TRANSIENT-PHASE OF ATP HYDROLYSIS BY MYOSIN SUB-FRAGMENT-1 ISOENZYMES

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1. Introduction

We have recently shown that isoenzymes of sub-fragment-1, prepared by chymotryptic digestion of rabbit skeletal-muscle myosin, may be separated on DEAE-cellulose according to their alkali light-chain content [1]. The steady-state ATPase activities of these two isoenzymes were identical in the absence of actin, but the actin-activated ATPase showed differences both in the maximum turnover rate of hydrolysis (k_{cat}) and the apparent affinity for actin (K_m), which are clearly related to the particular light chain present [1,2]. These results indicated differences in the elementary processes of ATP hydrolysis by these isoenzymes and it was therefore of interest to investigate the transient-phase of ATP hydrolysis for possible differences in the mechanism of ATP-cleavage. This study was further prompted in an attempt to resolve the current controversy concerning the amplitude of the transient-phase of phosphate production (the phosphate 'early burst') and its relationship to the possible existence of two distinct routes of ATP hydrolysis by myosin and its proteolytic sub-fragments. These two routes were postulated on the basis of a burst-amplitude of one mol P_i/mol myosin (i.e., 0.5 mol/sub-fragment-1) [3,4]. Measurement of the stoicheiometry of the phosphate burst is complicated by a number of factors which have been reviewed recently [5]. In particular, the presence of inactive enzyme will produce low values as has been shown in the case of cardiac myosin [6]. According to the mechanism of Lymn and Taylor [7] and extended by Bagshaw et al. [8], Mg·ATP is hydrolysed by myosin as follows:

$$M + \mathrm{ATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} M\cdot\mathrm{ATP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} M^*\cdot\mathrm{ATP}$$

$$\underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} M^{**}\cdot\mathrm{ADP}\cdot P_i \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} M^*\cdot\mathrm{ADP}\cdot P_i \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} M^*\cdot\mathrm{ADP} +$$

$$P_i \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} M\cdot\mathrm{ADP} \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} M + \mathrm{ADP}$$

where M denotes sub-fragment-1 and the asterisks are used to distinguish different intermediates (and also indicate species of enhanced protein fluorescence relative to M). The steady-state rate is controlled by $k_{+4} = 0.06\ s^{-1}$ and since the process of ATP-cleavage is readily reversible [9], the steady-state intermediate is an equilibrium-mixture of M^*·ATP and M^{**}·ADP·P_i. The presence of this equilibrium-mixture will also reduce the amount of ADP bound to the enzyme in the steady-state and hence produce a burst-amplitude of less than unity. The most recent studies of the phosphate 'early burst' by Taylor [10] have shown variation according to pH, temperature and ATP concentration, which may reflect differences in the equilibrium constant K_3. Nevertheless, at ATP concentrations greater than 10^{-5} M, the burst-amplitude was about 0.8 mol P_i/sub-fragment-1 site, suggesting that all the active sites were capable of hydrolysing ATP by this mechanism. Other papers supporting two different mechanisms for ATP-cleavage have claimed isolation of sub-fragment-1 species which have different burst-amplitudes consistent with the two distinct routes of

Abbreviations: S-1 (A1) and S-1 (A2) are sub-fragment-1 isoenzymes containing the alkali-1 and alkali-2 light-chains respectively

ATP hydrolysis [11,12]. Our separation of structurally different sub-fragment-1 isoenzymes offered an opportunity to examine the mechanism of ATP-cleavage.

2. Methods

Chymotryptic sub-fragment-1 was prepared as described previously and separated into two components, S-1 (A1) and S-1 (A2) according to the particular alkali light-chain [1]. These sub-fragment-1 isoenzymes contained no DTNB light-chains. The homogeneity of the preparations can be seen from gel-electrophoresis of the native proteins (fig.1).

Steady-state ATPase activities in the presence of Mg^{2+}-ions were carried out as described previously [6]. ATP-Binding was determined by the enhancement of protein fluorescence using a stopped-flow spectrofluorimeter built by Dr A. F. Fersht [13]. The maximum rate of ATP-binding, at pH 8.0, is 400 s^{-1} [8] but the observed rate decreases at lower pH-values [14]. Experiments were carried out, at pH 6.5, where the maximum rate is about 100 s^{-1}, giving little loss of fluorescence signal within the dead-time of the instrument. Experiments were carried out in 0.1 M NaCl, 0.05 M piperazine-*NN'*-bis (2 ethane sulphonate), 5 mM $MgCl_2$ at pH 6.5 and 25°C.

Hydrolysis of [γ^{32}P] ATP was determined using the quenched-flow apparatus built by Dr A. R. Fersht [15]. The equilibrium constant (K_3) was determined from single turnover experiments in which sub-fragment-1 (14.6 μM) was mixed with 4.5 μM [γ-^{32}P] ATP in 0.1 M KCl, 0.05 M Tris–HCl, 5 mM $MgCl_2$, pH 8.0, at 25°C and the reaction quenched with 7% $HClO_4$ as described previously [9]. The $^{32}P_i$ liberated was separated from substrate by thin-layer chromatography on polyethyleneimine-cellulose, and the amount of $^{32}P_i$ determined as a percentage of initial [γ-^{32}P] ATP. Time-zero points were obtained during the experiment by mixing the protein with quenching solution before addition of labelled ATP. The assay counts could then be corrected for $^{32}P_i$ contamination originally present in the stock ATP and that which arose during $HClO_4$ treatment. Multiple turnover experiments were also carried out to determine the steady-state rate of ATP-cleavage and the amplitude of the transient-phase of phosphate production.

Fig.1. Polyacrylamide gel electrophoresis of native sub-fragment-1 preparations under non-dissociating conditions in pyrophosphate/glycine buffers (see ref. [16] for details). A, mixed sub-fragment-1 from chymotryptic digest. B, S-1 (A1) and C, S-1 (A2) separated on DEAE-cellulose (with electrophoresis carried out for a longer time than in A).

Two techniques were used to assay the $^{32}P_i$ released. The first used the quenched-flow apparatus described above with chromatographic separation of substrate and products. The second method was carried out manually as follows:

(i) Samples of protein and radioactive ATP were mixed on a magnetic stirrer and at regular time intervals 100 μl aliquots were withdrawn and rapidly mixed with 100 μl of a quenching solution containing 1.0 N HCl, 0.35 M KH_2PO_4 on a Vortex mixer.

(ii) From a suspension of 2% (w/v) activated charcoal 100 μl was added to adsorb the nucleotides and 50 μl of the resultant mixture were taken for scintillation counting as were 50 μl of supernatant after centrifugation in a Beckman 'Microfuge'.

(iii) A water-miscible scintillant was used as described previously [6]. Zero-time blanks were determined as above for correction of the time course of ATP-cleavage and the experimental conditions were identical to those used for the single turnover experiments.

This method was originally described by Fersht and Kaethner [15].

3. Results and discussion

3.1. *Kinetics of ATP-binding*

Values for k_{cat} and K_m for the Mg^{2+}-dependent ATPase activity measured by the linked-assay system were 0.05 s^{-1} and 3.0 μM for S-1 (A1) and 0.04 s^{-1} and 2.6 μM for S-1 (A2). Figure 2 shows the increase

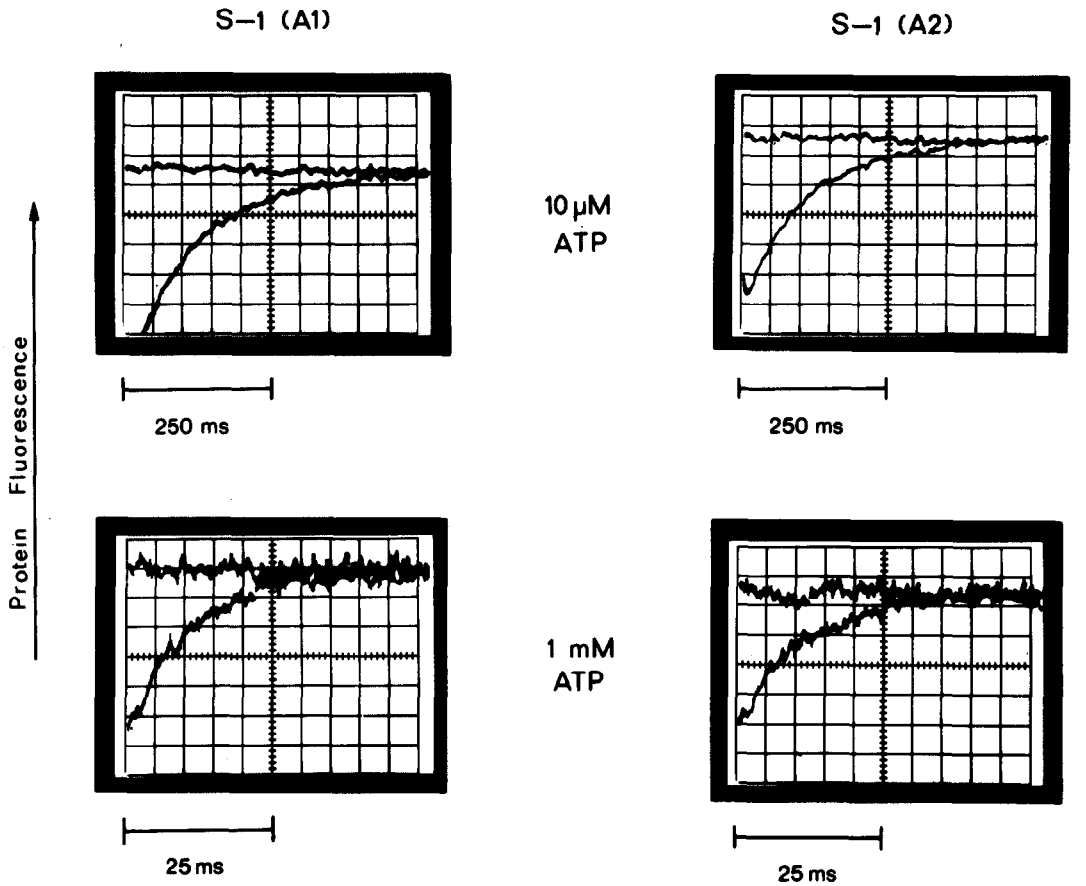

Fig.2. Stopped-flow records of protein fluorescence enhancement when sub-fragment-1 is mixed with ATP. One syringe contained 5.0 μM sub-fragment-1 and the other ATP (concentrations after mixing). For other details see Methods. The horizontal-traces show the protein fluorescence of the steady-state intermediate.

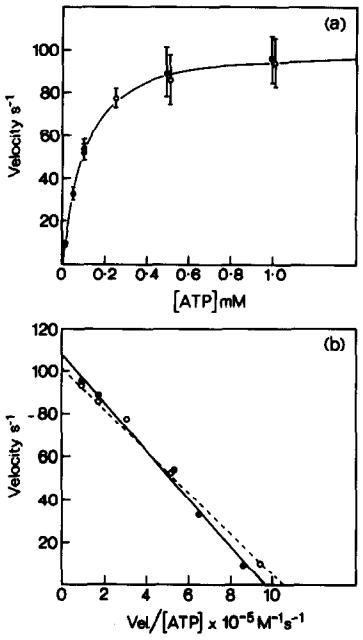

Fig.3. Kinetic analysis of the fluorescence enhancement of sub-fragment-1 on interaction with ATP. Data from stopped-flow traces (fig.2) were analysed as exponential processes to derive the velocity of reaction. (a) Plot of velocity against ATP concentration. (b) Eadie-Hofstee plot used to derive k_{+2} and K_1. S-1 (A1), (—•—). S-1 (A2), (—○—).

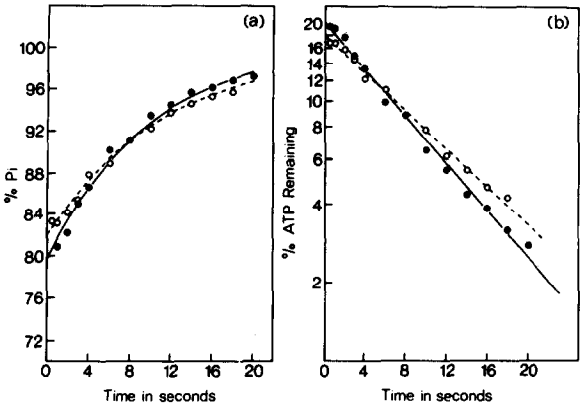

Fig.4. Production of $^{32}P_i$ from [γ-^{32}P]ATP catalysed by sub-fragment-1. One syringe contained 14.6 μM sub-fragment-1 and the other 4.5 μM ATP (concentrations after mixing). (a) Initial production of phosphate. (b) Semi-log plot of [γ-^{32}P]ATP remaining unhydrolysed. The lines in (a) were replotted from (b). S-1 (A1), (—•—). S-1 (A2), (---○---).

in protein fluorescence on ATP-binding to sub-fragment-1, the rate being described by a single exponential process. Figure 3 shows the dependence of these observed rates on ATP concentration. The data were analysed by Eadie-Hofstee plots (fig.3b) giving values of k_{+2} = 107 (± 4.6) s^{-1} for S-1 (A1) and 102 (± 3.3) s^{-1} for S-1 (A2). Corresponding values for the association constant K_1, were 9.0 X 10^3 M^{-1} and 10.4 X 10^3 M^{-1} respectively. Thus there is no significant difference between the ATP-binding characteristics of these two sub-fragment-1 isoenzymes. Other preparations of the isoenzymes gave similar results. These values compare with those obtained by Sleep and Taylor [14] for papain sub-fragment-1, at pH 6.5 and 20°C:

$$k_{+2} = 95\ s^{-1} \text{ and } K_1 = 9.5 \times 10^3\ M^{-1}.$$

3.2. *ATP-Cleavage during a single turnover of sub-fragment-1 ATPase*

The percentage $^{32}P_i$ cleaved when excess sub-fragment-1 was mixed with ATP was measured by quenched-flow (fig.4a). Under the conditions of the experiment, ATP-binding is very rapid with an estimated half-time of about 0.06 s [14] to produce an equilibrium-mixture of $M^*\cdot ATP \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} M^{**}\cdot ADP\cdot P_i$,

which decays at a slow exponential rate equivalent to $k_{+3} \cdot k_{+4}/(k_{+3} + k_{-3})$ (see Ref. [9] for details of the kinetic arguments). The semilog plot (fig.4b) shows an exponential rate of decay, 0.10 s^{-1} for S-1 (A1) and 0.083 s^{-1} for S-1 (A2), in agreement with the results previously reported for sub-fragment-1 produced by papain digestion [9]. Extrapolation of these measurements to zero time gives the proportions of ATP and ADP + P_i bound to sub-fragment-1 in the equilibrium-mixture. In both cases nearly 20% of the bound nucleotide is present as ATP from which values of K_3, 3.94 (for S-1 (A1)) and 4.58 (for S-1 (A2)), were obtained. Thus there is little difference in the equilibrium constant K_3 for the two sub-fragment-1 species, though both values were somewhat lower than those previously reported for papain-produced sub-fragment-1 (K_3 = 9) [9]. The discrepancy between these different results may be due to differences in the experimental methods used or to variation between chymotryptic- and papain-prepared sub-fragment-1.

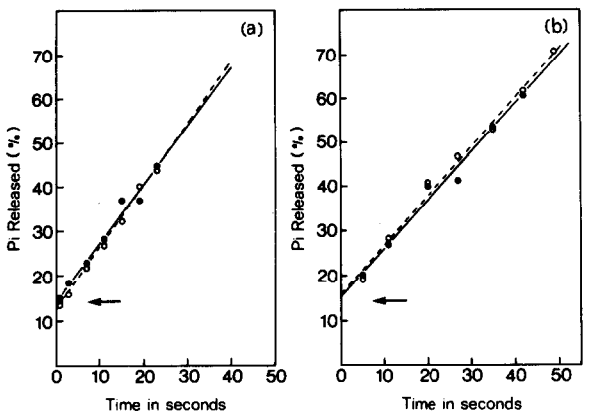

Fig.5. Production of $^{32}P_i$ from [γ-^{32}P]ATP catalysed by sub-fragment-1. (a) One syringe contained 4.8 μM sub-fragment-1 and the other 28.4 μM ATP (concentrations after mixing). (b) Manual quench technique under similar conditions. S-1 (A1), (——•——). S-1 (A2), (---o---). The arrow indicates a stoicheiometry of total bound-nucleotide, 1.0.

3.3. *ATP-Cleavage during multiple turnovers and phosphate early burst*

Multiple turnover experiments were carried out using both quenched-flow and manual quenching techniques (fig.5). The mean-values for the steady-state ATPase activities obtained from these experiments and others shown in table 1 were 0.064 s^{-1} for S-1 (A1) and 0.062 s^{-1} for S-1 (A2). These values are slightly higher than those reported above using the linked-assay system, but in good agreement with values published elsewhere [8]. Table 1 shows the amplitudes of the transient-phase of phosphate production for the two isoenzymes and values calculated for the stoicheiometry of total bound-nucleotide after correcting for the reversibility of ATP-cleavage. The mean-values obtained from these data were 0.99 ± 0.11 for S-1 (A1) and 1.00 ± 0.11 for S-1 (A2).

4. Conclusions

Transient kinetic experiments on the binding and cleavage of ATP by the two chemically distinct isoenzymes of sub-fragment-1, obtained after chymotryptic digestion of myosin, showed no significant differences between these two components. The stoicheiometry of bound-nucleotide in the transient-phase is 1.0 mol/mol of sub-fragment-1. We believe that values of unity have been obtained because of the high degree of purity of these preparations and their resultant high enzymatic activity. These results support the conclusions of Taylor [14] and those from other types of nucleotide-binding experiments (see review ref. [5]) that the stoicheiometry of nucleotide binding is 1 mol/mol sub-fragment-1. We feel that the observation of Tonomura and others [3,4] of much lower values for the stoicheiometry may be due in part to the presence of inactive protein or other impurities, similar to our own earlier experiments with cardiac sub-fragment-1 preparations [6].

Table 1
Amplitude of transient phase of ATP hydrolysis by sub-fragment-1 isoenzymes and stoicheiometry of bound nucleotide

	Quenched-flow method		Manual quench	
	Burst-amplitude (mol P_i/S-1)	Bound-nucleotide Total (mol/S-1)	Burst-amplitude (mol P_i/S-1)	Bound-nucleotide Total (mol/S-1)
S-1 (A1) No. 1	0.85	1.07	0.91	1.13
S-1 (A2) No. 2	0.72	0.88	0.89	1.08
S-1 (A1) No. 2	0.70	0.88	0.76[a]	0.95[a]
S-1 (A2) No. 2	0.69	0.84	0.85[a]	1.04[a]

The values for total bound nucleotide are obtained by correcting the phosphate burst for the reversibility of ATP-cleavage (values multiplied by $(K_3 + 1)/K_3$).

[a] These results are the mean of 4 different experiments on each sample

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