actin was used in these assays and was prepared from acetone powder of cardiac muscle (8). The concentration of cardiac actin was determined using $A_{280} = 1.15 \text{cm}^{-1}$. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out as described previously (12) and showed that the S1 was free of actin contamination.

The following chemicals were obtained from Sigma: ATP (Sigma grade vanadium-free used for kinetic experiments and grade 11 used for preparative procedures), chymotrypsin type 1-S, dithiothreitol, BTP, and lima bean trypsin inhibitor. MOPS was Ultra grade from Calbiochem-Behring. Ammonium sulfate absolute grade was obtained.
The transient kinetic apparatus and methods of data collection and analysis used in this work have been described (3). For each nucleotide concentration, 8–10 replicates of 200 data points were averaged and stored for further analysis. The first 100–150 data points were collected over a time span of approximately five half-times to characterize the fast reaction (see below); the remaining 50–100 data points were collected on a slower time scale. The protein was stored on ice until used; the drive syringes were filled and allowed to equilibrate to the appropriate temperature. A two-exponential non-linear least-squares fit was superior to a single-exponential fit for most of the ATP binding data with respect to the sum of the squared residuals, runs of residuals, and estimated parameter errors from the covariance matrix. Data for the fast-reacting component are analyzed in terms of a four-step mechanism for the binding and rapid hydrolysis of ATP (13).

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M + ATP \rightarrow^1 MATP \rightarrow^2 M^*ATP \rightarrow^3 M^{**ADP-P} \rightarrow^4 M^*ADP-P
\]

RESULTS

Each preparation of cardiac S1 was characterized by measurement of three parameters: \( k_{o.b} \) (in the usual reaction scheme, see Equation 1) was obtained from the steady-state Mg-ATPase activity and from single-turnover experiments (3); the actin-activated ATPase activity and the number of active sites/molecule of S1 were also estimated. Typical values for the steady-state ATPase \( k_{o.b} \) were 0.017 ± 0.002 s\(^{-1}\), for \( k_4 \) from single-turnover experiments 0.015 ± 0.002 s\(^{-1}\), and for actin-activated ATPase \( K_{\text{act}} \), actin = 36 μM and \( V_{\text{max}} \) = 6.6 s\(^{-1}\). These values agree quite well with those previously reported (1–3) and establish that the major functional properties of the S1 used are similar to those of previous preparations. However, in single-turnover experiments, the reaction curves were accurately first-order (Fig. 1a), unlike the situation previously reported (3). The number of active sites/molecule of S1 was found to be 0.8 ± 0.1 for the different preparations used. This number is in reasonable agreement with the value of 0.9 determined by Taylor and Weeds (1) from the amplitude of the phosphate burst on ATP binding corrected for reversibility of the ATP cleavage step.

Addition of ATP to cardiac S1 produced a rapid increase in protein fluorescence. A typical kinetic trace is given in Fig. 1b and indicates that the reaction was accurately pseudo first-order for approximately three half-lives with a very slow kinetic species \( k_{o.b} = 1 \text{ s}^{-1} \) accounting for 5–10% of the total fluorescence amplitude. The observed rate constants for the fast reaction calculated from these traces were plotted against ATP concentration. Two examples are shown in Fig. 2a. The corresponding amplitudes of the fast fluorescence change induced by ATP binding to cardiac S1 are shown in Fig. 2b; the values plotted have been corrected for the expected loss of amplitude due to ATP hydrolysis occurring during the dead time of the instrument (see “Materials and Methods”). The maximum amplitude of the corrected fast fluorescence change is typically 22–27%.

The transient kinetics associated with ATP hydrolysis were monitored in two buffer systems: MOPS (5 mM MOPS, 0.05 mM KCl, 5 mM MgCl\(_2\), 0.1 mM dithiothreitol, pH 7.0, \( \mu = 135 \text{ mM} \)) and BTP (50 mM BTP, 0.1 mM KCl, 10 mM MgCl\(_2\), 0.1 mM dithiothreitol, pH 7.0, \( \mu = 230 \text{ mM} \)). These conditions were chosen to allow comparison between previous data from this laboratory and that of a recent study in the kinetics of ATP hydrolysis by cardiac actomyosin (8). In view of the inability of cardiac S1, the investigations were performed at two different temperatures (15 and 20 °C) to determine whether temperature altered the kinetic behavior (see below). The rate constants for ATP hydrolysis are summarized in Table I. The value of the apparent second-order rate constant of ATP hydrolysis \( (K_{b}k_{4}) \) was decreased approximately 4–5-fold at higher ionic strength, while the maximum rate constant of the fluorescence change \( (k_4 + k_{-4}) \) was only slightly increased. These findings are similar to those reported by Johnson and Taylor (14) and Chock et al. (15) for skeletal myosin S1 and suggest that the initial association of Mg-ATP with the binding site involves charge neutralization. In a given buffer, the values for the apparent second-order ATP binding constant increased approximately 2-fold over the temperature.

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K_{b}k_{4} = \frac{1}{[ATP]} \text{and for } [ADP-P_i] \text{ at high } [ATP]
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range 15–20 °C. The maximum rate constant of the fluorescence change (k + k -3) increased by a factor of about 1.5 at both high and low ionic strength over the same temperature range. No large differences in the fraction of the fluorescence amplitude contributed by the fast reaction were observed at lower ionic strength and/or temperature (Table I). The rate constants and amplitudes (corrected for instrument dead-time) of the fluorescence transient following the mixing of cardiac S1 and ATP (Fig. 2) are qualitatively similar to those observed for skeletal S1 and ATP (14). This is consistent with the proposed three-step mechanism for the binding and rapid hydrolysis of ATP (with two steps involving changes in protein fluorescence) prior to the steady-state process (14). The observed rate constant at high ATP (2.5 mM) in MOPS is slightly less than that at lower concentrations of nucleotide (Fig. 2a). A similar decrease in kobs is seen with skeletal S1 (10 mM Tris/Mes, 0.1 M KCl, 20 °C; Ref. 14). The differences in the rate constants and amplitudes of the fluorescence transient (Fig. 2) in the two buffer systems studied (MOPS and BTP) suggest ionic-strength or buffer-ion effects on the individual rate constants of the reaction mechanism (Equation 1). These differences will be analyzed in detail in a later publication.

An important point relative to the kinetic heterogeneity is the contribution and nature of the slow phase. The relevant data are summarized in Table I and demonstrate that the contribution of the slow phase to the total fluorescence change is small (5–12% of the total) and is apparently independent of ATP concentration. The standard deviations given for kobs in Table I are for data over the ATP concentration range studied (15 μM to 2.5 mM). Although the standard deviations are large, due to the small signals, there are no trends that suggest dependence on ATP concentration as previously observed (2).

The inset in Fig. 2a gives the ATP concentration dependence of the slow rate constant for the slow kinetic species (MOPS, 15 °C) which is typical for the other conditions studied.

To test whether the transient kinetics of ATP hydrolysis were influenced by aggregation of cardiac S1 (2), observations were made at three protein concentrations (1, 2, and 4 μM after mixing). These data are summarized in Fig. 3 and within experimental error show no effect of protein concentration on the transient kinetics of ATP hydrolysis.

**DISCUSSION**

In the present study, we have observed that the time course of the fluorescence transient on addition of ATP to cardiac S1 is biphasic (1–3). The kinetic constants K1 and k + k -3 for the fast reaction correspond closely to those reported by Flamig and Cusanovich (3), under the same conditions, and are similar to those of Taylor and Weeds (1). These values are summarized in Table II. The slow fluorescence transient observed in these experiments is characterized by kobs ~1 s -1 at all tested concentrations of ATP (15 μM to 2.5 mM) and protein (1–4 μM S1). The contribution of the slow fluorescence transient was typically less than 10% of the total amplitude. It is difficult to attribute any mechanistic significance to the slow kinetic species. In typical stopped-flow studies of proteins, only three to four half-lives are reported and kinetic heterogeneity is routinely observed at long times. Cardiac S1 is known to be quite labile (1, 2), and therefore it seems likely that the slow phase of the fluorescence does not reflect a step in the mechanism of binding and hydrolysis of ATP. The fast phase of the fluorescence transient for cardiac S1 appears to obey the same kinetic mechanism that can be assigned to skeletal S1 (14). It should be stressed that the single-turnover experiments (Fig. 1a) demonstrate that k is kinetically homogeneous and is identical to k measured by ADP formation in steady-state experiments. Hence, only a single kinetic species appears to form products. The most plausible explanation for the origin of the slow kinetic species is that it is an unreactive conformer of cardiac S1 which slowly converted (kobs ~1 s -1) into the active form. This would explain the lack of effect of ATP concentration on kobs and is consistent with the observation that the rate constant for the slow phase is
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independent of S1 concentration (see below). This would be analogous to the well-established interconversion of ascorbate-reducible and -nonreducible cytochrome c at alkaline pH (16). There is no compelling reason to invoke a more complex reaction pathway such as a sequential mechanism with an additional step \( k_1 \) between steps 3 and 4 in Equation 1 or a branched pathway, although these cannot be rigorously excluded. Alternatively, the slow phase could be due to a small fraction of the S1, which has a low \( K_m \) for ATP (<15 \( \mu \)M ATP) and a maximum rate of fluorescence change \( (k_3 + k_{-3}) \) of ~1 s\(^{-1}\); a component of cardiac S1, which has a \( K_m \) for ATP of 4 \( \mu \)M and a low ATPase activity, has been observed by Taylor and Weeds (1). However, the results of single-turnover experiments indicate that \( k_4 \) would have to be identical for both kinetic species; thus, this possibility is unlikely.

Previously, the biphasic nature of the binding of nucleotides to cardiac S1 had been explained in terms of ATP-dependent reversible aggregation of two protein species (2, 3). Our observations that the rate constant of the slow fluorescence change is not dependent on either the ATP or protein concentration (up to 4 \( \mu \)M S1) are inconsistent with a rapidly reversible polymerization of the protein of the type reported for previous preparations (2); in those studies, the proportion of the higher molecular weight aggregate increased over the S1 concentration range used here \( (K_r = 2 \mu M) \). Higher concentrations of S1 may well show some aggregation; however, such studies are beyond the scope of this work where the emphasis has been on obtaining a preparation and conditions suitable for transient kinetic studies. The extent of the slow reaction is not altered by changing factors likely to modify the degree of aggregation of the protein; no large differences in the amplitude contributed by the slow reaction were observed at lower ionic strength or temperature.

Based on the experiments to date, high ionic strength and low temperature (15 °C) seem to be the most suitable conditions for investigating the mechanism of ATP hydrolysis by cardiac S1; under these conditions, the contribution of the slow phase to the total fluorescence transient is minimal (~5%).

In conclusion, cardiac S1 can be prepared which is predominantly a single kinetic species. This protein has kinetic properties which are very similar to those of the monomeric species of cardiac S1 observed previously (2), but are quantitatively distinct from those of S1 from rabbit skeletal muscle.

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REFERENCES

Bovine cardiac myosin subfragment 1. Transient kinetics of ATP hydrolysis.
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