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A transient kinetic study of enthalpy changes during the reaction of myosin subfragment 1 with ATP

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1. The enthalpy changes during individual reaction steps of the myosin subfragment 1 ATPase were studied with the use of a new stopped-flow calorimeter [Howarth, Millar & Gutfreund (1987) Biochem. J. **248**, 677–682]. 2. At 5 °C and pH 7.0, the endothermic on-enzyme ATP-cleavage step was observed directly ($\Delta H = +64$ kJ·mol^{-1}). 3. ADP binding is accompanied by a biphasic enthalpy change. 4. The release and uptake of protons was investigated by the use of two buffers with widely different heats of ionization. 5. Protons are involved in all four principal steps of the myosin subfragment 1 ATPase.

INTRODUCTION

A wide range of transient kinetic techniques have been applied to the study of the individual steps of the myosin-mediated hydrolysis of ATP. These investigations were aimed both at the elucidation of the chemical mechanism of the hydrolysis reaction and at the correlation of individual steps of this reaction with those of actin–myosin interaction and of the contractile process. Methods have been developed that can characterize these steps in systems at different levels of organization, from pure proteins in solution to intact fibres (Hibberd & Trentham, 1986).

The complementary information obtained from techniques that use different methods for the initiation of reactions, and different physical signals for monitoring intermediates, helps in the elucidation of the number and character of distinct steps involved.

The most detailed investigations have been carried out on the proteolytic subfragment S1 of myosin, which retains the ATPase and actin-binding functions of myosin. Stopped-flow techniques with fluorescence and absorbance monitors, as well as rapid sampling for subsequent chemical or isotopic analysis, have been used to provide information about the following principal steps in the hydrolysis pathway, where M denotes a single ATPase site of myosin or its subfragments (Trentham *et al.*, 1976; Taylor, 1979):

$$\begin{array}{ccccccc} & & \overset{1}{} & & \overset{2}{} & & \overset{3}{} & & \overset{4}{} & \\ \mathrm{M} & \rightleftharpoons & \mathrm{M\cdot ATP} & \rightleftharpoons & \mathrm{M\cdot ADP\cdot P_i} & \rightleftharpoons & \mathrm{M\cdot ADP} & \rightleftharpoons & \mathrm{M} & (1) \\ +\mathrm{ATP} & & & & & & +\mathrm{P_i} & & +\mathrm{ADP} & \end{array}$$

In the present paper we show how stopped-flow calorimetry, developed by Howarth *et al.* (1987), can make further important contributions to our understanding of the mechanism of the myosin ATPase and its relation to muscle contraction. The resolution of enthalpy changes during the steps outlined in reaction scheme (1), as well as the possible demonstration of additional ones, can contribute to our knowledge of the reaction in several ways. First, it is of interest to correlate the thermal events during the ATPase reaction with those studied in the extensive calorimetric investigations on live muscle fibres (for a review see Woledge *et al.*, 1985). A major objective of kinetic investigations on physiological systems is the temporal correlation of molecular events with the behaviour of the intact system. Secondly, the direct determination of the thermodynamic parameters (Gibbs energy, enthalpy and entropy) for individual steps of the reaction should contribute to our understanding of the nature of the events. And, thirdly, it is possible to obtain information about the rates and stoicheiometries of proton uptake and release during the interconversion of intermediates. It is shown in this paper that such data can be obtained by carrying out the same reaction in two buffer systems, one with a negligible and the other with a large heat of ionization. The calculation of changes in free [H^+] from such differential heat measurements provides a better procedure, less fraught with potential artifacts, than the use of indicators.

Some of the steps of reaction scheme (1) have been studied by Kodama & Woledge (1976) and Kodama (1985) with the use of conventional calorimetric techniques. Many of their conclusions were based on experiments with ATP analogues that are slowly hydrolysed, and are likely to form different complexes with myosin. However, as shown below, their conclusion about the endothermic nature of the ATP hydrolysis step is borne out by our direct observations.

The present paper demonstrates what can be learned about the individual reaction steps of the S1 ATPase by our technique in its present stage of development. A comparison of kinetic and thermodynamic information obtained from such studies with those obtained by other transient kinetic techniques will realize its full potential when experiments have been carried out under the wide range of conditions for which data are available.

EXPERIMENTAL

Chemicals

Myosin S1 was prepared by a chymotryptic digest of rabbit skeletal-muscle myosin, as described by Weeds &

Abbreviation used: S1, myosin subfragment 1.

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Taylor (1975). The two isoenzymes S1-A1 and S1-A2 were not separated for use in these experiments. S1 concentration was calculated by using $M_r = 115000$, $A_{280}^{1\%} = 7.9\ cm^{-1}$. ATP and ADP were obtained from Sigma Chemical Co. and were used without further purification. Their concentration was determined by using the molar absorption coefficient of $\epsilon_{259} = 15.4\ mM^{-1} \cdot cm^{-1}$.

Calorimetry

The stopped-flow calorimeter is described in detail in the accompanying paper (Howarth *et al.*, 1987). The Figures in the present paper are all difference records obtained with the use of water versus water as a blank, and are averages of three to five reactions. They simply represent the change in temperature with time; an increasing signal indicates warming (due to an exothermic process), and a decreasing signal indicates cooling (due to an endothermic process). The sensitivity of the thermopile was taken to be $56\ \mu V \cdot K^{-1}$, and the enthalpy change, ΔH (in $kJ \cdot mol^{-1}$), is calculated from the temperature change by using the relationship $\Delta H = \Delta T \cdot C_p / C_l$, where ΔT is the observed temperature change (mK), C_p is the product of the specific heat capacity and the density of the solution (taken as $4.2\ kJ \cdot l^{-1} \cdot K^{-1}$ at 5 °C) and C_l is the limiting concentration (mM). Concentrations are quoted throughout as reaction-chamber concentrations. The enthalpies of ionization of the buffers used were taken as $-36\ kJ \cdot mol^{-1}$ for imidazole and zero for cacodylate (CRC Biochemical Handbook, and confirmed with our calorimeter). Reactions with rates of less than about $1\ s^{-1}$ were corrected for heat loss by using a value of $0.017\ s^{-1}$ for the rate of heat loss, as described by Howarth *et al.* (1987). The records were digitized as 4000 12-bit points by a Nicolet 3091 digital oscilloscope, and stored and analysed on an Apple IIe microcomputer using standard non-linear fitting programs.

RESULTS

Kodama & Woledge (1976) were unable to observe the enthalpy change associated with the ATP-cleavage step, because it was much too fast for their batch calorimeter. The rate of the cleavage step at 20 °C is about $100\ s^{-1}$ (Johnson & Taylor, 1978), which is still too fast for the stopped-flow calorimeter, and the work described in the present paper was therefore performed at 5 °C. At this temperature the cleavage rate is between 5- and 10-fold slower (Johnson & Taylor, 1978), and so falls within the range of our instrument.

Working at low temperatures also lowers the value of the equilibrium constant K_2 (see reaction scheme 1). At 20 °C the phosphate burst has a value of 0.9 mol/mol, so $K_2 = 10$ (Bagshaw & Trentham, 1973), whereas at 5 °C the size of the burst is only 0.5 ± 0.1, so $K_2 = 1$ (Taylor, 1977; Kodama *et al.*, 1986). This means that at 5 °C only 50% of the ATP is cleaved rapidly during the phosphate burst, and the remainder is cleaved slowly at the same rate as the phosphate-release step. Steps 1–3 were investigated by mixing S1 with ATP, step 4 was investigated by mixing S1 with ADP, and an ADP-displacement experiment (S1·ADP versus ATP) was used to measure the net heat of ATP hydrolysis.

ATP versus S1, cacodylate buffer

In the first experiments ATP was mixed with an excess of S1, so that the individual steps could be observed, and the enthalpy changes could be determined accurately by using the nucleotide concentration. Under these conditions the product ADP does not dissociate from S1 to any significant extent, and the end point of the reaction is S1·ADP. Since cacodylate has a negligible heat of ionization, the observed temperature changes correspond to the actual reaction heats. The details and results of such a single-turnover experiments are shown in Fig. 1.

Three phases can be seen: a large rapid production of heat, followed by a small exponential endothermic phase that is complete in about 2 s, and finally a slow exponential exothermic phase. This last phase has an observed rate constant of $0.024\ s^{-1}$ (after correction for heat loss), and so correlates with the phosphate-release step (step 3). This compares with an observed rate of $0.036\ s^{-1}$ obtained by Bagshaw & Trentham (1974) at 5 °C at pH 8, and gives a value of $0.048\ s^{-1}$ for k_{+3} [since $k_{+3} = k_{obs.}\ (K_2 + 1)/K_2$].

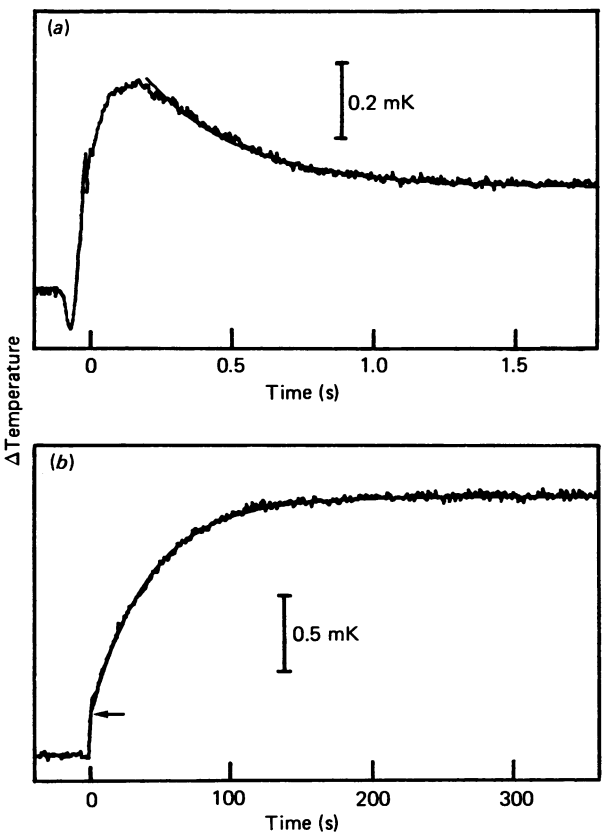

Fig. 1. S1 versus ATP (single turnover) in cacodylate buffer

Conditions: 50 mM-sodium cacodylate buffer, pH 7.0, 0.1 M-KCl, 5 mM-$MgCl_2$, 185 μM-S1, 125 μM-ATP, 5 °C (all concentrations quoted throughout as reaction-chamber concentrations). Time zero marks the point at which flow stops. The same reaction is displayed on two time scales, and the record in (*b*) has been corrected for heat loss. The transient events in (*a*) are too fast to be seen on the slow time scale, but the net temperature rise in (*a*) (of 0.26 mK or $-8\ kJ \cdot mol^{-1}$) can be seen as a burst in (*b*) (marked by arrow). The exponential fitted to the endothermic phase in (*a*) has a rate constant of $2.95\ s^{-1}$, and the exponential fitted to the exothermic phase in (*b*) has a rate constant of $0.024\ s^{-1}$ and an amplitude (from the arrow) of 1.38 mK ($-46\ kJ \cdot mol^{-1}$).

There is a net rise of 0.26 mK (-8 kJ·mol^{-1}) to the end of the endothermic phase, which is the sum of the enthalpy changes of steps 1+2. However, the individual enthalpy changes of these two steps cannot be resolved in this experiment because the temperature changes cancel out during the first 200 ms. This problem can be overcome by using a high concentration of ATP, so that the fast exothermic binding phase is largely over before the endothermic cleavage step begins. The drawback with this approach is that, in order to calculate ΔH, the S1 concentration is required. This cannot be measured as accurately as the nucleotide concentration, since there is always an inactive fraction of S1. Chemical measurements of ATP binding give values of 70–85% activity (Barman *et al.*, 1983); a value of 80% was assumed for our experiments. The results of such an experiment are shown in Fig. 3.

The initial heating is now faster, confirming that this is due to the binding step. The endothermic phase is also faster, with a rate constant of 7–8 s^{-1}. This is similar to previous estimates of the rate of ATP cleavage from different techniques. Johnson & Taylor (1978) obtained a rate of 20 s^{-1} for the fluorescence enhancement under the same conditions, and Sleep & Taylor (1976) measured the rate of the phosphate burst by quench-flow as 7 s^{-1} at 3 °C in the presence of 40 mM-KCl.

The rate of the ATP-cleavage step is difficult to measure accurately by fluorescence, because the signals from the binding and the cleavage steps are difficult to separate. Both binding and cleavage give increasing fluorescence signals, so the observed rate of the slower step will tend to be an overestimate of the true value. With heat measurements, on the other hand, the signals are in opposite directions, leading to an underestimate for the rate of cleavage. If the endothermic signal in our results were reporting a slower step than ATP cleavage, then a lag phase might be expected, but no such lag is observed. The simplest assumption is therefore that this phase is indeed the ATP-cleavage step.

The amplitude of this phase is 0.78 mK, which corresponds to an observed enthalpy change, $\Delta H_{2(\text{observed})}$, of +32 kJ·mol^{-1}. Since $K_2 = 1$, this observed enthalpy change is due to the cleavage of only 50% of the ATP, so ΔH_2 is twice this, i.e. +64 kJ·mol^{-1}. The enthalpy changes during the other two steps can now be calculated. The net heat rise up to the end of the endothermic phase (Fig. 1*a*) is -8 kJ·mol^{-1}, which is the sum of $\Delta H_1 + \Delta H_{2(\text{observed})}$. This latter term is +32 kJ·mol^{-1}, so $\Delta H_1 = -40$ kJ·mol^{-1}. The observed enthalpy change for the phosphate-release step is -46 kJ·mol^{-1} (Fig. 1*b*), but this also includes the enthalpy change due to the remaining 50% of ATP cleavage. This is the same as the observed enthalpy change during the cleavage step, and so contributes a cooling of 32 kJ·mol^{-1}, so $\Delta H_3 = -78$ kJ·mol^{-1}. These thermodynamic data are summarized in Table 1.

Table 1. Summary of thermodynamic data for the hydrolysis of ATP by S1 at 5 °C at pH 7.0

All enthalpy changes are quoted in units of kJ·mol^{-1}. The observed enthalpy changes are corrected as described in the text by using the following relationships:

$$\Delta H_1 = \Delta H_{1+2(\text{obs.})} - \Delta H_{2(\text{obs.})}$$
$$\Delta H_2 = \Delta H_{2(\text{obs.})} \cdot (1 + K_2)/K_2$$
$$\Delta H_3 = \Delta H_{3(\text{obs.})} - (\Delta H_{2(\text{obs.})}/K_2)$$
$$\Delta H_4 = \Delta H_{4a} + \Delta H_{4b}$$

and by assuming that $K_2 = 1$. The net enthalpy change is obtained by experiments (Fig. 5), and so is not necessarily exactly the same as the sum of the four steps. ΔH (Kodama, 1985) is taken from Table 2 of that reference and is for 4 °C at pH 8.0. Δ(protons) data are quoted as mol of H$^+$/mol of ATP, and are calculated by using -36 kJ·mol^{-1} for the enthalpy of ionization of imidazole.

	1 ATP binding	2 ATP cleavage	3 Phosphate release	4 ADP release (a)	4 ADP release (b)	Net (1+2+3+4)
Sodium cacodylate buffer						
$\Delta H_{\text{observed}}$ (kJ·mol^{-1})		-8 / +32	−46	+7	+29	−19
$\Delta H_{\text{corrected}}$ (kJ·mol^{-1})	−40	+64	−78	+36		−19
ΔH (Kodama, 1985) (kJ·mol^{-1})	−65	+67	−73	+54		−17
Imidazole/HCl buffer						
$\Delta H_{\text{observed}}$ (kJ·mol^{-1})		+16 / +24	−68	+18	0	−34
$\Delta H_{\text{corrected}}$ (kJ·mol^{-1})	−8	+48	−92	+18		−34
Δ(protons) (mol/mol)	0.9 H$^+$ uptake	0.4 H$^+$ release	0.4 H$^+$ release	0.3 H$^+$ uptake	0.8 H$^+$ release	0.4 H$^+$ release
	0.5 H$^+$ uptake			0.5 H$^+$ release		

ATP versus S1, imidazole buffer

The ATP-binding experiments were repeated in imidazole buffer. In this case the observed temperature changes are composed of the actual reaction heat plus any heat due to the involvement of protons. The stoicheiometry of proton release or uptake can be calculated from the known enthalpy of ionization of imidazole. The results (shown in Figs. 2 and 3) are similar to those in cacodylate. The observed rates of both the endothermic phase (8.1 s^{-1}) and the exothermic phase (0.026 s^{-1}) in imidazole are similar to the rates of the corresponding phases in cacodylate (7.7 s^{-1} and 0.024 s^{-1} respectively), indicating that the reaction mechanism is not altered.

The enthalpy changes for the individual steps can therefore be calculated in the same way as used previously for cacodylate. The amplitude of the endothermic phase (0.56 mK; Fig. 3*b*) corresponds to an observed enthalpy change, $\Delta H_{2(\text{observed})}$, of $+24$ kJ·mol^{-1}, and so $\Delta H_2 = +48$ kJ·mol^{-1} in imidazole buffer (since $K_2 = 1$). This is smaller (i.e. more negative) than the corresponding value in cacodylate, indicating that a fraction of a proton (0.4 mol of H^+/mol of ATP cleaved) is released with this step.

The phosphate-release step has an amplitude of 2.43 mK (Fig. 2*b*), so $\Delta H_{3(\text{observed})} = -68$ kJ·mol^{-1}, and this includes $+24$ kJ·mol^{-1} due to ATP cleavage, so $\Delta H_3 = -92$ kJ·mol^{-1}. This too is more negative than the value in cacodylate, and so corresponds to a release of 0.4 mol of H^+/mol with this step. But perhaps the most striking feature of the results in imidazole buffer is the large initial cooling (Fig. 2*a*). The magnitude of this cooling was variable, but the net cooling to the end of the slow endothermic phase at about 1 s is -0.57 ± 0.15 mK, which corresponds to an observed enthalpy change, $\Delta H_1 + \Delta H_{2(\text{observed})}$, of $+16$ kJ·mol^{-1}. Since $\Delta H_{2(\text{observed})} = +24$ kJ·mol^{-1}, then ΔH_1 in imidazole is -8 kJ·mol^{-1} compared with -40 kJ·mol^{-1} in cacodylate.

It should be noted that just mixing S1 in imidazole buffer with imidazole buffer in the absence of ATP (i.e.

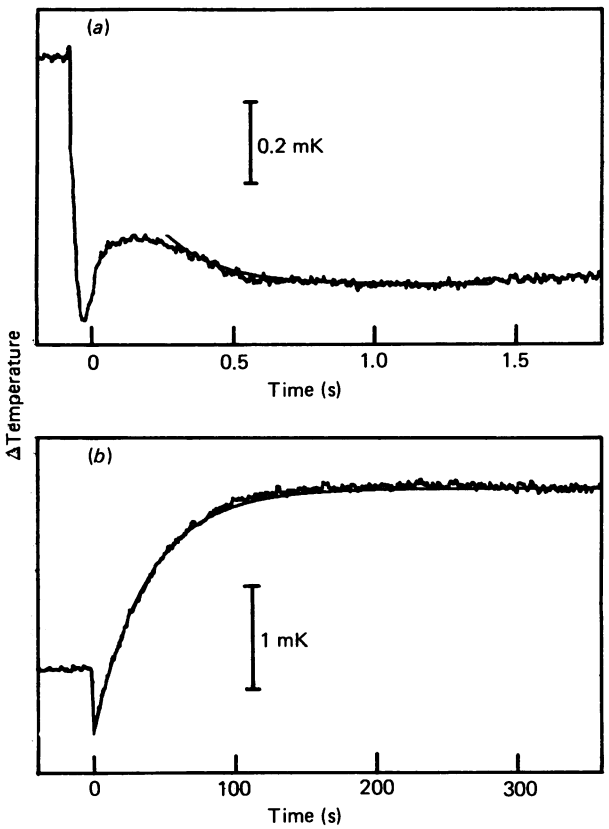

Fig. 2. S1 versus ATP (single turnover) in imidazole buffer

Conditions: 50 mM-imidazole/HCl buffer, pH 7.0, 0.1 M-KCl, 5 mM-$MgCl_2$, 200 μM-S1, 150 μM-ATP, 5 °C. The same reaction is displayed on two time scales, and the record in (*b*) has been corrected for heat loss. Only the net decrease in temperature in (*a*) (of -0.57 mK or $+16$ kJ·mol^{-1}) is seen in (*b*) as the initial decrease at time zero. The exponential fitted to the endothermic phase in (*a*) has a rate constant of 5.4 s^{-1}, and the exponential fitted to the exothermic phase in (*b*) has a rate constant of 0.026 s^{-1} and an amplitude of 2.43 mK (-68 kJ·mol^{-1}).

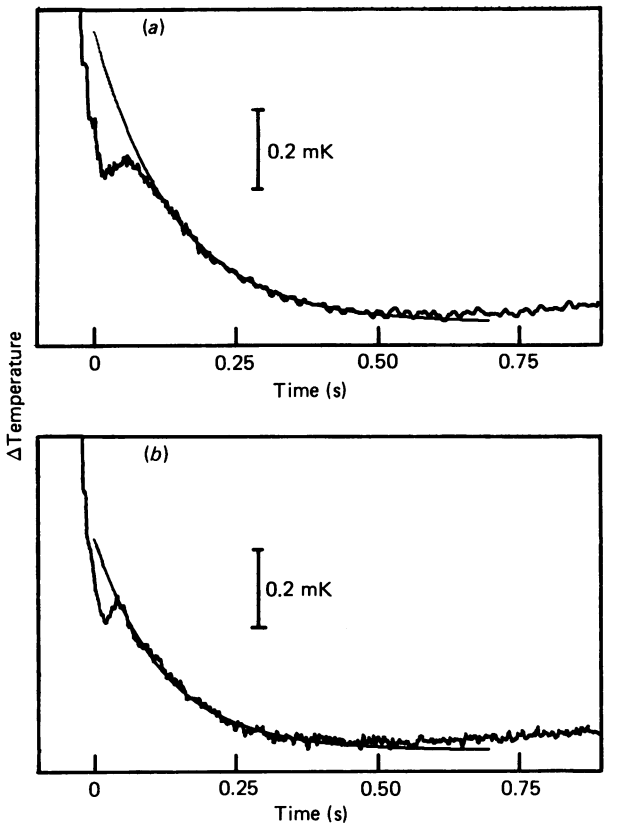

Fig. 3. ATP-cleavage step, high ATP concentration

(*a*) Cacodylate buffer. Conditions: as for Fig. 1 but with 100 μM-S1, 1 mM-ATP. The fitted exponential has a rate constant of 7.07 s^{-1} and an amplitude (extrapolated back to zero time) of 0.78 mK (32 kJ·moi^{-1}). (*b*) Imidazole buffer. Conditions as for Fig. 2 but with 100 μM-S1, 3 mM-ATP. The fitted exponential has a rate constant of 8.13 s^{-1} and an amplitude (extrapolated back to zero time) of 0.56 mK (24 kJ·mol^{-1}). The actual S1 concentration measured by A_{280} was 125 μM in both cases, but for the calculation of ΔH it was assumed that 80% was active. The start point of these records is off scale because these multiple-turnover reactions continue to produce heat for about 30 min, and the baseline from the previous push had not equilibrated to zero before the next reaction was initiated.

just diluting S1) also resulted in a small instantaneous cooling of some 4 kJ·mol^{-1}. This was not seen with cacodylate buffer, and so suggests that the cause of this cooling is a proton uptake. This could be due to changes in the hydration sphere surrounding the protein molecule, or disruption of S1–S1 interactions caused by shearing in the mixing chamber or by dilution. Whatever the reason, this cooling (which has already been subtracted from the record shown in Fig. 2) was quite variable and probably accounts for the variation in the cooling with ATP. The variation, however, is not enough to alter the striking conclusion that there is an uptake of almost 1 mol of H^+/mol with the binding step.

ADP versus S1

The enthalpy change associated with the ADP-release step was measured by using the reverse reaction, ADP binding to S1. Fluorescence studies have indicated that ADP binding is followed by two first-order transitions with maximum rates of 180 s^{-1} and 15 s^{-1} at 4 °C (Trybus & Taylor, 1982). The calorimetric records for ADP binding to S1 in cacodylate are in agreement with this (Figs. 4*a* and 4*b*). There is an initial rapid heat production (of 0.5 mK or −29 kJ·mol^{-1}) followed by a smaller exothermic exponential phase with a rate constant of 11.3 s^{-1} and an amplitude of 0.12 mK (−7 kJ·mol^{-1}). The total enthalpy change is −36 kJ·mol^{-1}.

However, the same reaction in imidazole buffer has different characteristics (Fig. 4*c*). There is no initial rapid rise, but only a single exponential exothermic process with a rate constant of 4.6 s^{-1} and an enthalpy change of −18 kJ·mol^{-1}. If we assume that the actual reaction mechanism is the same in the two buffers, then these differences must be due to the involvement of protons. Since no burst heat is observed in imidazole, this indicates that the initial heat rise of 29 kJ·mol^{-1} observed in cacodylate is cancelled out by an approximately equal cooling due to the uptake of protons. If this is correct this corresponds to an uptake of 0.8 mol of H^+/mol.

The rates of the slow phases were variable in both buffers, but they did not increase with increasing ADP concentration, and the rate was always 2–5-fold slower in imidazole. The reason for this disrepancy is unknown. It is unlikely that the same step has different rates in the two buffers, but it is possible that two different steps are being measured. These could be successive steps in a sequential pathway (but then a lag would be expected in the imidazole record). Alternative steps in a branched pathway may also account for this discrepancy. However, for the purpose of this study the end result would be the same, and it will be assumed that the slow phases in the two buffers represent the same step (or sum of steps). The enthalpy changes therefore indicate a release of 0.3 mol of H^+/mol during the slow phase and a net uptake of 0.5 mol of H^+/mol accompanying ADP binding. This is summarized below:

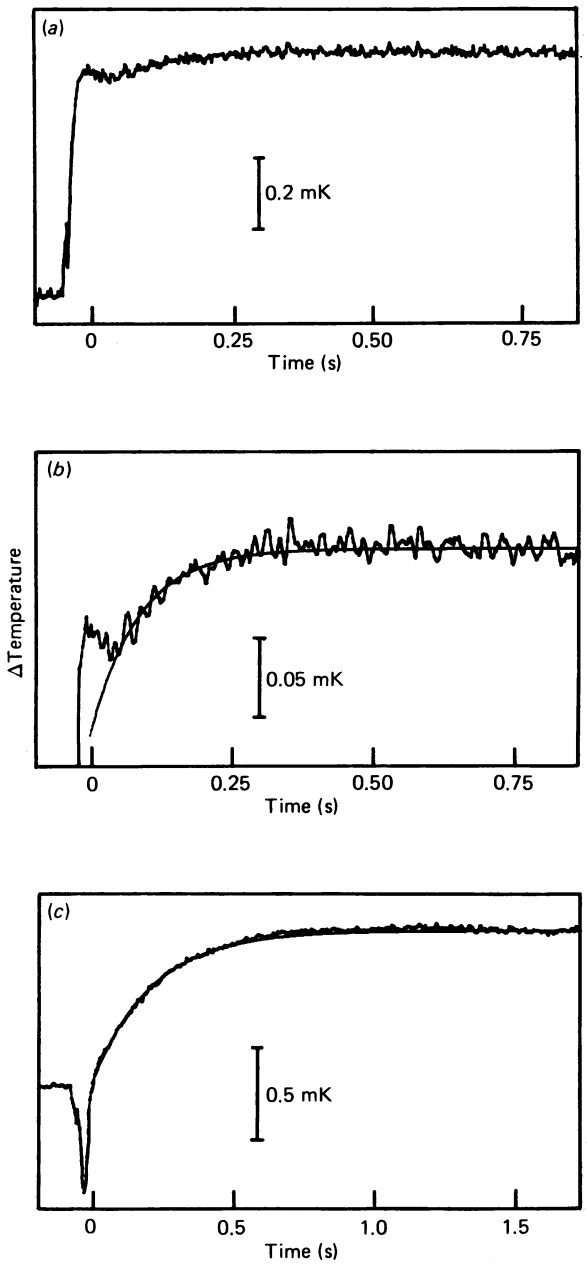

Fig. 4. ADP binding to S1

(*a*) Cacodylate buffer. Conditions: as for Fig. 1 but with 120 μM-S1, 80 μM-ADP. Total temperature rise is 0.67 mK (−36 kJ·mol^{-1}). (*b*) Enlargement of slow phase in (*a*). The fitted exponential has a rate constant of 11.3 s^{-1} and an amplitude (extrapolated back to zero time) of 0.12 mK (−7 kJ·mol^{-1}). The immediate post-trigger cooling phase in this record is an instrument artifact (discussed in Howarth *et al.*, 1987). (*c*) Imidazole buffer. Conditions: as for Fig. 2 but with 250 μM-S1, 200 μM-ADP. The rate constant is 4.58 s^{-1} and the amplitude is 0.86 mK (−18 kJ·mol^{-1}).

	M + ADP ⇌	M·ADP ⇌	M′ADP	Net
ΔH (cacodylate) (kJ·mol^{-1})	−29	−7		−36
ΔH (imidazole) (kJ·mol^{-1})	0	−18		−18
Δ(protons) (mol/mol)	0.8 H^+ uptake	0.3 H^+ release		0.5 H^+ uptake

(2)

ATP versus S1·ADP

In this experiment ATP is mixed with an excess of S1·ADP. ATP displaces ADP from S1·ADP, the rate of the displacement being given by:

$$k_{\text{disp.(observed)}} = \frac{k_{+4} \cdot k_{+3} \cdot K_2}{(1+K_2)[k_{+4}+k_{+3} \cdot K_2/(1+K_2)]}$$

The end point is S1·ADP, so the net reaction is the hydrolysis of ATP. The results of the displacement experiments are shown in Fig. 5. In both buffers there is an endothermic phase followed by the slow exothermic phosphate-release step. The rate of the slow phase is about 0.02 s^{-1}, which is slightly lower than the rates of the phosphate release step observed in the single-turnover experiments (0.024 s^{-1} in cacodylate and 0.026 s^{-1} in imidazole). This difference is to be expected, since k_{+3} and k_{+4} only differ by about a factor of 2 at this temperature (Bagshaw & Trentham, 1974), and so the endothermic displacement phase will tend to diminish the observed rate of the following exothermic process.

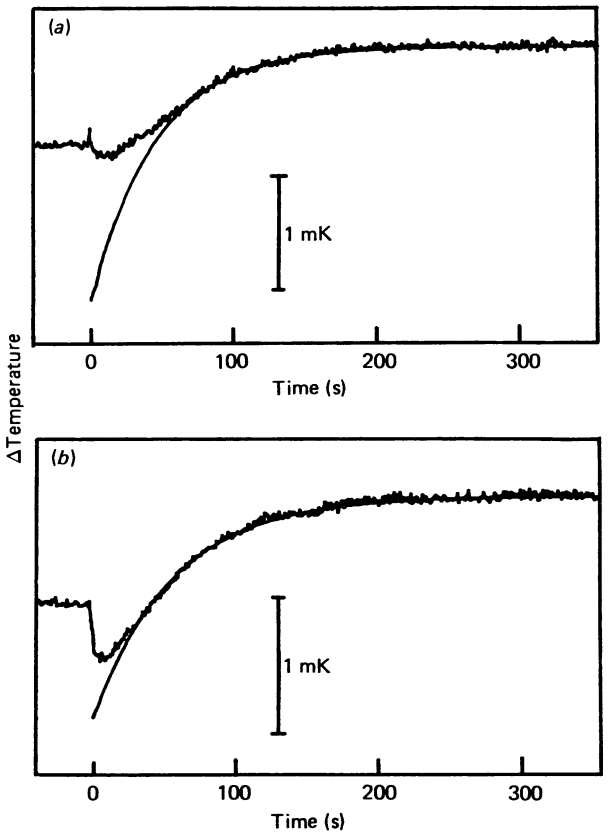

Fig. 5. ADP displacement by ATP

(*a*) Cacodylate buffer. Conditions: as for Fig. 1 but with 250 μM-S1, 300 μM-ADP, 200 μM-ATP. The exponential is fitted to the data from 80 s, and has a rate constant of 0.022 s^{-1} and an amplitude (extrapolated back to zero time) of 2.25 mK (−47 kJ·mol^{-1}). The net temperature rise is 0.9 mK (−19 kJ·mol^{-1}). (*b*) Imidazole buffer. Conditions: as for Fig. 2 but with 125 μM-S1, 150 μM-ADP, 100 μM-ATP. The exponential is fitted from 50 s and has a rate constant of 0.018 s^{-1} and an amplitude (extrapolated back to zero time) of 1.64 mK (−69 kJ·mol^{-1}). The net temperature rise is 0.8 mK (−34 kJ·mol^{-1}). Both these records are corrected for heat loss.

Extrapolating the fitted exponentials back to zero time gives values of −65 kJ·mol^{-1} and −47 kJ·mol^{-1} for the enthalpy changes in cacodylate and imidazole respectively, which are the same as those in the single-turnover experiments.

The net enthalpy change for ATP hydrolysis is obtained from the difference between the start and end points. This gives values for ΔH_{net} in cacodylate and imidazole of −19 kJ·mol^{-1} and −34 kJ·mol^{-1} respectively, which indicates that there is a net release of 0.4 mol of H^+/mol accompanying ATP hydrolysis under these conditions. An alternative way to measure the net heat of ATP hydrolysis would to be measure the steady-state rate of heat production. However, this method depends on knowing accurate values for the active S1 concentration, the turnover number ($k_{cat.}$) and the rate of heat loss, and consequently is subject to large cumulative errors. We have performed such multiple-turnover experiments in the stopped-flow calorimeter, and within experimental error the values for ΔH_{net} agree with those obtained by the displacement experiments.

In both buffers the endothermic displacement process has at least two phases. This is partly because the displacement is biphasic anyway (Trybus & Taylor, 1982), but mainly because of the effect of the heating due to the subsequent phosphate release. It is not therefore possible to obtain values for $k_{disp.}$ or $\Delta H_{disp.}$ by this method, but it is clear that the enthalpy change accompanying the displacement is strongly endothermic in both buffers.

DISCUSSION

Thermodynamic data

The enthalpy of hydrolysis of ATP has been measured on numerous occasions under many different conditions, and has a value of −20 kJ·mol^{-1} at 20 °C at pH 8.0 (Podolsky & Morales, 1956; Gajewski *et al.*, 1986). This compares with the value of −19 kJ·mol^{-1} obtained in our calorimeter at 5 °C at pH 7.0 in the presence of 0.1 M-KCl. The enthalpy changes for the individual steps of the S1 ATPase (summarized in Table 1) are also in broad agreement with previous estimates obtained by using conventional calorimetry (Kodama & Woledge, 1976; Kodama, 1985). The data from Table 2 of Kodama (1985) are listed in Table 1 for comparison with our results.

The Kodama data were obtained by using a batch calorimeter at 4 °C in Tris buffer, pH 8.0, and the proton stoicheiometry was measured separately in order to calculate ΔH for each reaction step. By using the slowly hydrolysed analogue adenosine 5′-[γ-thio]triphosphate to measure the heat of nucleotide binding, Kodama & Woledge (1976) deduced that the ATP-cleavage step should be endothermic. Kodama & Kometani (1986) have reported an endothermic phase on mixing ATP with S1 at 5 °C that they attribute to the cleavage step, but this process is over within 100 ms, and neither the rate nor the amplitude could be measured with any certainty. The present work therefore represents the first clear demonstration of the positive enthalpy change accompanying the on-enzyme cleavage of ATP by S1, and presents a direct method for its detailed study.

The enthalpy changes listed in Table 1 are subject to two main sources of error. Firstly there are errors due to

the uncertainty in the value of K_2, which will affect both ΔH_2 and ΔH_3. The observed enthalpy changes for these two steps are corrected by using $K_2 = 1$ (see the legend to Table 1), but this is a difficult constant to measure accurately, and the true value could vary from 0.5 to 1.5 under these conditions (Taylor, 1977). This would result in an error of about ± 10 kJ·mol^{-1} in the corrected values of ΔH_2 and ΔH_3. The second source of error is in the measurement of very rapid or 'burst' heats such as those that accompany nucleotide binding. As mentioned in the Results section, the size of these burst heats tend to be more variable than those that are produced more slowly, and indeed just mixing S1 with buffer can give rise to a small instantaneous heat in some circumstances. Since these vary rapid heats are produced during the dead time of the apparatus, this variation is probably due (at least in part) to asymmetry in the 'work heat' produced in the two mixing chambers (for a more detailed discussion of this problem see Howarth *et al.*, 1987). This error could amount to about ± 15 kJ·mol^{-1} in the values of ΔH_1 and ΔH_4.

Proton uptake and release

In addition to the thermodynamic data, the stopped-flow calorimeter has provided some interesting results relating to the involvement of protons in the individual steps of the S1 ATPase. In contrast with previous measurements, the present data are obtained in an unperturbed well-buffered system at physiological pH and ionic strength. The proton stoichiometry results can be summarized in the following modified form of reaction scheme (1):

$$\underset{}{\overset{1}{\underset{+\mathrm{ATP}}{\mathrm{M}}}} \rightleftharpoons \underset{0.9\ \mathrm{H^+}\ \text{uptake}}{\mathrm{M{\cdot}ATP}} \overset{2}{\rightleftharpoons} \underset{0.4\ \mathrm{H^+}\ \text{release}}{\mathrm{M{\cdot}ADP{\cdot}P_i}} \overset{3}{\rightleftharpoons} \underset{0.4\ \mathrm{H^+}\ \text{release}}{\mathrm{M'ADP}} \overset{4a}{\rightleftharpoons} \underset{0.3\ \mathrm{H^+}\ \text{uptake}}{\mathrm{M{\cdot}ADP}} \overset{4b}{\rightleftharpoons} \underset{+\mathrm{ADP}\ \ 0.8\ \mathrm{H^+}\ \text{release}}{\mathrm{M}} \quad (3)$$

$+P_i$

0.5 H^+ uptake

0.5 H^+ release

Net release = 0.4 mol of H^+/mol

It is clear from this summary that protons are involved in each step of the S1 ATPase, and that the reaction is essentially symmetrical in this respect. The proton uptake with ATP binding and cleavage (0.5 mol of H^+/mol) is released on ADP release, so that the net yield of protons (0.4 mol of H^+/mol) is equivalent to the release that occurs at the phosphate-release step. This agrees with the conclusion of Bagshaw & Trentham (1974). There is also a similarity between this and the symmetrical nucleotide-binding model proposed by Trybus & Taylor (1982). In their model (based on fluorescence measurements), the binding of both ATP and ADP to S1 is followed by two similar isomerizations, in the case of ATP the second isomerization being the cleavage step.

There are two discrepancies between the present study and previous results on the proton stoichiometries of the S1 ATPase. Firstly the net yield of 0.4 mol of H^+/mol of ATP hydrolysed is lower than expected. The net release of 1 mol of H^+/mol at pH 8 is perhaps the most common signal routinely used to assay the ATPase activity of myosin and its subfragments, and is the major reason why so many myosin studies are performed at pH 8 rather than pH 7, the more physiological pH. At pH 7 the theoretical yield decreases to about 0.7 mol of H^+/mol at low ionic strength (Edsall & Gutfreund, 1983), and Green & Mommaerts (1953) measured the yield as 0.58 mol of H^+/mol at 20 °C at pH 7.0 in the presence of 0.15 M-KCl. However, the proton yield is highly dependent on Mg^{2+} concentration as well as pH, since Mg^{2+} has a greater affinity for ATP than for ADP. At pH 7 and below there is a complex relationship between the proton yield and [Mg^{2+}], since the reactions

$$\mathrm{MgATP^{2-} + H_2O \rightleftharpoons HADP^{2-} + HPO_4^{2-} + Mg^{2+}}$$

and

$$\mathrm{MgATP^{2-} + H_2O + H^+ \rightleftharpoons HADP^{2-} + H_2PO_4^- + Mg^{2+}}$$

can both be significant (Alberty, 1968). These reactions release Mg^{2+} rather than H^+ and so decrease the proton yield. Thus a yield of 0.4 mol of H^+/mol is not unreasonable under the conditions used.

It should be noted that step 3 in reaction scheme (3) is actually a composite step, in which a rate-limiting isomerization of the ternary complex precedes the actual release of phosphate (Bagshaw & Trentham, 1974). It is not possible to determine from our data whether the proton release is associated with the first, second or both of these steps. However, Nosek *et al.* (1987) have suggested that it is $H_2PO_4^-$ rather than HPO_4^{2-} that is released from contracting skinned muscle fibres, since only the acidic form is effective in inhibiting contraction. Since the pK of phosphate is about 6.8 under these conditions, then release of $H_2PO_4^-$ would cause a release of about 0.6 mol of H^+/mol at pH 7.

The second discrepancy concerns the uptake of protons with nucleotide binding. Previous studies with pH indicators have shown that at pH 8 a rapid release of 0.2–0.3 mol of H^+/mol accompanies the binding of ATP, ADP and ATP analogues to myosin and its subfragments at 20 °C at pH 8 in the presence of 0.1–0.5 M-KCl (Bagshaw & Trentham, 1974; Chock & Eisenberg, 1974; Koretz & Taylor, 1975). A rapid proton release can also be observed with a pH electrode at pH 8 at 20 °C by comparing the pH change during single-turnover experiments in the presence and in the absence of ADP (Bagshaw & Trentham, 1974). However, since the rate of ATP cleavage is fast at this temperature, it is difficult to determine from these experiments whether the 'proton burst' with ATP is due to the binding step (suggested by Bagshaw & Trentham, 1974) or the cleavage step (suggested by Chock, 1979). Attempts to test this by measuring the pH-dependence of K_2 remain equivocal, since K_2 is constant between pH 8.5 and 7 but decreases below pH 7 (Taylor, 1977; Kodama *et al.*, 1986). Therefore what has previously been termed the 'proton burst' in fact comprises both the binding and cleavage steps with ATP, and both the fast and slow steps with ADP. Using this convention, we observe an uptake of 0.5 mol of H^+/mol as opposed to a release of about 0.3 mol of H^+/mol on nucleotide binding.

Previous studies have shown that the size of the proton burst is highly dependent on the conditions used, so this

may account for the discrepancy. The proton burst decreases to zero as the pH decreases to pH 6 at 20 °C (Koretz & Taylor, 1975), although it also increases slightly with decreasing temperature (Marsh *et al.*, 1977; Kodama, 1981). Marsh *et al.* (1977) have reported a proton uptake of 0.25 mol of H^+/mol at 25 °C at pH 8 in the presence of 0.1 M-KCl when potassium ADP binds to heavy meromyosin in the absence of bivalent cations. This is a non-physiological reaction, but it does indicate that changing the conditions (in this case the charge on the nucleotide) has a major effect on the proton burst. Furthermore, according to the two-state model proposed by Shriver (1986), at 5°C a second conformation of myosin becomes significantly populated. The two states of myosin are thought to behave differently in their interactions with nucleotides, and this could explain the difference in the size of the proton burst. Since the burst is composed of two phases, which under our conditions partly cancel each other, a large change in the proton burst could be due to a relatively small change in both phases or a large change in one.

However, preliminary pH-electrode experiments in our laboratory suggest that there is also a rapid proton release at 5 °C at pH 7. Although the stopped-flow calorimeter is able to measure the magnitude of very fast temperature changes (and therefore proton changes) that take place within the dead time, there are large errors in this sort of measurement, as already explained above. However, it would require an enormous error of at least 30 $kJ \cdot mol^{-1}$ for a proton release to appear to be such a large uptake. Furthermore, Sleep *et al.* (1981) have shown that with both ATP and ADP binding to S1 both the maximum rate (k_{+2} or k_{-4a} in reaction scheme 3 and the apparent second-order rate constant (k_{+1} or k_{-4b}) are dependent on pH, so protons must be involved in each of these steps. The rapid uptake of release of protons with nucleotide binding to S1 is clearly a complex phenomenon, and will require more detailed investigation.

One major purpose of this paper is to demonstrate the potentialities of time-resolved calorimetry to the study of enzyme mechanisms in general, and to the S1 ATPase in particular. More detailed investigations under a range of conditions are obviously necesssary to resolve some of the questions raised here. For example, pairs of buffers with widely differing enthalpies of ionization are available for pH values other than 7, and these would allow our transient kinetic studies of proton uptake and release to be extended over a wide range of conditions.

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