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THE USE OF PRESSURE PERTURBATIONS TO INVESTIGATE THE INTERACTION OF RABBIT MUSCLE MYOSIN SUBFRAGMENT 1 WITH ACTIN IN THE PRESENCE OF MgADP

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1. Introduction

A question of fundamental importance in the molecular interpretation of the mechanism of muscle contraction is the evaluation of the degree of bridge formation and its rate of change, during the different states associated with the hydrolysis of ATP by actomysin. The states of the system have been defined using rapid flow and oxygen exchange methods [1,2]. Rapid flow techniques have some theoretical and experimental limitations when viscous and turbid solutions have to be mixed prior to observation. Our use of the pressure relaxation technique, developed [3] to study the rates of equilibration of the attachment of myosin, subfragment 1 to actin, is intended to complement the above investigations.

Preliminary investigations indicated that the interaction of actin and myosin was sensitive to pressure perturbation over 1–200 atm. Ultracentrifuge measurements of the actin and myosin binding constant also suggested that this was sensitive to pressure [4]. The current operating pressure of our perturbation equipment has an upper limit of 300 atm. This is insufficient to produce a significant perturbation, over a wide range of protein concentration, at physiological ionic strength where the binding constant in the absence of adenine nucleotides is estimated as 10^7–10^8 M^{-1} [5]. The equilibrium can be perturbed sufficiently in 0.5 M KCl or at physiological ionic strength in the presence of saturating concentrations of ADP, when the binding constant is reduced to $\sim 10^6$ M^{-1} [6]. We present here a detailed study of the kinetics and thermodynamics of S1–actin interaction in the presence of ADP.

Abbreviations: S1, myosin subfragment 1; A, actin; ADP, D, adenosine diphosphate

2. Methods

2.1. *Proteins*

F actin was prepared from an acetone powder as in [7]. Concentrations were determined from $E^{1\%}_{280\ nm}$ = 11.08 cm^{-1} [8].

Myosin subfragment 1 was prepared from rabbit skeletal muscle myosin as in [9]. For most experiments the isoenzymes of S1 were not separated in order to maximise the yield of S1. Concentrations are quoted on the basis of M_r = 115 000 and $E^{1\%}_{280\ nm}$ = 7.9 cm^{-1}.

2.2. *Instrumentation and data treatment*

The pressure relaxation equipment has been described in [3]. Changes in light scattering were followed by changes in the turbidity using a tungsten lamp and a Farrand monochromator. The signal was monitored at 360 nm where there is no significant absorbance by either the proteins or nucleotide.

The use of turbidity as a measure of the association between actin and myosin S1 has been discussed in [10]. The signal is approximately linearly dependent upon the degree of occupancy of the actin filament by S1. Titration of S1 against actin under conditions where the protein concentrations are much greater than the dissociation constant of the reaction allows a molar extinction coefficient of 2.72×10^4 cm^{-1} to be measured (fig.1).

Kinetic data were stored on a Data Lab DL 905 transient recorder and analysed using a non-linear least squares fitting routine as in [11].

Temperature control was achieved by circulating water from a thermostatted bath through the base of the pressure-jump apparatus. The temperature in the bath was monitored throughout the course of an experiment and the sample temperature was mea-

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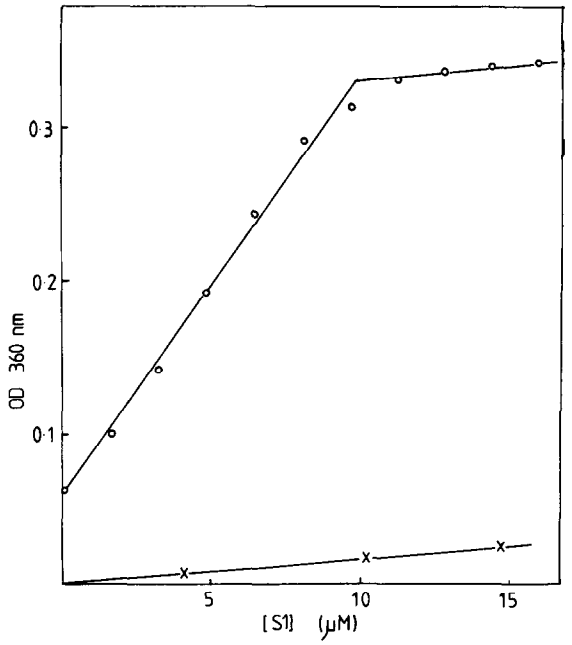

Fig.1. Turbidometric titration of S1 against actin (0.1 M KCl, 5 mM $MgCl_2$, 50 mM Tris, pH 8.0, 21°C): (x) titration of S1 into 3 ml buffer; (+) titration of S1 into 3 ml buffer with 10 μM actin.

sured at the beginning and end of each set of experiments by inserting the thermocouple of a Cormack digital thermometer into the sample chamber. The temperature remained constant during the course of experiments to within 0.2°C.

3. Results

The changes in turbidity produced by a rapid pressure change on an acto . S1 . ADP solution are shown in fig.2. The observed change in turbidity fitted a single exponential under all conditions investigated. No rapid jump in turbidity was seen on pressure release. The results shown in fig.2 were obtained using a pressure release valve which allows the pressure to return to 1 atm in <50 ms. Using a valve with a pressure release time of 100 μs gave identical results.

The variation of the observed rate with the concentrations of actin and S1 . ADP is shown in fig.3. The plots were obtained by assuming the reaction was of the form:

$$A + S1.D \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} A.S1.D \qquad (1)$$

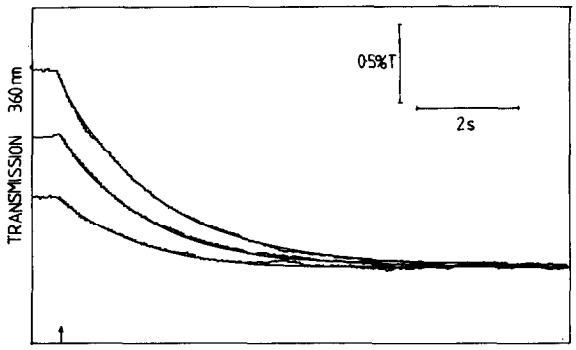

Fig.2. The pressure-induced changes in turbidity of acto . S1. ADP. The 3 traces represent the observed changes for pressure jumps of 175,125 and 75 atm. The arrow indicates the time at which pressure was released. Each trace is the average of 5 successive relaxations and the single exponential fit is superimposed. The observed rates were 0.56,0.65 and 0.62 s^{-1}, respectively. Reaction conditions: 0.1 M KCl, 5 mM $MgCl_2$, 50 mM Tris, pH 8.0, 21°C.

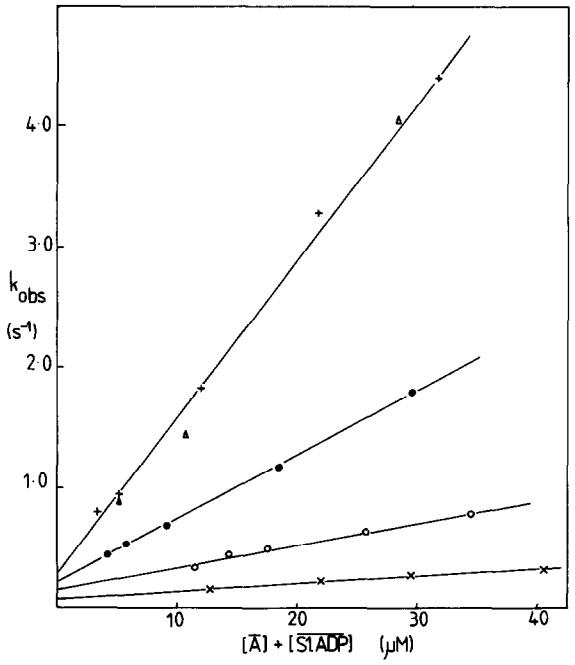

Fig.3. The concentration and temperature dependence of the observed rate of the pressure-induced relaxations of acto . S1 . ADP. Buffer conditions as in fig.2. Typically the total [actin] was held constant between 8–10 μM and [S1] varied to give the equilibrium concentrations shown except for results Δ where S1 was held at 10 μM and [actin] varied: (+,△) 25.8°C; (•) 20.7°C; (○) 14.1°C; (X) 5.7°C.

Table 1

pH	[KCl]	°C	k_{+1} ($M^{-1}.s^{-1}$)	k_{-1} (M^{-1})	K_1 (M^{-1})
8	0.1	5.7	5.49×10^3	0.10	5.49×10^4
8	0.1	9.0	9.79×10^3	0.11	8.9×10^4
8	0.1	14.1	1.81×10^4	0.15	1.21×10^5
8	0.1	20.7	5.39×10^4	0.19	2.84×10^5
8	0.1	25.8	1.31×10^5	0.28	4.68×10^5
7	0.1	20.7	1.10×10^5	0.14	7.86×10^5
8	0.5	20.5	4.75×10^3	0.17	2.79×10^4
8[a]	0.5	20.2	1.99×10^5	0.21	9.48×10^5
8[b]	0.01	5.9	9.10×10^4	0.10	9.10×10^5
8[c]	0.01	5.9	6.06×10^4	0.10	6.06×10^5

[a] Acto–S1 in the absence of ADP; [b] S1–A1 20 mM Tris–HCl; [c] S1–A2 20 mM Tris–HCl

All solutions contained 5 mM $MgCl_2$, 2 mM ADP: pH 8, 50 mM Tris–HCl; pH 7, 50 mM imidazole–HCl

A value of K_1 ($= k_{+1}/k_{-1}$) was assumed and the equilibrium concentrations of actin and S1.ADP calculated. The data was then analysed in terms of eq. (2):

$$k_{obs} = k_{+1}(\overline{S1.ADP}] + [\overline{A}]) + k_{-1} \quad (2)$$

(where bars indicate final equilibrium concentrations) to give estimates of k_{+1} and k_{-1}. An iteration procedure was then carried out until the assumed K_1 and k_{+1}/k_{-1} agreed to within 10%. The observed rates and amplitudes were independent of [ADP] over 0.5–2 mM.

The variation of the observed rates with temperature is shown in fig.3 and the dependence of k_{+1}, k_{-1} and K_1 with temperature, ionic strength and pH is listed in table 1. Preliminary results with acto-S1 in the absence of ADP are included for comparison. Thermodynamic parameters derived from the variation of the rate constants with temperature are shown in table 2.

4. Discussion

The data in table 1 show the marked ionic strength dependence of the actin–S1.ADP reaction which has been noted by several workers for other actin and myosin interactions [10,13]. The data show that this is primarily due to the effect upon k_{+1} suggesting a significant contribution from charged groups in the binding reaction. The difference at 0.5 M KCl between acto-S1 in the presence and absence of ADP is also primarily due to changes in k_{+1}. No significant difference in the binding of the 2 isoenzymes of S1 was observed at 0.1 M KCl and so the isoenzymes were not separated except for those experiments in table 1 which were performed at 10 mM KCl. Under these conditions a small difference in the binding of the isoenzymes was observed and this was also due to differences in k_{+1}.

The values of the dissociation constant obtained here are in general agreement with results obtained under slightly different conditions [6].

The data in table 2 show that the binding reaction has a very high temperature dependence which is indicative of a conformational change in the proteins. The large value of ΔS° is not unusual for protein–protein interactions [14] and suggests that the reaction is largely entropy controlled. A large entropy

Table 2
Thermodynamic data for the actin and S1.ADP interaction

	k_{+1}	k_{-1}
E_a(kcal/mol)	25.7	8.5
$H^\dagger$ 25°C(kcal/mol)	25.1	7.9
$S^\dagger$ (cal/K/mol)	96.1	8.1
$G^\dagger$ 25°C(kcal/mol)	−3.52	5.5

K_d 25°C	ΔG°	ΔH°	ΔS°
2.39 μM	7.7 kcal/mol	−17.05 kcal/mol	−82.9 cal/K/mol

Reaction conditions as for fig.3

change may be expected for a reaction which has a significant volume change, as a large volume change normally reflects changes in the ordering of water due to changes in the number of exposed charged or hydrophobic groups. The changes in turbidity on actin and S1 . ADP recombining after a pressure release, gave very good fits to a single exponential and the results were analysed in terms of the single-step binding mechanism of eq.(1). Several pieces of evidence suggest that the binding reaction may be more complex.

As described by eq.(1), the association of the 2 proteins is a diffusion-controlled reaction, but the association rate constant is smaller than the expected 10^6–10^7 $M^{-1} . s^{-1}$ for a diffusion-controlled association between 2 proteins [13]. Indeed, at physiological ionic strength stopped-flow studies of the binding of actin and S1 suggest a value of 10^6 $M^{-1} . s^{-1}$ [10]. The rate constant obtained here is >20-times lower.

Similarly, the activation energy of a diffusion controlled reaction is normally of the order of 4 kcal/mol and the activation energy for the binding reaction here is >5-times higher. Whilst the temperature dependence of the viscosity of an acto–S1 solution might be expected to yield a larger than normal activation energy, this is unlikely to be >10 kcal/mol.

The simplest model which can account for the data is a 2 step association reaction where a first-order isomerisation is preceded by a faster diffusion controlled reaction:

$$A + A1 . D \underset{k_{-a}}{\overset{k_{+a}}{\rightleftharpoons}} A.S1.D \underset{k_{-b}}{\overset{k_{+b}}{\rightleftharpoons}} A.\overset{*}{S}1.D \qquad (3)$$

where $K_a = k_{+a}/k_{-a}$.

As single exponential traces are observed with no rapid turbidity change on pressure release, this 2 step model is only compatible with the data under the following conditions: $k_{+a}, k_{-a} >> k_{+b}, k_{-b}$, turbidity of A.S1.D = turbidity of $A.\overset{*}{S}1.D$ and the only significant pressure sensitivity must be on step b. Under these conditions:

$$k_{obs} = \frac{K_a\, k_{+b}\, ([\bar{A}] + [\overline{S1.D}])}{1 + K_a\, ([\bar{A}] + [\overline{S1.D}])} + k_{-b}$$

and at low concentrations of $[\bar{A}] + [\overline{S1.D}]$ as used in these experiments this simplifies to:

$$k_{obs} = K_a\, k_{+b}\, ([\bar{A}] + [\overline{S1.D}]) + k_{-b}$$

Using this model $K_1 = K_a\, K_b$ and the data at 20°C, pH 8.0 and 0.1 M KCl give $k_{-b} = 0.19\ s^{-1}$, $K_a\, k_{+b} = 5.4 \times 10^4\ M^{-1} . s^{-1}$ such that $k_{+b} \geqslant 5\ s^{-1}$ and $K_a = \leqslant 10^4\ M^{-1}$.

Models including an isomerisation in one of the proteins prior to the diffusion controlled binding reaction, cannot be entirely eliminated, for example:

$$S1.D \underset{}{\overset{1}{\rightleftharpoons}} \overset{*}{S}1.D + A \underset{}{\overset{2}{\rightleftharpoons}} A.\overset{*}{S}1.D$$

If step 2 is much faster than step 1 and only step 1 is pressure sensitive, then this model would predict single exponential perturbations, but the observed rate would decrease with increasing concentrations of $\overline{\overset{*}{S}1.D} + \bar{A}$. If step 1 is much faster than step 2, perturbation of either step would give rise to single exponential traces but:

$$k_{obs} = \frac{k_{-2} + k_{+2}\, (k_{-1}\, [\overline{\overset{*}{S}1.D}] + k_{+1}\, ([\overline{\overset{*}{S}1.D}] + [\bar{A}]))}{k_{+1} + k_{-1}}$$

This has the same form as eq.(1) and produces values of the rate constant and activation energy of the diffusion controlled reaction similar to those for eq.(1).

Thus a two step model of this type is only compatible with the data if step 1 and step 2 are closely coupled such that the reaction appears as a single exponential.

One further piece of evidence that the acto . S1 . ADP binding reaction is more complex, comes from an analysis of the amplitude of the observed perturbations.

The relation between the perturbation amplitude and the pressure change is given by:

$$\Delta \ln K = -\frac{\Delta V . \Delta P}{RT}$$

where ΔP and $\Delta \ln K$ are the induced change in pressure and the resulting change in equilibrium constant, ΔV is the molar volume change of the reaction. If the change in extinction coefficient for the reaction is known, then $\Delta \ln K$ and hence ΔV can be calculated from the amplitudes. However, analysis of the ampli-

tudes of the perturbations gave differing values of ΔV depending upon the initial concentrations of actin and S1. ADP. As the rates of the reactions observed were slow compared to the pressure release time, and the data give very good fits to a single exponential, the error in the amplitudes is small. This suggests that the model used to calculate $\Delta \ln K$ is incorrect; however, the amplitude data are not satisfactorily fitted by a 2 step model either. Values of ΔV, assuming a 1 step model, varied from 22–39 ml/mol ΔV decreasing as the protein concentration increased. Assuming a 2 step model ΔV varied from 18–29 ml/mol.

One possible explanation for the variability of ΔV calculated from the amplitude data is that the extinction coefficient used was inappropriate over the whole concentration range. As shown in fig.1, S1–actin binding does not give a strictly linear change in turbidity so that the extinction coefficient used may only be accurate for 20–80% of saturation of actin. The variability in the calculated values of ΔV does appear to be too large to be accounted for by the error in the extinction coefficient, suggesting that the association reaction may be more complex.

These results show the potential of the method of pressure relaxation for studying the rate of equilibration of the attachment of S1 to actin. We have shown the method can readily measure the forward and reverse rate constants for the binding reaction in the presence of ADP, and that the binding reaction is highly dependent upon ionic strength and temperature. We are now developing the technique to allow the study of the rate of equilibration of the rigor complex and the other kinetically defined states of the actomyosin ATPase.

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