A novel pressure-jump apparatus for the microvolume analysis of protein–ligand and protein–protein interactions: its application to nucleotide binding to skeletal-muscle and smooth-muscle myosin subfragment-1

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Reactions involving proteins frequently involve large changes in volume, which allows the equilibrium position to be perturbed by changes in pressure. Rapid changes in pressure can thus be used to initiate relaxation in pressure; however, this approach is seldom used, because it requires specialized equipment. We have built a microvolume (50 μ l) pressure-jump apparatus, powered by a piezoelectric actuator, based on the original design of Clegg and Maxfield [(1976) *Rev. Sci. Instrum.* **47**, 1383–1393]. This equipment can apply pressure changes of ± 20 MPa (maximally) in time periods as short as 80 μ s and follow the resulting change in fluorescence signals. The system is relatively simple to use with fast (approx. 1 min) exchange of samples. In the present study, we show that this system can perturb the binding of

2'(3')-O-(*N*-methylantraniloyl)-ADP to myosin subfragment-1 (S1) from skeletal and smooth muscles. The kinetic data are consistent with previous work, and in addition show that, although 2'(3')-O-(*N*-methylantraniloyl)-ADP binds with a similar affinity to both proteins, the increase in molar volume for the skeletal-muscle S1 binding to ADP is half of that for the smooth-muscle protein. This high-volume change for smooth-muscle S1 may be related to the ability of ADP to induce a 23° tilt in the tail of S1 bound to actin.

Key words: ligand binding, molar volume change, pressure relaxation, strain sensing.

INTRODUCTION

Protein–protein and protein–ligand interactions constitute the fundamental molecular events that underlie physiological response. Most of the physiologically relevant molecular events in proteins occur on the time scale of 0.01–1000 ms. Therefore to understand these molecular processes, we must use methods that work in this time domain. Although well-established rapid-mixing methods (stopped flow and quenched flow) have elucidated many processes that occur on a time scale longer than 1–2 ms, faster events can only be studied by more specialized methods.

Protein–protein interactions, protein–ligand interactions and protein isomerizations frequently involve large changes in volume due to changes in the amount of water bound on the protein surface. Thus the equilibrium position of such processes can be perturbed by the changes in hydrostatic pressure. The use of rapid-pressure perturbations to study protein–protein and protein–ligand interactions has made a unique contribution to the study of proteins because of its high time resolution (80 μ s) and the mild nature of the applied step changes [1]. However, it has been a method used only by a few specialists. We have now constructed a simple-to-use apparatus based on the original design by Clegg and Maxfield [2], in which a piezoelectric crystal stack applies fast (approx. 100 μ s) reversible pressure jumps of up to 20 MPa (200 atmospheres) to a 50 μ l sample of protein solutions. The small volume of solution required and the fact that the protein can be repeatedly monitored and then recovered after the measurements mean that the studies can be completed with microgram quantities of protein. Thus this apparatus promises to be a major new tool for biophysical studies of protein reactions of biological relevance.

The cyclical interaction of myosin subfragment-1 (S1) with nucleotide and actin forms the molecular basis of the force-generating mechanism in the myosin family of molecular motors. We have previously used rapid-pressure perturbations to study the interaction of S1 with actin in the presence and absence of ADP [3–5]. On the basis of these studies, we proposed that a rapid isomerization of the acto-S1 complex, which occurs with a large volume change, is closely coupled with the force-generating event of the actomyosin ATPase cycle [6].

The interaction of ADP with S1 and actoS1 has been a subject of renewed interest in recent years, because of the discovery of an ADP-induced structural change in myosin-decorated actin filaments [7] only when smooth-muscle myosin S1 or brush-border myosin I are used but not when skeletal-muscle S1 is used. This has led to extensive discussion of the role of such ADP-induced conformational changes in the force-generating mechanism [8–11]. We had previously examined the interaction of ADP with skeletal S1 in the absence of actin and found no measurable perturbation of the reaction by pressure [12]. With the help of the new machine, we can now detect such a change when using a fluorescent analogue of ADP, namely 2'(3')-O-(*N*-methylantraniloyl)-ADP (mant-ADP). The results reveal new features of the interaction.

Values for the kinetic properties of mant-ADP interacting with rabbit skeletal muscle-derived S1 (skS1) or chicken gizzard-derived smooth-muscle S1 (smS1) obtained using the new apparatus compare favourably with values already obtained using stopped flow, but using much less material. Analysis of the amplitude data allowed estimates of the molar volume change ΔV° to be made. Surprisingly, smS1, despite having a very similar K_D value, exhibited roughly twice the volume change of the skS1. This result may directly reflect differences in the

Abbreviations used: mant-ADP, 2'(3')-O-(*N*-methylantraniloyl)-ADP; mant-dADP, (3')-O-(*N*-methylantraniloyl)-2'-deoxy-ADP; S1, myosin subfragment-1; skS1, rabbit skeletal muscle-derived S1; smS1, chicken gizzard-derived smooth muscle S1.

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structural transitions that occur on nucleotide binding and release. Some of this work was presented previously at the Biophysical Society Annual Meeting in February 2002 [13].

METHODS

Pressure perturbation theory

Pressure perturbation works by using pressure to alter the free energy of a reaction (ΔG°) in which there is a difference in volume (ΔV°) between the reactants and products. So, for a ligand-binding reaction, as shown in Scheme 1,

If the reactants $A + B$ have a total volume V and the bound product $A \cdot B$ has a volume $V + \Delta V^\circ$, then the forward reaction must perform work $P\Delta V^\circ$ against the external pressure to proceed. The pressure perturbation function is thus given by the differential of the work done with respect to the change in pressure:

$$\Delta V^\circ = (\Delta V^\circ / RT) \Delta P \quad (1)$$

The equilibrium constant K may be defined as an association constant:

$$K = [A \cdot B] / [A][B] \quad (2)$$

Substituting $\Delta G^\circ = -RT \ln K$ in the pressure perturbation function and integrating, a useful expression is derived:

$$\Delta K / K = -\Delta V^\circ \Delta P / RT \quad (3)$$

in which $\Delta K / K$ (approximately $\Delta \ln K$) is the small fractional change in the equilibrium constant over a pressure step ΔP . Thus a positive value for ΔV° , in this convention (which will be used throughout), denotes a decrease in the equilibrium association constant upon the application of pressure leading to dissociation of the complex.

A change in pressure can also lead to a perturbation of the rate constant k of a reaction. In terms of transition state theory, this is most simply understood as a volume change on formation of the activated complex and referred to as the molar activation volume $\Delta V^\ddagger$. This is analogous to the temperature dependence of reaction rates and activation enthalpy.

Proteins and mant-ADP

skS1 was prepared by the method of Weeds and Taylor [14]. The isoenzyme A1 was used for these experiments. smS1 was prepared as described by Cremo and Geeves [9]. Mant-ADP was prepared by the method of Hiratsuka [15]. All S1 experiments were performed in the following buffer at pH 7 [100 mM KCl/20 mM Mops/5 mM $MgCl_2$ /5 mM dithiothreitol/1 mM sodium azide].

Pressure-jump apparatus

The apparatus is based on the original design of Clegg and Maxfield [2], except that the internal volume is decreased to 50 μ l and the solution is exchanged via two simple valves, making the system much simpler to operate. The basic design of the apparatus is shown in Figure 1. The sample (50 μ l) is held in an

Figure 1 Schematic diagram of the pressure-jump apparatus

(Main) The sample (50 μ l) is held in an optical chamber consisting of a sapphire ring (inner diameter 2 mm $\times$ outer diameter 5 mm $\times$ 5 mm high). The ends of the chamber are closed with the help of a pressure sensor at one end and a polyimide membrane at the other. Pressure is applied when the membrane is deformed by the piston attached to a piezoelectric translator (Physik Instrumente P-245.70 or P-245.30). The sample is exchanged via two valves. (Section) Fluorescence was excited by monochromatic light from a light guide, and emission is collected on to a photomultiplier at 90° to the incident light by a collimating lens and an appropriate filter. HV, high voltage piezo power switch; Q, charge amplifier; PM, photomultiplier.

optical chamber consisting of a sapphire ring (inner diameter 2 mm $\times$ outer diameter 5 mm $\times$ 5 mm high). The ends of the chamber are closed by a pressure sensor (601A; Kistler Instrumente AG, Winterthur, Switzerland) at one end and a piston attached to a piezoelectric translator (P-245.70 or P-245.30; Physik Instrumente GmbH, Waldbronn, Germany) at the other. The sample is isolated from the piston by a thin membrane [0.003 inch (1 inch = 2.54 cm) thick polyimide film; Kapton® VN, Du Pont, Kensulat Ltd, U.K.]. The sample is exchanged via two specially designed low-volume pressure valves.

In the current apparatus, the pressure-induced changes in the solution properties are monitored by fluorescence (Figure 1). In principle, a second window could be added to allow absorbance measurements, but in practice there are space limitations and the short path length is not ideal for measuring absorbance changes. A second chamber including absorbance windows is under construction. Excitation light is provided by a 100 W Xe/Hg lamp (Hamamatsu Photonics, Enfield, U.K.), which is passed into a monochromator before delivery to the cell via a quartz light guide. The exit of the light guide (4 $\times$ 1 mm) abuts directly on to the sapphire ring. Emission light is collected on a photomultiplier (R1463; Hamamatsu Photonics, Enfield, U.K.) at 90° to the incident light via a lens and a suitable cut-off filter to eliminate scattered excitation light. The output from the photomultiplier is amplified and collected by a fast (1 MHz, 12-bit) A/D converter (DAS 50; Keithley Instruments Reading, U.K.) running under Windows 3.1 on a PC using software written in C++ (J. Huber, Max-Planck-Institut, Dortmund).

Samples are loaded by first filling the sapphire ring via the inlet port, then the outlet port is closed and the sample is prepressurized to between 0.5 and 4 MPa. This has the effect of removing elasticity from the system and deforming the membrane into full direct contact with the piston. Data collection is triggered by the computer, followed a short time later (10 % of sampling

Figure 2 Fluorescence and pressure transient of 1 μM fluorescein in 20 mM phosphate buffer observed after fast pressure jumps at pH 6.4

The piezostack was activated for 0.1 ms after triggering the data collection system. The rise (a) and fall (b) of the measured pressure change (single shot) and the fluorescein response (average of 4000 pressure jumps) are shown. The pressure change was complete in $< 100 \mu\text{s}$ with a slight ripple on the signal, which decayed over the next 0.4 ms. After this time period the signal was stable. The fluorescence response occurred at the same time as the pressure change and showed a similar oscillation.

time) by triggering of the high voltage applied to the piezocrystal stack (Figure 2). A rolling average is employed to make best use of the 1 MHz data acquisition of the A/D converter; typically 500 data points per transient are stored to disk. After the completion of the transient, a wait period is allowed for stabilization before the data collection is again triggered followed by the pressure fall signal. Thus transients resulting from both the increase and decrease in pressure are collected and stored separately at a maximum acquisition rate of 12.5 transients/s. The software allows data from as many as 1000 transients to be averaged and stored to disk for later analysis. (Note that the signal/noise ratio improves linearly with the square root of the number of repeats.) Sample stability may be monitored using the equilibrium fluorescence. If significant photobleaching occurs, the sample can be speedily exchanged, another measurement performed and the results averaged later.

Measurement details

Mant fluorescence was excited at 365 nm and collected via a KV 389 emission filter (Schott GmbH, Mainz, Germany). For fluorescein, an excitation wavelength of 488 nm was used with an OG 530 emission filter (Schott GmbH). In both cases, 1.0 mm slit widths were used, which provided a bandwidth of 2.5 nm, although the excitation beam is dominated by the mercury lines at 365 and 486 nm. In all cases, the background signal (dark current plus stray light) was obtained using a buffer and regarded as zero fluorescence. All measurements presented here, except the data in Figure 8, were obtained using the smaller (P-245.30) piezostack, which is capable of generating pressure steps of up to 10 MPa but is faster than the larger actuator.

Fluorescence titrations of fluorescein, in either phosphate or imidazole buffer, were performed by diluting the stock solution of fluorescein to 1 μM in buffer and by adjusting the pH, although sampling periodically. This provided samples of fluorescein in buffer that could be tested in the pressure jump. Each solution was monitored in the pressure-jump apparatus to obtain the equilibrium signal before the pressure jumps were performed. The high-tension supply to the photomultiplier was kept constant during these measurements, so as to maintain the same photomultiplier sensitivity, and the buffers were used as blanks for the equilibrium measurements.

RESULTS

Performance tests

The fluorescence spectrum of fluorescein is known to be highly pH-dependent, owing to the variety of protonation equilibria it exhibits [16]. The protonation of phosphate anions has been shown to exhibit a considerable volume change ($24 \text{ ml} \cdot \text{mol}^{-1}$) and is therefore a pressure-sensitive pH buffer [17]. Protonation equilibria are generally very fast when compared with the speed of the piezoactuators in our pressure-jump apparatus, hence fluorescein in phosphate buffer can be used to test the speed and sensitivity of the system.

The performance of the equipment is illustrated in Figure 2, where the fluorescence of 1.0 μM fluorescein was monitored in 20 mM phosphate buffer at pH 6.4, after pressure jumps of 7.0 MPa. The pressure transient was also recorded for direct comparison. The data recording was initiated before the pressure jump was applied at $t = 0$. The pressure trace shows a short lag of $15 \mu\text{s}$ (which is consistent with the propagation of a pressure pulse) followed by a pressure increase (Figure 2a), which was essentially complete $80 \mu\text{s}$ after triggering the system. A slight ripple in the pressure transient was observed, but it decayed away after 0.4 ms (which is consistent with resonance within the sample chamber).

After this time period, the pressure signal remained constant for at least 100 s. The pressure release followed a similar profile to that of the pressure increase (Figure 2b). In contrast with the pressure record, which was from a single pressure jump, the fluorescence trace is the average result of 4000 pressure jumps. The fluorescence transient begins with a rapid fall (approx. $1 \mu\text{s}$) in fluorescence, followed by a slower increase, which began at $t = 0$ and was complete in $< 100 \mu\text{s}$. The rapid fall of the fluorescence transient proved to be an electrical artifact caused by the approx. 1 kV signal applied to the piezoactuator and is present in the absence of any light. The fluorescence signal followed the same time course as the pressure signal, including small oscillations (indicating the presence of low-amplitude pressure waves), as expected if it is caused by a shift in the pH of the phosphate buffer.

The amplitude of the pressure-induced change in fluorescence contains information about the size of the pressure-induced change in pH and any direct effect of pressure on the fluorescein. The two effects can be distinguished by comparing the transients

Figure 3 Fluorescein pH titrations showing total fluorescence and pressure jump-induced fluorescence change

Fluorescence titration of 1 μM fluorescein in 20 mM imidazole buffer ($\square$) (with 100 mM KCl and 5 mM MgCl_2) and in 20 mM phosphate buffer ($\bullet$) (top). Plot of the fluorescence change versus pH for the same solutions, subjected to a +7 MPa pressure jump at 20 $^\circ\text{C}$ (bottom). Fitting to both sets of data simultaneously gave pK values of 6.28 and 4.8, with the ΔV° values of ionization -9 and $-19 \text{ ml} \cdot \text{mol}^{-1}$ respectively in imidazole plus a compression effect of $M_B^{-1} = 0.046\% \cdot \text{MPa}^{-1}$. The phosphate buffer caused an additional pH jump of -0.029 , which is equivalent to a ΔV° value of $-24 \text{ ml} \cdot \text{mol}^{-1}$ for ionization.

observed in phosphate buffer as a function of pH with those in imidazole, a pressure-insensitive pH buffer. Figure 3 shows the pH dependence of the fluorescein fluorescence in both buffers and the amplitudes of the pressure-induced fluorescence transients. The titration curve of fluorescein is analytically complicated and was considered to consist of two ionizing species, which could be adequately represented using the following formula:

$$\frac{F}{F_{\max}} = 1 - \left\{ Q \frac{[\text{H}^+]}{K_1 + [\text{H}^+]} + (1 - Q) \frac{[\text{H}^+]}{K_2 + [\text{H}^+]} \right\} \quad (4)$$

where Q is the relative fluorescence quench upon ionization of the first species. The pK values of the two ionizing species were estimated to be 6.28 and 4.8 by fitting the titration curve in tandem with the subsequent amplitude analyses, and are marked with arrows on the upper panel of Figure 3. These values are compatible with previous measurements, after correction for ionic strength and given the complex internal equilibria in the fluorescein system [16].

The lower panel of Figure 3 shows the fluorescence change upon compression of the samples. In imidazole buffer (Figure 3, $\square$), the fluorescence increased with increase in the pressure, whereas below pH 7, the phosphate-buffered samples decreased their fluorescence on the application of pressure. This is expected if pressure decreases the pH of the phosphate buffer, leading to a loss of fluorescence. If the pH remains constant in imidazole buffer, then the increase in fluorescence indicates a shift in the pK of fluorescein. At $\text{pH} > 9$, the observed amplitude is dominated by the increase in concentration due to the volume change upon compression of the sample (indicated as the inverse bulk modulus of water, $M_B^{-1} = 0.046\% \cdot \text{MPa}^{-1}$).

Detailed analysis of the data allows an estimate of the molar volume changes associated with phosphate and fluorescein ionization. The method of analysis is given in detail in the Appendix.

Briefly, it consisted of making adjustments to eqn (4) to account for the shifts in pK values for the fluorescein (due to their respective volume changes, which are marked on the lower panel of Figure 3 as ΔV_1 and ΔV_2) as well as the change in pH of the phosphate buffer (shown as ΔpH), and an additional term is introduced to account for the compression effect. The two volume changes resulting from the analysis were 9 and 19 $\text{ml} \cdot \text{mol}^{-1}$ for the protonation reactions of fluorescein, with a pH shift of -0.029 due to ionization of the phosphate buffer. This represents a value of 24 $\text{ml} \cdot \text{mol}^{-1}$ for the ΔV° of the primary protonation reaction of phosphate and agrees well with the value of a previous study (also 24 $\text{ml} \cdot \text{mol}^{-1}$ [17]).

Nucleotide binding to skS1

The fluorescence of a mant group covalently attached to the 2'- or 3'-hydroxyl group of ADP increases its fluorescence by a factor of 2.2 on binding to S1. It binds with an affinity of 0.1 μM , i.e. 5–10-fold tighter than the unmodified ADP [18], and therefore provides a very sensitive monitor of the association/dissociation of ADP with S1. The mant-ADP exists in two forms (2'- and 3'-labelled) that are in slow equilibrium with each other. The use of mant-labelled deoxy-ADP avoids the complications that may arise from the mixed isomers, and it also has a lower affinity for S1 (approx. 0.5 μM versus approx. 0.1 μM for both skS1 and smS1).

Figure 4 shows examples of fluorescence transients, resulting from pressure jumps of varying step sizes between 0.6 and 10 MPa applied to a solution of 5.5 μM skS1 and 5.0 μM of (3')-O-(N-methylantraniloyl)-2'-deoxy-ADP (mant-dADP). The fluorescence decreased by approx. 2% for a 10 MPa pressure increase, indicating that increased pressure caused a dissociation of the mant-dADP-S1 complex. The fluorescence was stable and fully recovered on pressure release. There was also a 0.035% $\cdot \text{MPa}^{-1}$ fast change in fluorescence at the beginning of the transient. This was consistent with instantaneous compression as mentioned in the previous section, inasmuch as the amplitude of this fluorescence change was proportional to the total fluorescence of the sample. The main fluorescence transients were fitted by a single exponential function and the results of this analysis plotted (Figure 5). The amplitude was linearly dependent on the size of the applied pressure step (slope = 1.7%/10 MPa for both pressure application and release). The reciprocal relaxation time was independent of the pressure step size ($\tau^{-1} = 3.9 \text{ s}^{-1}$), for both pressure application and release within the accuracy of the determination, although the presence of this relaxation clearly demonstrates that there is a positive volume change for this reaction. The combination of linear dependence of amplitude on pressure and good single-exponential fits to the fluorescence transients indicates that the change in concentration is small compared with the total concentration of the reactants.

The concentration dependence of mant-nucleotide interacting with S1 was also investigated to analyse the kinetic mechanism of this reaction. Figure 6 shows examples of fluorescence transients in which 6.0 MPa pressure jumps were applied to solutions containing skS1 in excess of mant-ADP. As the total concentration of the free reactants increased from the lowest (A) to the highest (D), the reciprocal relaxation time increased, consistent with a second-order binding process, and the amplitudes decreased. The simplest reaction is as shown in Scheme 2:

Scheme 2

Figure 4 Example of pressure-jump induced fluorescence transients for a skS1/mant-dADP solution: effect of pressure step size

Fluorescence transients in a solution of $5.0 \mu\text{M}$ mant-dADP and $5.5 \mu\text{M}$ skS1 after pressure jumps of five different step sizes, all of which returned to 2 MPa. The data shown are the mean of 5–10 transients and a single exponential fit is drawn through the data.

Figure 5 Effect of pressure step size on relaxation parameters

A plot of the dependence of the reciprocal relaxation time and amplitude of the fluorescence transients in a solution of $5 \mu\text{M}$ mant-dADP and $5.5 \mu\text{M}$ skS1 against the size of the applied pressure jump. The reciprocal relaxation time was independent of the applied pressure step, whereas the amplitude was linearly dependent on the pressure step. Slope 1.7% per 10 MPa, equivalent to a 4.4% change in concentration of S1–dADP.

where k_{on} is the second-order binding rate constant, k_{off} the first-order dissociation rate constant and the ** on the bound species indicates the 2-fold enhancement in the fluorescence of bound mant-ADP. The following equation describes the concentration dependence of the reciprocal relaxation time:

$$\tau^{-1} = k_{\text{on}}\{[\overline{\text{M}}] + [\overline{\text{D}^*}]\} + k_{\text{off}} \quad (5)$$

where the overbars indicate that the reactant concentrations are the equilibrium concentrations. The concentration of bound S1-nucleotide (and therefore the equilibrium reactant concentra-

tions) is given by the following solution to a quadratic, in total concentrations $[\text{M}]_{\text{T}}$ and $[\text{D}^*]_{\text{T}}$:

$$[\overline{\text{MD}^{**}}] = \{([\text{M}]_{\text{T}} + [\text{D}^*]_{\text{T}} + K_{\text{D}}) - \sqrt{([\text{M}]_{\text{T}} + [\text{D}^*]_{\text{T}} + K_{\text{D}})^2 - 4[\text{M}]_{\text{T}}[\text{D}^*]_{\text{T}}}\} / 2 \quad (6)$$

where K_{D} is the dissociation constant (i.e. the inverse equilibrium binding constant) and is given by $K_{\text{D}} = k_{\text{off}}/k_{\text{on}}$. Eqns (5) and (6) can be combined to yield a single equation, describing the dependence of the reciprocal relaxation time on the total concentration of the reactants:

$$\tau^{-1} = k_{\text{on}}\sqrt{([\text{M}]_{\text{T}} + [\text{D}^*]_{\text{T}} + K_{\text{D}})^2 - 4[\text{M}]_{\text{T}}[\text{D}^*]_{\text{T}}} \quad (7)$$

The results of this analysis for skS1 in excess of mant-ADP are shown in Figure 7(a) (filled symbols). The good single-exponential fits to the fluorescence transients are consistent with a slight change in the total concentration of the reactants. The values of τ^{-1} were linear over the range of reactant concentrations that was accessible to this technique. The data are therefore consistent with the single-step reversible binding model. There was no significant difference between the relaxations determined from pressure increase ($k_{\text{on}} = 2.05 \pm 0.10 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ and $k_{\text{off}} = 0.35 \pm 0.20 \text{ s}^{-1}$, where the errors represent statistical confidence limits obtained from fitting) and pressure release ($k_{\text{on}} = 2.06 \pm 0.10 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ and $k_{\text{off}} = 0.31 \pm 0.20 \text{ s}^{-1}$). Differences between rate constant values (and hence K_{D}) were not resolved for this system, due to their high relative error, which means that for this system the total volume change ΔV° for the reaction could not be estimated from the rate data.

Figure 8 shows the results of repeating this experiment using the large piezostack (P-245.70) to generate 16 MPa pressure steps. In this case, the reciprocal relaxation times were significantly retarded at elevated pressure, compared with the low-pressure cases, and there was a significant difference between the values for k_{on} ($k_{\text{on}} = 2.11 \pm 0.03 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ at 20 MPa versus $k_{\text{on}} = 2.68 \pm 0.07 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ at 4 MPa), which allowed an estimate of $\Delta V^{\ddagger} = +36 \pm 6 \text{ ml} \cdot \text{mol}^{-1}$ to be made for the activation volume. The values for k_{off} , however, were still not sufficiently well resolved to permit estimation of the change in K_{D} value, and hence of ΔV° , from the rate data ($k_{\text{off}} = 0.24 \pm 0.07 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ at 20 MPa versus $k_{\text{off}} = 0.26 \pm 0.20 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ at 4 MPa).

Analysis of the amplitude data does allow the pressure-induced changes of concentration of bound mant-ADP to be estimated.

Figure 6 Examples of transients showing the effect of changing concentrations of skS1 and mant-ADP

Observed transients after a 6 MPa pressure jump on a solution of skS1 with mant-ADP. The best-fit single exponentials are shown superimposed and the data shown are: (A) 0.7 μM S1, 0.6 μM mant-ADP, mean of three measurements; (B) 1.2 μM S1, 1.0 μM mant-ADP, mean of three measurements; (C) 2.5 μM S1, 1.0 μM mant-ADP, mean of five measurements; (D) 4.0 μM S1, 1.0 μM mant-ADP, mean of ten measurements.

Figure 7 Concentration dependence of relaxation parameters for mixtures of mant-ADP/mant-dADP with skS1/smS1

The dependence of the reciprocal relaxation time and amplitude of the transient on the calculated free concentrations of S1 and (a) mant-ADP, (b) mant-dADP for data with skS1 (filled symbols) and smS1 (open symbols). Values of fitted parameters are given in Table 1. The results of decreasing pressure are indicated by ∇ , $\blacktriangledown$ and smooth line fits, whereas the results of increasing pressure are indicated by $\triangle$, $\blacktriangle$ and dashed line fits.

The analysis of the amplitudes for the 6 MPa relaxations are shown in Figure 7(a) (lower panel). The amplitude of the fluorescence change was used to calculate the change in the concentration of the bound mant-ADP, and thus the pressure-induced change in K_D value. This was used to calculate the molar volume change of the reaction from eqn (4) (see the Appendix for details) and gave a value, $\Delta V^\circ = 58.0 \pm 0.9 \text{ ml} \cdot \text{mol}^{-1}$.

The results of similar measurements and analysis using mant-dADP are shown in Figure 7(b) and summarized in Table 1. The results show that mant-dADP binds with a 3–4-fold lower affinity ($K_D = 0.54 \mu\text{M}$), due to a 40% lower second-order association rate constant and a > 2-fold higher dissociation rate

constant. The molar volume change for the reaction remains unchanged.

Comparison between skS1 and smS1

The same measurements were made for both mant-ADP and mant-dADP with smS1 (Figure 7, open symbols), and the results are summarized in Table 1. The affinity of the nucleotides for smS1 was very similar to those for skS1, with the same difference between mant-ADP and mant-dADP to those for skS1, i.e. a 5-fold weaker affinity for the deoxy-nucleotide. The fitted rate constants show greater differences, with both the association

Table 1 Results of the analysis of data from Figure 7

Results of the analysis of rate constant and amplitude data (Figure 7) for skeletal- and smooth-muscle S1 binding to mant-ADP and mant-dADP.

	Skeletal S1 binding to mant-ADP		Smooth S1 binding to mant-ADP		Skeletal S1 binding to mant-dADP		Smooth S1 binding to mant-dADP	
P_{∞} (MPa)	4	10	4	10	4	10	4	10
$K_1 k_{+2} = k_{on}$ ($s^{-1} \cdot \mu M^{-1}$)	2.06 ± 0.10	2.05 ± 0.10	1.52 ± 0.05	1.30 ± 0.05	1.26 ± 0.06	1.13 ± 0.06	0.95 ± 0.20	0.83 ± 0.20
$k_2 = k_{off}$ (s^{-1})	0.31 ± 0.20	0.35 ± 0.20	0.16 ± 0.03	0.14 ± 0.03	0.67 ± 0.20	0.73 ± 0.20	0.52	0.44
$1/K_1 K_2 = K_D$ (μM)	0.15	0.17	0.10	0.11	0.54	0.65	$0.55 \pm 0.02^*$	$0.53 \pm 0.02^*$
ΔV° ($ml \cdot mol^{-1}$)	58.0 ± 0.9		112 ± 7		60 ± 3		129 ± 3	

* $1/K_1 K_2$ value obtained from amplitude data. **Figure 8** The dependence of the reciprocal relaxation time on the free concentrations of S1 and mant-ADP, for 16 MPa pressure jumps

The results of decreasing pressure are indicated by ▼ and smooth line fits, whereas the results of increasing pressure are indicated by ▲ and dashed line fits. The results of analysis were (4 MPa) $K_1 k_{+2} = 2.68 \pm 0.07 s^{-1} \cdot \mu M^{-1}$, $k_2 = 0.26 \pm 0.20 s^{-1}$ and (20 MPa) $K_1 k_{+2} = 2.11 \pm 0.03 s^{-1} \cdot \mu M^{-1}$, $k_2 = 0.24 \pm 0.07 s^{-1}$.

and dissociation rate constants being approx. 30% lower for smS1.

What is striking in the data presented in Figure 7 is that the amplitudes of the perturbations are significantly larger for smS1 compared with the skS1. Given that the K_D values are essentially the same and the fluorescence changes on nucleotide binding are the same (we found $Q = 2.2$ for both proteins, in agreement with [9,19]), this must mean that the molar volume change for ADP binding is larger for smS1. The analysis shows that the ΔV° for ADP binding to smS1 is approximately twice that of skS1 for both mant-ADP and mant-dADP, with values of 120 and 60 $ml \cdot mol^{-1}$.

All of the results shown here were obtained using protein in excess of nucleotide. A duplicate set of data was also obtained in which the nucleotide was in excess (results not shown). For mant-dADP, there was little difference between the two data sets, as expected based on Scheme 2. However, when mant-ADP was used, the fitted values for k_{on} were between 60 and 80% lower than when excess protein was used (a control without protein present resulted only in a fast transient, consistent with compression of the mant-ADP sample). Mant-ADP is known to exist as two (2' and 3') isoforms. The simplest explanation of the results is that the two forms of mant-ADP are in competition for the nucleotide-binding pocket and have different affinities for S1, as seen for kinesin [20,21]. It is also possible that the two

isoforms have different fluorescent yields when bound to S1, as reported previously for cy-3 analogues of ADP [22]. The analysis of the relaxation kinetics with two competing ligands is complex and is beyond the scope of the present study.

DISCUSSION

Performance of equipment

The design of the new pressure-jump apparatus allows small samples of 50 μl to be subjected to 10 MPa pressure jumps, which are complete in 80 μs using a P-245.30 transducer. The speed of the response is directly related to the size of the crystal stack used. Smaller stacks have a lower mass and a faster response time but can apply only small pressure steps. The P-245.70 stack has three times the amplitude of movement (120 μm versus 40 μm), and we have used it to apply 20 MPa pressure steps in 150 μs . Smaller stacks are available, e.g. the P802.00 is theoretically capable of making jumps in $< 20 \mu s$, but this design would restrict the jump size to 1.5 MPa. The low sample volume of the current apparatus, the high sensitivity of the fluorescence signal (1% signal changes can be routinely monitored) and the simple solution-exchange system will make the apparatus available for a wide range of studies on proteins. A preliminary description of the apparatus was published previously [23], where its use for the study of protein-folding reactions was demonstrated. Jacob et al. [23] also showed that the apparatus could be used at temperatures of up to 80 $^\circ C$ and with viscous solvents such as 95% ethylene glycol. More recently [24], this apparatus has been used to directly measure the fast (approx. 1000 s^{-1}) transition between the 'open' and 'closed' nucleotide-binding states of a single tryptophan mutant of the *Dictyostelium* sp. myosin head, demonstrating the capability of this apparatus to detect small, fast fluorescence changes.

S1-nucleotide binding measurements

The improved performance of the apparatus compared with previous pressure-jump systems [1,2] allowed measurements to be made of the pressure-induced dissociation of mant-ADP and mant-dADP from S1 and the subsequent re-association reaction on pressure release. We previously looked at this reaction using the oil-based systems built in Bristol [1] and were unable to detect a significant signal change for the reaction, because signal resolution was limited by the small number of repeats possible with this system.

All of the transients were fitted satisfactorily by a single exponential equation, indicating that the perturbation of the reaction was relatively small. This was confirmed by the linear dependence of the fluorescence amplitude of the reaction on the size of the applied pressure step (Figure 5). For this experiment, there was no significant difference in the observed relaxation time

of the reactions due to either the application or release of pressure, nor was there a significant correlation between the observed relaxation time and the applied pressure step. If the equilibrium position ($K_D = k_{\text{off}}/k_{\text{on}}$) is perturbed by pressure, then k_{on} , k_{off} or both should be perturbed by pressure. In this experiment, the total free reactant concentration was chosen to be relatively low (approx. 3 μM) to give high-amplitude reactions where both k_{on} and k_{off} contribute to the reciprocal relaxation time. Assuming an activation volume $\Delta V^\ddagger$ of approx. 50 $\text{ml} \cdot \text{mol}^{-1}$, the expected change in reciprocal relaxation time at the maximum pressure change of 10 MPa would be of the order of 10%, which is at the limit of resolution for this type of experiment. In fact, data presented below suggest a lower activation volume. Hence, the activation volume could not be resolved under these circumstances.

In contrast with the data in Figure 5, analysis of the pressure sensitivity of the second-order binding constant (Figures 7 and 8) did show consistently that the data collected for pressure jump from 4 MPa up to 10 and 20 MPa were slower than those recorded for jumps returning to 4 MPa. The extent to which k_{off} was thus retarded by pressure is difficult to quantify, but for k_{on} the pressure differential calculated from Figure 8 was estimated at $1.5 \pm 0.3\% \cdot \text{MPa}^{-1}$. This corresponds to an activation volume of $36 \pm 6 \text{ ml} \cdot \text{mol}^{-1}$, but the uncertainty in the lines fitted to the reciprocal relaxation times means that comparisons between skS1 and smS1 are not significant.

Although all of the data are consistent with a single-step binding reaction, there is considerable evidence, at least in the case of skS1, that the reaction is at least a two-step process [25,26]. The following scheme can be used for a two-step process in which ADP is in rapid equilibrium with a myosin-ADP complex ($\text{M} \cdot \text{D}^*$), which then undergoes an isomerization, resulting in a change in both tryptophan fluorescence and mant fluorescence as shown in Scheme 3:

In this scheme, the first step is a fast equilibrium diffusion-controlled reaction with k_{+1} typically $\geq 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, with an affinity K_1 of 10^3 – 10^4 M^{-1} [27,28]. Under these circumstances, the overall second-order binding constant [k_{on} in Scheme 2 and eqn (5)] is given by $k_{\text{on}} = K_1 k_{+2}$ and the dissociation rate constant is given by $k_{\text{off}} = k_{-2}$. The formation of an encounter complex between S1 and ADP has previously been observed [26] and later confirmed with mant-labelled nucleotides [18]. A two-step binding reaction can give rise to two relaxations. However, for a K_D value of 0.1 μM , if $K_1 = 10^4 \text{ M}^{-1}$ then $K_2 = 10^3$; for the concentrations used here, the occupancy of the intermediate state $\text{M} \cdot \text{D}^*$ is always very low. Only a single relaxation is therefore expected to be observed.

The results show that there are only slight differences in the K_D value for mant-ADP with skeletal-muscle and smooth-muscle S1, and these differences are reflected in slight changes in both $K_1 k_{+2}$ and k_{-2} . The values for mant-dADP are also similar for the two proteins. However, mant-dADP binds to both proteins with a 4–5-fold weaker affinity compared with mant-ADP. This is reflected in changes in both $K_1 k_{+2}$ and k_{-2} . The lower affin-

ity of mant-dADP for the proteins produces enhanced fluorescence amplitudes and hence better-defined parameters for this nucleotide, but does not significantly alter the value of ΔV° , which is independent of the nucleotide.

Values for ΔV° are derived solely from fits to the amplitude data (see the Appendix) and are calculated assuming a single-step binding mechanism. Provided K_2 is high (i.e. occupancy of the $\text{M} \cdot \text{D}^*$ state is low under all conditions), then the calculation remains valid. The ΔV° values cannot be assigned to a particular step or distributed between steps, but show simply that there is a total positive volume change of the sizes shown between ($\text{M} + \text{D}^*$) and $\text{M} \cdot \text{D}^{**}$. This is 2-fold higher for smS1 than for skS1 in the case of both nucleotides.

These molar volume changes are quite high in comparison with, e.g., protonation of a simple salt such as phosphate (24 $\text{ml} \cdot \text{mol}^{-1}$ [17]), or denaturation of a cold-shock protein ($-60 \pm 20 \text{ ml} \cdot \text{mol}^{-1}$ [23]). Volume changes often result from the displacement of structured water from a protein or ligand surface either directly by the binding or indirectly via induced changes in conformation. The much higher value for smS1, as compared with skS1, suggests that there is a significant difference in the induced conformational changes that occur with smS1. This is in contrast with the published ΔV° value for ADP binding to acto-S1 from skeletal muscle ($59 \pm 13 \text{ ml} \cdot \text{mol}^{-1}$ [12]), which is very similar to that for mant-ADP binding to skS1. The affinities of ADP for S1 and actoS1 are very different (0.1 μM versus 100 μM for acto-skS1). The values of ΔV° may therefore reflect water, which is displaced directly on binding to ADP. The additional volume change in smS1 may reflect displacement of water molecules during an additional isomerization of the nucleotide binding site. It is now well established that there is an extra 20–25° tilt in the light-chain-binding domain (the putative lever arm) of smooth-muscle myosin upon ADP binding/release compared with skeletal-muscle myosin when bound to actin [7,8]. It has been proposed that this tilt is evidence of a strain-sensing mechanism, whereby ADP release is inhibited by tension, with a consequent increase in the duty ratio [9]. It is not clear, however, if the additional isomerization seen in ADP binding to acto-smS1 (where the K_D value is 20 μM , i.e. five times tighter than for acto-skS1) is present in the absence of actin. However, studies of fluorescence lifetimes have demonstrated the existence of two smS1-ADP states, both with and without actin, which are thought to be due to the presence of an equilibrium mixture of pre-swing and post-swing states [8]. It is therefore possible that the difference in ΔV° for ADP-S1 binding, between skS1 and smS1, is due to the additional conformational change. It is therefore possible that this transition can be investigated further by pressure perturbation studies. For example, comparison between smS1 and other types of myosin may provide a simple method of establishing if the additional conformational change is present.

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APPENDIX

Analysis of fluorescence changes in fluorescein

Using the titration curve of fluorescein, it was possible to analyse the fluorescence amplitudes in imidazole (which exhibits a

negligible volume change on ionization) and thus find values for the molar volume change associated with the two ionization reactions of fluorescein. In imidazole buffer, the fluorescence under compression may be expressed by including terms for the

perturbation to the pK values:

$$F/F_{\max} = 1 - \{Q[H^+]/(K_1 + \Delta K_1 + [H^+]) + (1 - Q)[H^+]/(K_2 + \Delta K_2 + [H^+])\} \quad (A1)$$

Eqn (3) yielded values -9 and $-19 \text{ ml} \cdot \text{mol}^{-1}$ for the molar volume changes associated with these two ionization reactions. From this information, it was possible to calculate a value for the pH shift, and therefore for volume change, of phosphate ionization using the data measured in phosphate buffer. Thus the fluorescence output after compression for fluorescein in phosphate buffer is given by the following formula:

$$F/F_{\max} = 1 - \{Q \times 10^{(pH + \Delta pH)} / (K_1 + \Delta K_1 + 10^{(pH + \Delta pH)}) + (1 - Q)10^{(pH + \Delta pH)} / (K_2 + \Delta K_2 + 10^{(pH + \Delta pH)})\} \quad (A2)$$

where ΔpH is the pH shift caused by the shift in pK_a value of the phosphate buffer (because the composition of the buffer did not change, $\Delta(pK_a) = \Delta pH = -0.029$). A value for the ΔV° was calculated from ΔK_a and it was $24 \text{ ml} \cdot \text{mol}^{-1}$, which is consistent with the literature value [17].

Additionally, the decrease in volume of the sample caused a commensurate increase in fluorescein concentration, consistent with the compressibility of water ($M_B^{-1} = 0.046 \% \cdot \text{MPa}^{-1}$). The total change in fluorescence signal on compression was therefore calculated using the following formula:

$$\Delta F = (F_{\text{final}} - F_{\text{initial}}) + (\Delta P \cdot F_{\text{final}} M_B^{-1} / 100) \quad (A3)$$

Using this equation for the data obtained with imidazole and phosphate along with the titration data, the fitting procedure was able to converge well, providing the parameter estimates shown above.

Analysis of the fluorescence amplitude in mant binding

It was also possible to analyse the fluorescence amplitude in the mant-labelled nucleotide-binding experiments to provide estimates for the overall molar volume change ΔV° for the reaction. The fluorescence enhancement on binding mant-ADP to S1 ($Q = 2.2$, from equilibrium measurements and [9,19]) provided a relationship between the amplitude and the change in concentration Δc :

$$\% \Delta F/F = (F_{\text{final}} - F_{\text{initial}}) / F_{\text{final}} = (Q - 1) \Delta c / ([D^*] + Q[MD^{**}]) \quad (A4)$$

The concentration of bound mant-ADP could be calculated using eqn (6), and substituting $K_D = K_D + \Delta K_D$ in eqn (6) allowed the change in concentration ($\Delta c = [MD^{**}]_{\text{final}} - [MD^{**}]_{\text{initial}}$) to be modelled as a function of the molar volume change ΔV° and the equilibrium dissociation constant [using eqn (3)]. Adjustment of the modelled parameters (ΔV° , and on one occasion K_D) so that the simulated curve moved to the extreme edge of the data allowed the confidence limits to be estimated for these parameters. Where errors are shown on K_D rather than k_{off} , this indicates that k_{off} was derived by adjusting the K_D value within the amplitude fit and recalculating k_{off} from the product $k_{\text{on}} \cdot K_D$.

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