Protein–protein interactions and their contribution in stabilizing frog myosin

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1. INTRODUCTION

An understanding of the ATPase kinetics of muscle myosin and of its proteolytic fragment, subfragment 1, is important for the interpretation of the contractile characteristics of muscle in biochemical terms. Since the physiological investigation of contraction has largely concentrated on frog muscle, the study of frog muscle myosin and subfragment 1 is particularly relevant. Frog muscle myosin and subfragment 1 are considerably more labile than the equivalent rabbit proteins [1, 2]. This presents serious problems when characterizing the ATPase mechanism or other properties of the proteins. Here, we show how activity can be maintained or restored following partial inactivation. The methods rely on protein–protein interactions and so afford some insight into the role of such interactions in stabilizing myosin and enabling it to take up an active ATPase conformation.

An important characteristic of myosin and subfragment 1 is the rapid phosphate liberation observed upon addition of ATP to the contractile proteins [3]. This phosphate burst, measured in a quenched-flow experiment, is protein-bound phosphate which is released upon acid denaturation of the protein [4]. The amount of bound phosphate is a function of the equilibrium constant for the ATP hydrolysis step. For frog muscle myosin and subfragment 1 at 0°C, the rate constant for the cleavage step is fast compared to the rate of product release, and the equilibrium constant for the cleavage step is 18 [5]. Thus during the transient phase, there will be 0.94 mol phosphate formed/mol active site. This stoicheiometry has been used here to determine the amount of active protein in a myosin or subfragment 1 preparation.

In this study of the phosphate burst, we show that addition of actin to inactivated frog subfragment 1 can restore the ATPase activity, to give phosphate burst amplitudes as high as 0.8 mol P_i/mol subfragment 1. Traditionally, myosin is studied at high ionic strength so as to keep it in solution. Under these conditions, the phosphate burst amplitude showed that frog myosin was only partially active, and that inactivation was relatively fast. However, when the phosphate burst of myosin was studied at low ionic strength, namely conditions under which myosin formed filaments, it was found that the ATPase sites were nearly fully active, and that inactivation of the myosin at low ionic strength was slower than at high ionic strength.

2. MATERIALS AND METHODS

2.1. *Proteins*

Frog myosin was obtained from all hind leg muscles of *Rana temporaria*, and frog subfragment 1 was obtained by chymotryptic digestion of myosin in the absence of divalent metals and purified on DEAE-cellulose [1]. Proteins were observed by electrophoresis on SDS–polyacrylamide gels as in [1]. Some degree of actin contamination of the myosin was always present, but actin contamination was less than stoicheiometric. Subfragment 1 preparations were actin-free. Except where indicated,

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subfragment 1 used in these experiments contained a mixture of the A1- and A2-alkali light chains and was devoid of DTNB light chain. Rabbit actin was prepared as in [6]. It appeared as a single band on SDS gel electrophoresis and was free of contaminant ATPase activity. Protein concentrations were determined spectrophotometrically using a Zeiss M4 QIII spectrophotometer and the following extinction coefficients:

– for myosin and subfragment 1,

$A_{280}^{1\%} = 5.6\ cm^{-1}$ and $7.1\ cm^{-1}$, respectively [1];

– for actin, $A_{280}^{1\%} = 11.0\ cm^{-1}$ [7].

2.2. *Quenched flow measurements*

All measurements were carried out at 0°C and pH 7.0. The molarity of active frog myosin or subfragment 1 was estimated from the amplitude of the P_i burst, and incorporating a correction of 5.3% to account for the reversibility of the ATP hydrolysis step. Quenched-flow measurements using frog muscle proteins [5] were performed using an apparatus based on the design in [8] but with a double-push mode, permitting measurement of the reaction at times from 50 ms upwards. The apparatus, reactant syringes and reaction lines were immersed in an ice/water bath for temperature regulation at 0°C. The amplitude of the phosphate burst was determined by extrapolating the steady-state hydrolysis rate to zero time. Hydrolysis was assayed by measuring $^{32}P_i$ after adsorption of the nucleotide onto charcoal. Assay conditions are given in the figure legends.

3. RESULTS

3.1. *Effect of actin on the magnitude of the rapid phosphate liberation of frog subfragment 1*

The time course of a typical phosphate burst experiment with 28.5 μM initial [ATP] is shown in fig.1. In the absence of actin, the phosphate burst of frog subfragment 1 had an amplitude of 11.0% of the total ATP present. With the correction for the reversibility of the cleavage step, such a phosphate burst amplitude corresponded to 3.3 μM active subfragment 1 or 22.3% of the total subfragment 1. Calculating the steady state hydrolysis rate following the burst on the basis of 3.3 μM active subfragment 1, 5.23 nmol . mg active protein $^{-1}$. min $^{-1}$ ($k_{cat} = 0.010\ s^{-1}$) was obtained. The transient phosphate liberation was fitted by an exponential with a rate constant of $8.0\ s^{-1}$.

In the presence of 32 μM actin, the amplitude of the phosphate burst increased to 24.8% of the total nucleotide, indicating that the active subfragment 1 had increased to 50.3% of the total enzyme. On this basis, the steady state hydrolysis rate was 7.88 nmol . mg active protein $^{-1}$. min $^{-1}$ ($k_{cat} = 0.015\ s^{-1}$).

The higher steady-state hydrolysis rate of actosubfragment 1 reflects a predictably low degree of actin activation for 32 μM actin and 0.1 M KCl. The transient phosphate liberation was fitted by an exponential with a rate constant of $5.2\ s^{-1}$.

In general, the magnitude of the phosphate burst observed for frog subfragment 1 in the absence of actin was 0.301 ± 0.068 mol P_i/mol total subfragment 1 heads ($n = 6$, range 0.119–0.516) giving on average 31.7% active subfragment 1. The variability

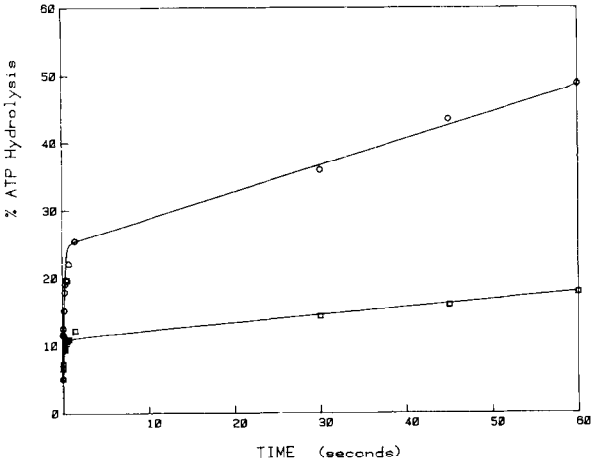

Fig.1. Transient phosphate liberation of frog subfragment 1 in the presence and absence of rabbit actin. Conditions: 14.8 μM subfragment 1, 25 μM initial MgATP, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 50 mM Tris–HCl (pH 7.0), at 0°C; (○) as above but with 32 μM actin. The solid lines were fitted to the data using a non-linear regression program. The end point for the phosphate burst was determined by extrapolating the steady-state ATPase rate to zero time. In the absence of actin, the observed rate constant for the process was 8.0 s^{-1} and 5.2 s^{-1} after addition of actin. The difference in rate constants is not significant.

in burst size depended on both the protein preparation and the age of the protein. The rate constant for the time course of the phosphate burst was proportional to the substrate concentration, with a second order rate constant similar to the value of $1.1 \times 10^5\ M^{-1}\ s^{-1}$ given in [5].

Addition of F-actin to subfragment 1 caused an increase in the size of the phosphate burst, but did not significantly affect its time course. In the presence of a molar excess of actin, the amplitude of the phosphate burst increased to 0.57 ± 0.09 mol P_i/mol total subfragment 1 heads (n = 6, range 0.34–0.80) indicating that on average 60.2% of the subfragment 1 heads were active.

The second order rate constant for the phosphate liberation in the presence of actin was $(1.47 \pm 0.18) \times 10^5\ M^{-1}\ s^{-1}$ (n = 6 for 8–93 μM ATP). Thus, the presence of actin increased the proportion of active heads by a factor of 1.90 ± 0.31 (n = 6). Actin could not restore activity to all subfragment 1 heads, but actin was proportionally more effective in restoring activity to subfragment 1 with low initial activity.

The relationship between the burst amplitude and the actin concentration was investigated. The burst amplitude increased with actin concentration, up to an actin concentration close to the subfragment 1 concentration, at which point a plateau was reached. Higher actin concentrations did not increase the burst amplitude further.

In an experiment with frog subfragment 1 that contained either the A1- or the A2-alkali light chain, it was found that the A1- and the A2-subfragment 1 were initially 54.3% and 52.9% active, and that upon addition of a 2-fold molar excess of actin (4 μM), the burst amplitudes increased similarly for the A1- and A2-subfragment 1, giving 84.6% and 78.6% active protein, respectively.

Storage of subfragment 1 in 0.1 M KCl at pH 7.0 on ice resulted in relatively fast denaturation, so that after 48 h, only 10–15% of the sites remained active. Addition of actin to subfragment 1 after 48 h resulted in up to a 3-fold increase in the active protein concentration. Storage of subfragment 1 in the presence of actin however was preferable to maintain a high proportion of active sites.

During the preparation of subfragment 1 from frog myosin, the proportion of active sites was reduced 20–40% by the chymotrypsin treatment. This loss of active sites (which was not observed for rabbit myosin) was largely reversed by addition of actin.

3.2. *The effect of ionic strength and filament formation on the rapid phosphate liberation of frog myosin*

The amplitude of the phosphate burst of frog myosin dissolved in 0.5 M KCl was measured. Its amplitude showed that immediately following preparation, ~60% of the heads were active. However, if the phosphate burst amplitude of fresh myosin was measured in 0.1 M KCl, after exposure of the myosin to 0.5 M KCl for up to 2 h, 90–100% of the myosin heads were active. Thus any myosin denaturation that occurred after relatively short incubation in high salt was reversible.

In addition, low salt conditions improved storage since incubation of frog myosin in 0.1 M KCl at 0°C resulted in 89% active heads after 48 h, compared to 39% active heads for myosin stored for the same duration in 0.5 M KCl. In this case however, most of the denaturation was irreversible.

In neither of the above situations did addition of a molar excess of actin increase the amplitude of the phosphate burst of partially inactivated myosin, in

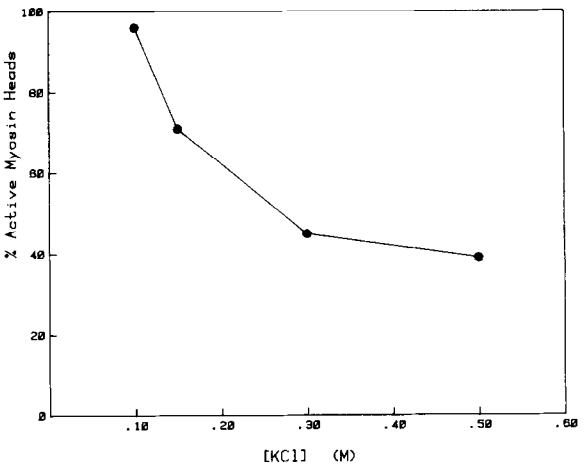

Fig.2. The relationship between the proportion of active myosin heads and [KCl]. The phosphate burst was measured by extrapolation to zero time of the myosin steady-state rate measured over 160 s at 0°C. Initial ATP was 29.4 μM; 2 mg/ml total myosin were dissolved in 25 mM Tris–HCl, (pH 7.0), 5 mM $MgCl_2$, 0.1 mM dithiothreitol with the [KCl] shown in the figure.

contrast to what had been found for subfragment 1.

The role of the salt concentration on the proportion of active sites was investigated further (fig.2.): 48 h old myosin was used so that a large proportion of myosin was inactive at high ionic strength. The increase in the proportion of active sites was most pronounced for salt concentrations at which myosin formed filaments.

Normally dithiothreitol was present (0.1–1.0 mM) during protein preparations and during subsequent experiments. However, no effect was observed on the size of the phosphate burst when dithiothreitol was removed or replaced by glutathione. This was somewhat surprising since modification of thiol groups is a common cause of protein inactivation. The denaturation could not be ascribed to the presence of chloride since similar results were obtained if KCl was replaced by potassium propionate.

4. CONCLUDING REMARKS

It was shown above that the activity of partially inactivated frog subfragment 1 can be restored and maintained by the addition of rabbit actin and that frog myosin can be partially reactivated by allowing myosin to form filaments. The restoration of active sites by actin indicates that actin stabilizes subfragment 1. This result is consistent with the reduction of the subfragment 1 flexibility by actin as revealed by NMR studies [9]. Our results suggest that the myosin filament plays an important role in stabilizing the myosin heads. The link between filament formation and the proportion of active sites suggests that interactions between the head and the tail of the molecule, or between two adjacent heads on a myosin filament contribute to the stability of the active site. Possibly this stabilization by filaments contributes to maintaining myosin as a viable ATPase in vivo. Upon aging, both myosin and subfragment 1 showed a degree of irreversible damage which might be attributed to aggregation as observed by analytical ultracentrifugation in [5].

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