The mechanism of inhibition of the Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum by the cross-linker *o*-phthalaldehyde

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Labelling the Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum with *o*-phthalaldehyde (OPA) results in loss of ATPase activity at a 1:1 molar ratio of label to ATPase. The affinity of the ATPase for Ca^{2+} is unaffected, as is the E1/E2 equilibrium constant. The rate of dissociation of Ca^{2+} from the Ca^{2+} -bound ATPase is also unaffected and Mg^{2+} increases the rate of dissociation, as for the unlabelled ATPase. Effects of Mg^{2+} on the fluorescence intensity of the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin are also unaffected by labelling with OPA, consistent with the fluorescence change reporting on Mg^{2+} binding at the gating site on the ATPase. The affinity of the ATPase for ATP is reduced by labelling, as is the rate of phosphorylation. The rate of phosphorylation is independent of

the concentration of ATP above $25 \mu\text{M}$ ATP, so that the slow step is the first-order rate constant for phosphorylation by bound ATP. The rate of the back reaction between phosphorylated ATPase and ADP is little affected, suggesting that the slow step in phosphorylation could be the slow conformation step before phosphoryl transfer. The rate of dephosphorylation of the phosphorylated ATPase is also decreased, suggesting that a similar conformation change could be involved in the dephosphorylation step. The rate of the Ca^{2+} transport step appears to be unaffected by labelling. The net result of these changes is that the labelled ATPase is present predominantly in a Ca^{2+} -free, phosphorylated form at steady state in the presence of ATP.

INTRODUCTION

In the preceding paper we showed that *o*-phthalaldehyde (OPA) reacted with the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum (SR) with a 1:1 stoichiometry, cross-linking leading to loss of ATPase activity [1]. The isoindole group on the ATPase, formed as a result of cross-linking a Cys and a Lys residue on the ATPase, was fluorescent with a fluorescence emission maximum at 402 nm, characteristic of an isoindole ring in a hydrophobic environment. Cross-linking of the ATPase by OPA was purely intramolecular with no sign of any intermolecular cross-linking [1].

The kinetics of the ATPase are usually discussed in terms of the E1–E2 model shown in Scheme 1. It is proposed that, in the E1 conformation, the ATPase possesses two outward-facing Ca^{2+} -binding sites of high affinity. Following binding of Ca^{2+} and ATP to the ATPase, the ATPase is phosphorylated and undergoes a conformation change to E2PCa_2 from which Ca^{2+} is lost to the lumen of the SR. Dephosphorylation of E2P then allows recycling to E1 [2]. This scheme proposes two distinct forms of the phosphorylated ATPase, E1PCa_2 and E2PCa_2 , with the two Ca^{2+} -binding sites alternating between outward and inward exposure. Other experiments are, however, consistent with two pairs of Ca^{2+} -binding sites on the ATPase and a single phosphorylated form of the ATPase, phosphorylation of the ATPase resulting in transfer of Ca^{2+} ions from one pair of sites

(the sites exposed to the cytoplasm) to the other pair of sites (the sites exposed to the lumen of the SR) [3].

MATERIALS AND METHODS

SR was prepared from rabbit skeletal muscle and labelled with excess OPA to give a 1:1 molar ratio of isoindole to ATPase as described in the previous paper [1]. ATPase activities were measured in 40 mM Hepes/KOH, pH 7.2, 100 mM KCl, 5 mM MgSO_4 , 2.1 mM ATP and maximally stimulating Ca^{2+} [1]. *p*-Nitrophenylphosphatase activity was measured in 40 mM Hepes/KOH, pH 7.4, 100 mM KCl, 20 mM Mg^{2+} , 1.01 mM EGTA, 0.91 mM Ca^{2+} , at *p*-nitrophenylphosphate concentrations between 5 and 20 mM. The liberation of *p*-nitrophenol was monitored at 410 nm, and activities were calculated using an absorption coefficient of $6000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for *p*-nitrophenol.

The time dependence of phosphorylation of the ATPase by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at 25°C was determined using a Biologic QFM-5 system as described in Starling et al. [4]. The time dependence of dephosphorylation of the unlabelled ATPase phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was determined using the triple-mixing capability of the Biologic QFM-5 system [4]. SR (0.2 mg of protein/ml) was incubated in 20 mM Hepes/Tris, pH 7.2, containing 5 mM MgSO_4 , 100 mM KCl and $100 \mu\text{M}$ CaCl_2 . This was mixed with an equal volume of the same buffer containing $100 \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After 200 ms, the mixture was mixed with an equal volume of 5.0 mM unlabelled ATP in the same buffer, followed by quenching as described above. For the labelled ATPase, where dephosphorylation was much slower, the experiment was carried out in exactly the same way but with manual mixing, the initial phosphorylation step being for 5 s.

The time-dependence of ADP-induced dephosphorylation was determined by first incubating labelled or unlabelled SR vesicles (0.2 mg of protein/ml) in 50 mM Mops, pH 7.2, containing

Scheme 1

5 mM MgSO_4 , 100 mM KCl, 50 μM CaCl_2 and 50 μM [γ - ^{32}P]ATP for approx. 1 min. This was then mixed in a 1:1 ratio with the same buffer containing 5 mM MgSO_4 , 100 mM KCl, 10.08 mM CaCl_2 , 10 mM EGTA, and 3 mM ADP (giving a final Ca^{2+} concentration of 44 μM), followed by quenching as described above.

Equilibrium levels of phosphorylation of the ATPase by [^{32}P]P_i were determined in 150 mM Mes/Tris, pH 6.2, containing 5 mM EGTA, 10 mM Mg^{2+} and 10 mM P_i, at 25 °C and a protein concentration of 0.2 mg/ml, as described in Baker et al. [5]. Steady-state levels of phosphorylation of the ATPase by [γ - ^{32}P]ATP were determined by incubation of SR (0.4 mg of protein/ml) in 40 mM Hepes/Tris, pH 7.2, containing 100 mM KCl, 5 mM Mg^{2+} and 1 mM Ca^{2+} , at 25 °C. Samples were quenched after 5 s with 12 % trichloroacetic acid/0.2 M phosphoric acid. The precipitate was collected on Whatman GF/C filters, washed three times with quenching solution and then counted.

Binding of ATP to the ATPase was determined as described by Dupont [6]. SR (0.5 mg of protein) was collected on a filter (Whatman GF/C) and then washed with [^{14}C]ATP in 150 mM Mops/Tris, pH 7.2, containing 100 mM KCl, 5 mM Mg^{2+} and 0.5 mM EGTA under vacuum and the filter counted. Blanks were determined by filtering the same solution in the absence of ATPase. Binding of $^{45}\text{Ca}^{2+}$ to the ATPase was measured using the double labelling method described in Starling et al. [7].

Rapid kinetic fluorescence measurements were performed using a Biologic SFM-3 fluorimeter with BioKine V3.20a software for data acquisition and analysis [8]. Tryptophan fluorescence was excited through a monochromator set at 290 nm and emitted light was collected at 90 ° and filtered by a high-pass cut-off filter with 50 % transmission at 320 nm (UV 32, Andover Corporation).

The time dependence of phosphorylation-induced $^{45}\text{Ca}^{2+}$ release from the ATPase was determined using a Biologic RFS-04 rapid filtration system, at room temperature (typically 20 °C). SR (0.4 mg of protein/ml) was incubated in buffer (150 mM Mops/KOH, pH 7.0, 100 mM KCl, 20 mM Mg^{2+}) containing 4 % (w/w protein) A23187, 100 μM $^{45}\text{Ca}^{2+}$ and 500 μM [^3H]sucrose and loaded on to a Millipore HAWP filter (0.45 μm) and then rapidly perfused with the same buffer containing 100 μM $^{45}\text{Ca}^{2+}$, 500 μM [^3H]sucrose and 2 mM ATP. The filter was dried overnight in air and counted in OptiPhase 'HiSafe' 3. The amount of [^3H]sucrose trapped on the filter was used to calculate the wetting volume of the filter, and the amount of $^{45}\text{Ca}^{2+}$ calculated to be in this volume was subtracted from the total $^{45}\text{Ca}^{2+}$ on the filter to give that bound to the ATPase. Equilibrium binding of $^{45}\text{Ca}^{2+}$ to the ATPase was determined in the same way.

The ATPase was labelled with nitrobenzo-2-oxa-1,3-diazole (NBD) or 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) as described [9]. Fluorescence measurements were performed at 25 °C using an SLM Aminco 8000C fluorimeter. Tryptophan fluorescence was observed with excitation and emission wavelengths of 290 and 340 nm, respectively; the fluorescence intensities of NBD- and of DMC-labelled ATPase were monitored with excitation and emission wavelengths of 430 and 520 nm, and 350 and 425 nm, respectively.

RESULTS

As shown in the preceding paper [1], labelling the ATPase with OPA results in total loss of ATPase activity at a 1:1 molar ratio of isoindole:ATPase. In the following experiments we characterize

Table 1 Effect of modification of the ATPase with OPA on Ca^{2+} binding

SR (0.4 mg of protein/ml) was incubated in 150 mM Mops/Tris, pH 7.2, containing 5 mM Mg^{2+} , 100 mM KCl, 50 μM $^{45}\text{Ca}^{2+}$ and, where indicated, 4 % (w/w protein) A23187 and absorbed on to Millipore filters (control). The filters were then perfused with the same buffer containing 2 mM ATP for 1 min. The level of Ca^{2+} bound to the ATPase was determined as described in the Materials and methods section.

Conditions	Ca^{2+} bound (nmol/mg of protein)	
	SR	OPA-labelled SR
Control + A23187	7.9	6.3
Buffer + ATP	83.4	6.7
Buffer + ATP + A23187	4.5	0.2

the basis for this loss of activity, using the ATPase labelled with excess OPA to give a 1:1 molar ratio of isoindole to ATPase.

Ca^{2+} binding

The level of $^{45}\text{Ca}^{2+}$ binding to the ATPase (7.9 nmol of Ca^{2+} bound/mg of protein; Table 1) compared with the maximum level of phosphorylation of the ATPase by ATP (approx. 3–4 nmol [EP]/mg of protein; see Figure 2) gives a stoichiometry of Ca^{2+} binding of two Ca^{2+} ions bound per active ATPase molecule. This stoichiometry is not significantly changed by reaction with OPA (Table 1). The affinity of the ATPase for Ca^{2+} can be monitored very conveniently by following the change in tryptophan fluorescence intensity between the Ca^{2+} -bound and Ca^{2+} -free forms of the ATPase [10,11]. The pCa values for 50 % Ca^{2+} binding measured in this way in 50 mM Hepes/Tris at pH 7.0 are 5.68 and 5.71 for the unmodified and modified ATPase, respectively (results not shown). The rate of dissociation of Ca^{2+} from the ATPase can be determined from the decrease in tryptophan fluorescence intensity observed when the Ca^{2+} -bound ATPase is mixed with EGTA in a stopped-flow fluorimeter [8,12]. As reported previously [8], and as shown in Figure 1, the rate of dissociation of Ca^{2+} is increased by addition of Mg^{2+} . Labelling with OPA had no effect on the rates of dissociation of Ca^{2+} measured in either the absence or the presence of Mg^{2+} , the dissociation rates being 10.7 s⁻¹ and 11.9 s⁻¹ for the unlabelled and labelled ATPase respectively in the absence of Mg^{2+} and 25.7 s⁻¹ and 27.6 s⁻¹ for the unlabelled and labelled ATPase respectively in the presence of 20 mM Mg^{2+} . We conclude that labelling with OPA does not affect the properties of the Ca^{2+} -binding sites on the ATPase.

E1/E2 equilibrium

Shifts in the E1/E2 equilibrium for the ATPase can be monitored from changes in the intensity of the fluorescence of NBD-labelled ATPase. It has been shown that the E1 and E2 conformations are states of high and low fluorescence intensity, respectively [11]. Labelling the ATPase with OPA had no significant effect on the fluorescence of the NBD-labelled ATPase or on its pH dependence (results not shown). We conclude that OPA labelling has no effect on the E1/E2 equilibrium of the ATPase.

ATP binding, phosphorylation and dephosphorylation

In contrast to the lack of effect on Ca^{2+} affinity, labelling the ATPase with OPA resulted in a significant reduction in the

Figure 1 Effect of OPA-labelling on the rate of Ca^{2+} dissociation from the ATPase

Shown are the time dependencies of the decrease in tryptophan fluorescence intensity seen when unlabelled SR (**A**) or OPA-labelled SR (**B**) incubated in buffer (100 mM Mops/80 mM Tris, pH 7.2) containing $100 \mu\text{M}$ Ca^{2+} is mixed with an equal volume of the same buffer containing 2 mM EGTA, in the absence (a) or presence (b) of 20 mM Mg^{2+} .

affinity of the ATPase for MgATP, from the value of $7 \mu\text{M}$ found for the unlabelled ATPase (Figure 2A). It is not possible to extend the binding studies to ATP concentrations higher than approx. $100 \mu\text{M}$ because at very high ratios of unbound to bound ATP, the data become unreliable. However, if it is assumed that the stoichiometry of binding of ATP is unchanged by labelling with OPA, then the data obtained for the OPA-labelled ATPase can be fitted to a single-binding-site equation with a K_d value of $50 \mu\text{M}$ (Figure 2A). Labelling with OPA also resulted in a shift in the ATP-dependence of the steady-state level of phosphorylation of the ATPase (Figure 2B).

A very marked reduction in the rate of phosphorylation of the ATPase by ATP was observed on labelling (Figure 3). For the unlabelled ATPase, the rate of phosphorylation was $79 \pm 8 \text{ s}^{-1}$ with $100 \mu\text{M}$ ATP. For the OPA-labelled ATPase rates of phosphorylation of $1.9 \pm 0.2 \text{ s}^{-1}$, $2.6 \pm 0.7 \text{ s}^{-1}$ and $1.6 \pm 0.2 \text{ s}^{-1}$ were observed at 25, 50 and $100 \mu\text{M}$ ATP, respectively (Figure 3); the relatively large scatter in the results follows from the slowness of the reaction which is then difficult to study by the quench flow technique. However, the observation of no consistent increase in rate with increasing concentration of ATP suggests that the measured rate corresponds to the first-order rate constant for the phosphorylation of the ATPase by bound ATP. Petithory

Figure 2 Effect of OPA-labelling on ATP binding (**A**) or the ATP-dependence of steady-state phosphorylation (**B**)

(**A**) SR was incubated with $[^{14}\text{C}]\text{ATP}$ in 150 mM Mops/Tris, pH 7.2, 100 mM KCl, 5 mM MgSO_4 , 0.5 mM EGTA, and the level of bound ATP determined by filtration: $\circ$, unlabelled SR; $\square$, labelled SR. The solid lines show fits to a single binding site with K_d values of 7 and $50 \mu\text{M}$ for the unlabelled and labelled ATPase, respectively. (**B**) The steady-state level of phosphorylation of the ATPase was determined by incubation of unlabelled SR ($\circ$) or labelled SR ($\square$) (0.4 mg of protein/ml) in buffer (150 mM Mops/Tris, pH 7.2, 100 mM KCl, 5 mM Mg^{2+}) containing the required concentration of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 5 s. The reaction was quenched by addition of 12% trichloroacetic acid and 0.2 M phosphoric acid and the level of phosphorylation determined.

and Jencks [13] have shown that, for the native ATPase, a conformation change following binding of ATP to the ATPase is rate-controlling for phosphorylation, and that phosphoryl transfer from the bound ATP to the ATPase is fast; the low rate of phosphorylation for the labelled ATPase could follow from a reduction in the rate of either of these steps.

Addition of ADP to the phosphorylated ATPase in Ca^{2+} -loaded SR vesicles leads to biphasic dephosphorylation attributed to rapid formation of enzyme-bound ATP followed by a slow dissociation of the bound ATP [14]:

Applying the quasi-equilibrium approach [15] this model predicts a burst size α given by

$$\alpha = [1 + K_{\text{int}}(1 + K_d^{\text{ADP}}/[\text{ADP}])]^{-1}$$

and a rate constant k for the slow phase given by

$$k = \alpha k_{\text{off}}$$

where K_d^{ADP} is the dissociation constant for ADP, K_{int} is the equilibrium constant for phosphorylation on the ATPase and k_{off} is the rate constant for dissociation of ATP. The fraction of the phosphorylated ATPase reacting rapidly with ADP (the burst size) decreased only slightly from 0.57 to 0.46 on labelling the

Figure 3 Effect of OPA-labelling on rate of phosphorylation of the ATPase

SR (0.2 mg of protein/ml), labelled with OPA (open symbols) or unlabelled (closed symbol), was incubated in buffer (150 mM Mops/Tris, pH 7.2, 100 mM KCl, 5 mM Mg^{2+} , 100 μM Ca^{2+}) and mixed in a 1:1 ratio with the same buffer containing $[\gamma\text{-}^{32}P]ATP$ to give final concentrations of 25 (○), 50 (□), or 100 (△, ▼) μM . The solid lines show fits to single-exponential processes with the rates given in the text.

Figure 4 Effect of OPA-labelling on the rate of reaction of the phosphorylated ATPase with ADP

Labelled (□) or unlabelled (○) SR (0.2 mg of protein/ml) was incubated in buffer (50 mM Mops/Tris, pH 7.2, 100 mM KCl, 5 mM Mg^{2+}) containing 50 μM Ca^{2+} and 50 μM $[\gamma\text{-}^{32}P]ATP$ for approx. 1 min and then mixed in a 1:1 ratio with the same buffer containing 10.08 mM $CaCl_2$, 10.0 mM EGTA and 3 mM ADP, followed by quenching at the given times. The broken lines show extrapolations to time zero, giving the rapidly reacting fractions.

ATPase with OPA (Figure 4); any changes in K_{int} or K_d^{ADP} on labelling are therefore small. The rate constant for the slow phase was unaffected by labelling (8.3 and 9.0 s^{-1} for the unlabelled and labelled ATPase, respectively), suggesting that the rate of dissociation of ATP is unaffected by labelling.

The rate of dephosphorylation of the phosphorylated ATPase was determined by first phosphorylating the ATPase with $[\gamma\text{-}^{32}P]ATP$ followed by mixing with an excess of non-radioactive ATP (Figure 5). The rate of dephosphorylation was observed to decrease from $11.6 \pm 2.5 s^{-1}$ for the unlabelled ATPase to $0.12 \pm 0.06 s^{-1}$ for the labelled ATPase; the accuracy of the latter rate is relatively low because it had to be determined using manual mixing of the samples, since it was too low to be determined using the rapid quench system.

Figure 5 Effect of OPA-labelling on the rate of dephosphorylation of the phosphorylated ATPase

The rate of dephosphorylation of the unlabelled SR (○) was followed by using a rapid quench system. The enzyme syringe contained SR (0.2 mg of protein/ml) in 20 mM Hepes/Tris (pH 7.2), 5 mM Mg^{2+} , 100 mM KCl and 100 μM Ca^{2+} . This was mixed in a 1:1 ratio with the same buffer containing 100 μM $[\gamma\text{-}^{32}P]ATP$. The mixture was incubated for 200 ms and then mixed in a 1:1 ratio with the same buffer containing 5.0 mM unlabelled ATP. The reaction was quenched at the times indicated. The rate of dephosphorylation of the labelled SR (□) was determined under the same conditions, using manual mixing. The solid lines are fits to single exponentials with the rates given in the text.

Table 2 Effect of OPA-labelling on phosphorylation of the ATPase by P_i

SR (0.2 mg of protein/ml) was incubated in 150 mM Mes/Tris, pH 6.2, containing 5 mM EGTA, 10 mM P_i and 10 mM Mg^{2+} for the given time, then quenched and the level of phosphoenzyme determined.

Incubation time (s)	[EP] (nmol/mg of protein)	
	Unlabelled	OPA-labelled
15	2.06	0.03
30	1.95	0.01

At acid pH values in the absence of Ca^{2+} and the presence of Mg^{2+} the ATPase can be phosphorylated by P_i [2]. As shown in Table 2, labelling the ATPase with OPA completely eliminates phosphorylation under these conditions.

Ca²⁺ transport

Measurements of the level of Ca^{2+} binding to the ATPase in the presence of ATP indicate whether the ATPase accumulates under steady-state conditions in Ca^{2+} -free or Ca^{2+} -bound forms. When SR vesicles were incubated with $^{45}Ca^{2+}$ in the presence of the Ca^{2+} ionophore A23187 and the level of Ca^{2+} binding to the ATPase determined as described under the Materials and methods section, a binding of 7.9 nmol/mg of protein was obtained. If the same experiment was repeated but in the presence of 2 mM ATP, the level of bound $^{45}Ca^{2+}$ dropped to 4.5 nmol/mg of protein (Table 1). The release of 3.4 nmol of $^{45}Ca^{2+}$ /mg of protein indicated that, at steady state in the presence of ATP, approx. 43 % of the ATPase was in the Ca^{2+} -free form E2P or E2 (Scheme 1). Further, since the phosphorylation experiments show that the ATPase is fully phosphorylated under these

Figure 6 Effect of OPA-labelling on the rate of the Ca²⁺ transport step

SR (0.4 mg of protein/ml) was incubated in buffer (150 mM Mops/KOH, 100 mM KCl, 20 mM Mg²⁺, pH 7.0) containing 100 μ M ⁴⁵Ca²⁺ and 4% (w/w protein) A23187 and adsorbed on to Millipore filters. These were washed for the given times with the same buffer containing 100 μ M ⁴⁵Ca²⁺ and 2 mM ATP, and the level of Ca²⁺ bound to the ATPase determined as described in the Materials and methods section. Results are shown for unlabelled ($\square$) and labelled ($\circ$) SR. The solid lines show single-exponential fits with the rates given in the text.

conditions (Figure 2), we can conclude that 43 % of the ATPase is present as E2P. For OPA-labelled SR, however, incubation with ATP in the presence of A23187 releases essentially all the bound Ca²⁺, suggesting that all the ATPase at steady state is present in the E2P form. If the same experiment is carried out in the absence of Ca²⁺ ionophore, a large accumulation of Ca²⁺ is observed with unlabelled SR vesicles, attributable to continuous transport of Ca²⁺ by the ATPase. However, for OPA-labelled SR, the level of Ca²⁺ accumulation is equal to the initial level of Ca²⁺ bound to the ATPase (Table 1), suggesting that the ATPase can undergo only a single turnover before it accumulates as E2P, whose rate of dephosphorylation is very low for the labelled ATPase.

If the rate of phosphorylation of the ATPase by ATP in the presence of Ca²⁺ is much higher than the rate of dissociation of Ca²⁺ from the phosphorylated ATPase (the E1PCa₂ $\rightarrow$ E2P step in Scheme 1) then the rate of this step can be measured by pre-equilibrating the ATPase with ⁴⁵Ca²⁺ and then perfusing it on Millipore filters with ⁴⁵Ca²⁺ and 2 mM ATP. As shown in Figure 6, for the unlabelled ATPase about half the initially bound Ca²⁺ is released under these conditions, consistently with the data in Table 1, with a rate constant of 11.5 ± 2.2 s⁻¹. In terms of Scheme 1, the reaction under these conditions can be represented by:

Figure 7 Effect of OPA-labelling on fluorescence responses of DMC-labelled ATPase

Shown are the changes in fluorescence intensity for DMC-labelled ATPase before (a,c,e) or after reaction with OPA (b,d,f). Labelled SR was incubated in 50 mM Hepes, pH 6.0, 200 mM sucrose and 20 mM Mg²⁺ added followed by: (a,b), 10 mM P_i; (c,d), 50 μ M ATP; (e,f), 100 μ M ADP. Traces are uncorrected for dilution effects, which are less than 1%.

For the unlabelled ATPase, the rate of phosphorylation is very much higher than the measured rate of release of Ca²⁺, and so the measured rate represents the rate of the Ca²⁺ transport step, E1PCa₂ $\rightarrow$ E2P [16]. For OPA-labelled SR, the rate of Ca²⁺ dissociation is lower, fitting to a rate constant of 4.5 ± 0.4 s⁻¹ (Figure 6). This rate can be compared with that of phosphorylation of the OPA-labelled ATPase which has a rate of between 1.9 and 2.6 s⁻¹ for ATP concentrations up to 100 μ M (Figure 3).

Table 3 Effect of OPA-labelling on *p*-nitrophenylphosphatase activity

SR (12 μ g of protein/ml) was incubated in 40 mM Hepes/KOH, pH 7.4, 100 mM KCl, 20 mM Mg²⁺, 1.01 mM EGTA, 0.91 mM Ca²⁺ and 4% (w/w protein) A23187, with the given concentrations of *p*-nitrophenyl phosphate. Liberation of *p*-nitrophenol was monitored at 410 nm.

System	<i>p</i> -Nitrophenyl phosphate concn. (mM)...	<i>p</i> -Nitrophenylphosphatase activity (units/mg of protein)	
		2	20
Unlabelled SR	0.05	0.05	0.11
OPA-labelled SR	0.03	0.03	0.07

A higher rate for ATP-induced Ca^{2+} dissociation than for phosphorylation is unexpected. This could be due to the much higher (2 mM) concentration of ATP used in the Ca^{2+} dissociation experiments than in the phosphorylation experiments, or it could represent experimental variability between preparations of the ATPase [16a]. We conclude that the low rate of phosphorylation-induced Ca^{2+} release from the labelled ATPase can be attributed to a low rate of phosphorylation, and that any effects on the rate of the Ca^{2+} transport step are likely to be small.

p-Nitrophenyl phosphatase activity

The ATPase can also be driven by simple energy-rich phosphate donors such as *p*-nitrophenyl phosphate [2]. It has been suggested that phosphorylation of the ATPase by acetyl phosphate may not involve the conformation change on the ATPase involved in the reaction with ATP, but that dephosphorylation is the same for the ATPase phosphorylated by either ATP or acetyl phosphate [17]. Labelling the ATPase with OPA results in a 32–37% decrease in *p*-nitrophenyl phosphatase activity (Table 3). This is consistent with the very low rate of dephosphorylation of the phosphorylated ATPase reported above for the ATPase labelled with OPA.

Fluorescence responses

In previous studies we have shown that the fluorescence intensity of the ATPase labelled with Br-DMC is sensitive to binding of Mg^{2+} , nucleotides such as ADP and non-hydrolysable analogues of ATP, and to phosphorylation by either ATP in the presence of Ca^{2+} or P_i in the absence of Ca^{2+} [18]. As shown in Figure 7, labelling with OPA had no effect on the magnitude of the fluorescence response of the DMC-labelled ATPase to Mg^{2+} , but the response to P_i was eliminated; this is consistent with the lack of phosphorylation of the ATPase by P_i shown in Table 2. Addition of ATP to DMC-labelled ATPase in the presence of Ca^{2+} leads to phosphorylation of the ATPase, and a fluorescence response with a magnitude comparable with that seen on phosphorylation with P_i . The magnitude of the fluorescence response to 50 μM ATP for DMC-labelled ATPase labelled with OPA was reduced (Figure 7), consistently with the reduced steady-state level of phosphorylation (Figure 2B). Addition of ADP to DMC-labelled ATPase results in a decrease in fluorescence intensity smaller than that seen on phosphorylation with ATP [18]. As shown in Figure 7, the fluorescence response of DMC-labelled ATPase to the addition of 0.1 mM ADP was unaffected by labelling with OPA.

DISCUSSION

OPA labelling of the ATPase affected the rates of phosphorylation and dephosphorylation of the ATPase but not the rates of Ca^{2+} binding or Ca^{2+} transport. The affinity of the ATPase for Ca^{2+} and the rates of Ca^{2+} dissociation from the ATPase were unaffected by labelling with OPA (Figure 1), and OPA labelling also had no effect on the E2/E1 equilibrium for the ATPase (results not shown). Labelling did, however, decrease the affinity of the ATPase for ATP (Figure 2) and markedly decreased the rates of phosphorylation and of dephosphorylation (Figures 3 and 5), although the rate of the back-reaction between the phosphorylated ATPase and ADP was largely unchanged (Figure 4). Phosphorylation of the ATPase by P_i in the absence of Ca^{2+} was also abolished (Table 2).

It has been suggested that, following binding of Ca^{2+} and ATP

to the ATPase, the ATPase undergoes a conformational change in which the terminal phosphate group of the ATP molecule bound in the nucleotide-binding domain is aligned with Asp-351 in the phosphorylation domain of the ATPase [13]. This conformational change is then followed by rapid phosphoryl transfer from ATP to Asp-351. A variety of fluorescence experiments with trinitrophenyl nucleotides are also consistent with a conformational change on the ATPase following phosphorylation with P_i in which the hydrophobicity of the nucleotide-binding site increases [19–21]. The observation that labelling with OPA had only minor effects on the back-reaction with ADP suggests that the phosphoryl transfer step is largely unaffected by labelling. The decrease in the rate of phosphorylation by ATP could then follow from a change in the conformational step that normally follows binding of ATP to the Ca^{2+} -bound ATPase. Inhibition of ATPase activity is seen only following cross-linking of the ATPase with OPA [1] and it is therefore likely that it is cross-linking which is responsible for inhibition of the conformational change rather than modification of the reactive Cys residue. The observation that the rate of dephosphorylation of the phosphorylated ATPase is also reduced by labelling with OPA (Figure 5) suggests that a corresponding conformational change is involved before dephosphorylation can occur. In summary, it is suggested that OPA labelling holds the ATPase in a conformation in which the exposure of the nucleotide-binding site to water is reduced, perhaps by a movement towards the phosphorylation domain, to give a conformation analogous to that found in the phosphorylated, Ca^{2+} -bound form of the ATPase. Dephosphorylation of the phosphorylated OPA-labelled ATPase by phosphoryl transfer to ADP will then be normal. Dephosphorylation of the phosphorylated OPA-labelled ATPase by hydrolysis to give P_i will be slow because cross-linking prevents the conformation change on the phosphorylated ATPase following loss of bound Ca^{2+} which would normally increase the accessibility of the phosphorylated Asp residue to water. Phosphorylation of the labelled ATPase by ATP would be slow if ATP was unable to bind to the cross-linked ATPase in a conformation suitable for rapid phosphoryl transfer to Asp-351.

Cross-linking the ATPase with glutaraldehyde has been reported to stabilize a phosphorylated, Ca^{2+} -bound form of the ATPase [22]. Similarly, reaction of the Ca^{2+} -ATPase with *N*-ethylmaleimide results in occlusion of Ca^{2+} in a phosphorylated, ADP-sensitive conformation [23]. These results suggest that modification with glutaraldehyde or *N*-ethylmaleimide results in a decrease in the rate of the Ca^{2+} transport step ($\text{E1PCa}_2 \rightarrow \text{E2P}$). OPA-labelled ATPase is, however, present largely in a phosphorylated Ca^{2+} -free form at steady state in the presence of ATP (Table 1), suggesting that in this case the Ca^{2+} transport step is not blocked. This is consistent with the observation that the labelled ATPase can still transport Ca^{2+} (Table 1). The release of Ca^{2+} from the labelled ATPase on addition of ATP is slower than for the unlabelled ATPase, but this can be attributed to the low rate of phosphorylation for the labelled ATPase (Figure 6).

The decrease in the rate of dephosphorylation of the phosphorylated ATPase (Figure 5) and the lack of phosphorylation of the ATPase by P_i (Table 2) following cross-linking by OPA suggest that a conformational change is also required before the phosphorylated ATPase can react with water with release of a phosphate group. Since it has been argued above that labelling with OPA has little effect on the Ca^{2+} transport step, this conformational change would then occur following formation of E2P and could be the mirror-image of the change occurring on the ATPase before phosphoenzyme formation.

These experiments also help to confirm our previous assignments of ligand-induced changes in the fluorescence intensity of DMC-labelled ATPase. We have suggested that the effect of Mg²⁺ on the fluorescence intensity of DMC-labelled ATPase follows from binding of Mg²⁺ at a gating site on the ATPase, controlling the rate of dissociation of Ca²⁺ from the Ca²⁺-bound ATPase [8,11]. As shown in Figure 7, the magnitude of the response of DMC-labelled ATPase to the addition of Mg²⁺ is unaffected by labelling with OPA and, as shown in Figure 1, labelling with OPA also had no effect on the Mg²⁺-induced increase in the rate of dissociation of Ca²⁺ from the ATPase. Labelling with OPA did, however, eliminate the response to P_i in the absence of Ca²⁺ and reduce the response to ATP in the presence of Ca²⁺, consistently with the lack of phosphorylation under the former conditions (Table 2) and the reduced level of phosphorylation under the latter conditions (Figure 2).

The experiments reported here suggest that effects at the phosphorylation site on the ATPase are not directly related to effects at the gating site on the ATPase since modification with OPA affects phosphorylation and dephosphorylation with no effect on Ca²⁺ dissociation or its Mg²⁺-dependence. This is in contrast to experiments in which the ATPase was reconstituted into bilayers of dipalmitoylphosphatidylcholine in the gel phase where decreases in the rates of phosphorylation and dephosphorylation were also observed, but where Mg²⁺ was found to have no effect on either the rate of Ca²⁺ dissociation from the ATPase or on the fluorescence intensity of DMC-labelled ATPase [24].

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