Labelling the Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum with the cross-linker *o*-phthalaldehyde

Yamin M. KHAN, Matthew WICTOME, J. Malcolm EAST and Anthony G. LEE

Department of Biochemistry and Institute for Biomolecular Sciences, University of Southampton, Southampton SO16 7PX, U.K.

The Ca^{2+} -ATPase in the sarcoplasmic reticulum of skeletal muscle reacts with *o*-phthalaldehyde (OPA) to form a fluorescent isoindole product. The stoichiometry of labelling of the ATPase is 9 nmol of isoindole/mg of ATPase, corresponding to a 1:1 molar ratio of isoindole:ATPase. There is no evidence for any intermolecular cross-linking. Isoindole formation is faster in the presence of methylamine, but the stoichiometry of labelling is unchanged, whereas in the presence of 2-mercaptoethanol the level of labelling is much higher. It is concluded that OPA reacts with a single Cys residue (defining the specificity of the reaction) in a fast step, subsequent reaction with a Lys residue to form the

isoindole being rate-controlling. Labelling the ATPase with OPA in the absence of methylamine leads to total loss of ATPase activity, whereas in the presence of methylamine, the decrease in ATPase activity on reaction is small. We conclude that the loss of ATPase activity probably follows from formation of the intramolecular cross-link rather than from the initial modification of the Cys residue. Reaction with OPA is not affected by the presence of ATP, ADP or Ca^{2+} , so that the reactive Cys is not part of a ligand-binding site. The fluorescence emission spectrum of the labelled ATPase indicates a hydrophobic environment for the isoindole ring.

INTRODUCTION

Phosphorylation of the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum (SR) by ATP leads to the transport of two Ca^{2+} ions across the membrane. The binding sites for Ca^{2+} on the ATPase have been located in the transmembrane region of the ATPase, involving putative transmembrane α -helices 4, 5, 6 and 8 [1]. The position of the ATP-binding site is less well defined. Labelling of Lys-515 by fluorescein isothiocyanate is blocked by the presence of ATP, suggesting that Lys-515 is part of, or close to, the ATP-binding site [2,3]. Fluorescence energy transfer experiments have located Lys-515 towards the top surface of the ATPase, a long way from the Ca^{2+} -binding sites [4,5]. Long-range interactions are therefore involved in coupling phosphorylation of Asp-351 by ATP to changes in the orientations and affinities of the Ca^{2+} -binding sites. Nevertheless, conformational changes on the ATPase during the transport process appear to be small. Thus distances between a number of labelled residues on the ATPase are very similar in two of the major conformational states of the ATPase, the Ca^{2+} -bound state E1 Ca_2 and E2.vanadate, a state analogous to the phosphorylated intermediate E2P [6].

One approach to studying the effects of conformational changes on a protein is through intramolecular cross-linking, which can, in suitable cases, by cross-linking two regions or domains, prevent their relative movement. Ross and MacIntosh [7,8] have shown that glutaraldehyde reacts rapidly with the Ca^{2+} -ATPase to form an intramolecular cross-link and more slowly to form intermolecular cross-links. The intramolecularly cross-linked form was detected by its altered mobility on SDS/PAGE. Intramolecular cross-linking led to a decrease in affinity of the ATPase for ATP, a decrease in the rate of phosphorylation of the ATPase, and a build-up of the ATPase under steady-state conditions in a Ca^{2+} -bound, phosphorylated form [9]. A disadvantage of glutaraldehyde for such studies is its relative non-specificity. Here we report on the use of *o*-phthalaldehyde (OPA) as a cross-linker. This cross-links a sulphhydryl and an ϵ -amino group to form a fluorescent isoindole adduct [10–12]. It has been

suggested that, for reaction, the separation between the -SH and - NH_2 groups must be about 3 Å [12]. This structural requirement might be expected to lead to structural specificity in intramolecular cross-linking, with a reduced possibility of intermolecular cross-linking. OPA also has the advantage that, while it is itself non-fluorescent, the isoindole formed is highly fluorescent, making the reaction easy to monitor. Here we show that OPA reacts with the Ca^{2+} -ATPase with 1:1 stoichiometry, leading to inhibition of activity.

MATERIALS AND METHODS

SR from rabbit skeletal muscle and the purified Ca^{2+} -ATPase were prepared as described in Michelangeli et al. [13]. ATPase activities were determined at 25 °C by using a coupled enzyme assay in a medium containing, unless otherwise specified, 40 mM Hepes/KOH (pH 7.4), 5 mM MgSO_4 , 100 mM KCl, 2.1 mM ATP, 1.1 mM EGTA, 0.53 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) and a maximally stimulating concentration of Ca^{2+} (free Ca^{2+} concentration of 10 μM) in a total volume of 2.5 ml. Free concentrations of Ca^{2+} were calculated using the binding constants for Ca^{2+} , Mg^{2+} and H^+ to EGTA given by Godt [14].

SR or the purified ATPase was suspended to 6 mg of protein/ml in buffer (50 mM Hepes/KOH, 200 mM sucrose, pH 7.0) at room temperature and incubated with OPA (270 μM) for up to 1 h; OPA was added from a 10 mM stock solution in methanol. Unreacted OPA was separated from the labelled ATPase by centrifugation through Sephadex G-50 columns pre-equilibrated with the above buffer [15]. Concentrations of protein were estimated from the absorbance at 280 nm in 1% SDS/5 mM KOH, using absorption coefficients of 1.2 and 1.0 l·g⁻¹·cm⁻¹ for the purified ATPase and SR, respectively [16] and concentrations of label were estimated from the absorbance at 335 nm, using an absorption coefficient of 7660 M⁻¹·cm⁻¹ [11]. Standard errors on estimations of labelling stoichiometry are estimated to be $\pm 10\%$. Fluorescence measurements of OPA-labelled ATPase were per-

formed at 25 °C using an SLM Aminco 8000C fluorimeter; fluorescence was observed with excitation and emission wavelengths of 338 and 400 nm respectively.

The ATPase was labelled with 7-nitro-1,2,3-benzo-oxadiazole (NBD), 5-[(2-[(iodoacetyl)amino]ethyl)amino]naphthalene-1-sulphonic acid (IAEDANS) or 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) as described [6,17,18]. Labelling with fluorescein maleimide (Fmal) was performed by incubation of SR (8 mg of protein/ml) in buffer (50 mM Hepes, pH 7.0, 80 mM KCl, 5 mM Mg²⁺, 200 mM sucrose) with a 10:1 molar ratio of Fmal to ATPase at 25 °C for 2 h. Unbound label was then removed by centrifugation through two columns of Sephadex G-50. Concentrations of bound Fmal were estimated using an absorption coefficient of 83000 M⁻¹·cm⁻¹ at 490 nm. SR was labelled with *N*-ethylmaleimide (NEM) using the same protocol.

RESULTS AND DISCUSSION

Incubation of the purified ATPase with a 5-fold molar excess of OPA for 30 min at pH 7.2, followed by separation of unreacted OPA on a Sephadex column, led to isoindole ring formation as shown by the appearance of absorption at 335 nm. The molar ratio of isoindole:ATPase estimated for the labelled ATPase from the absorption spectrum was 9 nmol of isoindole/mg of ATPase. This is equivalent to a molar ratio of isoindole:ATPase of 1:1, based on total ATPase protein. The maximal level of phosphorylation of the Ca²⁺-ATPase by ATP is typically about 0.5 mol [EP]/mol of ATPase, implying that only about 50 % of the ATPase in the preparation might be active [19]. Nevertheless, labelling ratios of 1:1 with respect to total protein are also obtained with other labels such as fluorescein isothiocyanate [20] and Br-DMC [21]. The same isoindole:ATPase labelling ratio was obtained using SR vesicles rather than purified ATPase (Table 1). As shown in Table 1, the stoichiometry of isoindole formation was unaffected by the presence of Ca²⁺, ATP or ADP.

The time course of isoindole formation for SR vesicles incubated at a molar ratio of OPA:ATPase of 5:1 at pH 6.0 is shown in Figure 1. Labelling of the ATPase under these conditions is slower, reaching a molar ratio of isoindole:ATPase of 0.7:1 after 30 min. Steady-state ATPase activity decreased in parallel with incorporation of label, with a 63 % decrease in activity after 30 min. Both the extent of labelling and the decrease in activity varied monoexponentially with time, described by a rate constant of 0.006 s⁻¹, consistent with labelling of a single site on the ATPase (Figure 1).

The fluorescence spectrum of the product showed excitation and emission maxima at 338 and 402 nm respectively (Figure 1), which is characteristic of an isoindole ring [11,12]. A correlation has been established between solvent polarity and the wavelength

Figure 1 Labelling of SR by OPA at pH 6.0

SR vesicles (6 mg of protein/ml) were incubated in 50 mM Hepes/KOH, 200 mM sucrose, pH 6.0, with a 5:1 molar ratio of OPA:protein (0.27 mM OPA) at 25 °C and samples taken at the given time to determine the level of labelling (□) and the ATPase activity (○). ATPase activities were measured at pH 7.2 at 25 °C with 100 mM KCl, 2.1 mM ATP and maximally stimulating concentrations of Ca²⁺, as described in the Materials and methods section. The lines show fits to a single exponential with a first-order rate constant of 0.006 s⁻¹. The insert shows the fluorescence excitation (broken line) and emission (solid line) spectra for OPA-labelled SR (3.6 μM) recorded in 50 mM Hepes/KOH, 200 mM sucrose, pH 8.0, at 25 °C. Excitation and emission spectra were recorded with emission and excitation wavelengths of 400 and 338 nm, respectively.

for maximum fluorescence emission [11,12]. The emission spectrum of the labelled ATPase is consistent with a very hydrophobic environment for the isoindole ring, equivalent to hexane. It has been shown that OPA modifies aldolase and cAMP-dependent

Figure 2 pH dependence of OPA labelling

SR (3.6 μM) was incubated with OPA (18 μM) in 50 mM Hepes/KOH, 200 mM sucrose, at the appropriate pH and the fluorescence intensity was recorded with excitation and emission wavelengths of 338 and 400 nm, respectively. pH values were as follows: a, 7.2; b, 7.4; c, 7.7; d, 7.9; e, 8.1; f, 8.2; g, 8.3.

Table 1 Ligand effects on the stoichiometry of labelling of SR by OPA

SR (10 mg of protein/ml) was incubated with OPA (454 μM) in 50 mM Hepes/KOH, 200 mM sucrose, pH 7.0, at 25 °C. After 1 h, unreacted OPA was separated from labelled SR on a Sephadex column, and the labelling ratio determined.

Conditions	Labelling ratio OPA:ATPase
1 mM EGTA	1.1:1
0.1 mM Ca ²⁺	1.0:1
1 mM EGTA + 5 mM ATP	0.8:1
0.1 mM EGTA + 5 mM Mg ²⁺ + 5 mM ATP	1.0:1
0.1 mM EGTA + 5 mM Mg ²⁺ + 2.5 mM ADP	1.0:1

Figure 3 pH dependencies of the rates and amplitudes of the fluorescence change with OPA

The data shown in Figure 3 for $\text{pH} > 7.0$ were fitted to single exponentials with the rates ($\circ$) and amplitudes ($\square$) shown. For $\text{pH} < 7.0$, amplitudes were fixed at that observed for $\text{pH} > 7.0$ and the rates obtained are plotted ($\circ$).

Figure 4 Effects of ATP and methylamine on the labelling reaction at pH 8.0

SR ($9.1 \mu\text{M}$) was incubated with OPA ($45 \mu\text{M}$) in 50 mM Hepes/KOH, 200 mM sucrose, pH 8.0 in the absence (solid line) or presence (broken line) of 0.1 mM MgATP, and the increase in fluorescence intensity at 400 nm recorded. The dotted line shows the fluorescence change recorded in the presence of 40 mM methylamine. Also shown are the fractional decreases in activity recorded in the absence ($\circ$) or presence ($\square$) of 40 mM methylamine.

protein kinase at the active site, with the isoindole ring again being located in a very hydrophobic environment [11,12].

The increase in fluorescence intensity at 400 nm provides a convenient method to monitor the reaction between SR and OPA. The rate of the reaction is very pH dependent, increasing markedly with increasing pH (Figure 2). Under all conditions, the fluorescence changes are monoexponential. For pH values higher than 7.0, a free fit of the data gives a constant amplitude of fluorescence change, with a rate that increases with increasing pH (Figure 3). For pH values less than 7.0, it is not possible to record the fluorescence for a long enough period of time to obtain a unique free fit to a single exponential rate. For pH values less than 7.0, amplitudes were therefore fixed at that

Figure 5 Reaction with OPA in the presence of methylamine

SR ($0.36 \mu\text{M}$) was incubated in 50 mM Hepes/KOH, 200 mM sucrose, pH 7.0, containing methylamine and the fluorescence intensity at 400 nm was monitored as a function of time following the addition of OPA ($1.8 \mu\text{M}$). The fluorescence traces were fitted to single exponentials and the rates ($\circ$) and amplitudes ($\square$) plotted as a function of the concentration of methylamine. The insert shows the fluorescence traces at methylamine concentrations (mM) of: a, 0.02; b, 0.2; c, 1.8; d, 18; e, 36.

obtained for pH values greater than 7.0, and the rates then obtained are also shown in Figure 3.

Reactions of OPA with amines are faster in alkaline than acid solutions [10], suggesting that reaction with a non-protonated Lys residue is favoured. The pH dependence of the rate of reaction (Figure 3) with the ATPase is too steep to be controlled by protonation of a single residue but the increase in rate observed at pH values above 7.5 suggests the involvement of a Lys residue with an unusually low pK value. The presence of such Lys residues in the ATPase has been suggested previously on the basis of labelling studies [22].

The presence of ATP had no effect on the rate of reaction (Figure 4); similarly, the rate of reaction was unaffected by the presence of Ca^{2+} or ADP (results not shown). These results contrast with those of Ross and McIntosh [8] who found that the ATPase was protected by ATP or ADP against cross-linking by glutaraldehyde.

The rate of the reaction of the ATPase ($0.36 \mu\text{M}$) with OPA at pH 7.0 was 0.002 s^{-1} , independent of the concentration of OPA between 0.4 and $14.4 \mu\text{M}$, which corresponds to a range of molar ratios of OPA:ATPase of 1:1 to 40:1 (results not shown). It has been established that isoindole formation results from an initial reaction of OPA with a Cys residue followed by reaction with a Lys residue [10,12]. The reaction being first-order is consistent with rapid reaction of the Cys residue and OPA to form a hemithioacetal followed by slow reaction with the Lys to form the fluorescent isoindole:

To investigate the possibility that more than one Cys residue on the ATPase was activated by OPA, but that only one went on to form an isoindole, the reaction of OPA with SR in the presence of methylamine was studied. As shown in Figure 5, isoindole formation was faster at pH 7.0 in the presence of methylamine, the rate increasing with increasing methylamine concentration, but the amplitude of the change remaining

constant. The data fit to single-exponential processes, with the rates given in Figure 5. Isoindole formation at pH 8.0 is also faster in the presence of methylamine, again with the final fluorescence intensity being unchanged (Figure 4). We conclude therefore that only a single Cys residue reacts with OPA and that the rate-controlling step is the subsequent reaction of the hemithioacetal with the Lys residue.

Whereas reaction with OPA in the absence of methylamine leads to total loss of activity, after 5 min at pH 8.0 in the presence of 40 mM methylamine, activity has only decreased by approx. 20% (Figure 4). Similarly, reaction with OPA at pH 6.0, at a molar ratio of OPA:SR of 5:1, leads to an 80% loss of activity after 60 min, but only a 20% loss of activity in the presence of 1.6 M methylamine. Thus modification of the Cys residue on the ATPase is not sufficient to cause inhibition of the ATPase; inhibition only occurs on formation of the isoindole. This is also consistent with the parallel time courses of inhibition and isoindole formation at pH 6.0 (Figure 1) and pH 8.0 (Figure 4). Inhibition could therefore either be due to modification of a Lys residue or to the formation of an intramolecular cross-link between the Cys and Lys residues involved in isoindole formation.

A very different result was obtained on reaction with OPA in the presence of 2-mercaptoethanol. In this case the initial rate of reaction was the same as in the absence of 2-mercaptoethanol, but the extent of the fluorescence increase was very much greater (results not shown). Thus at pH 8.0 reaction of ATPase (3.6 μ M) with OPA (18 μ M) in the presence of 20 mM 2-mercaptoethanol produced, after a 15 min reaction, a fluorescence intensity five times that observed in the absence of 2-mercaptoethanol, and whereas reaction in the absence of 2-mercaptoethanol was complete after approx. 5 min, in the presence of 2-mercaptoethanol fluorescence intensity was still increasing steeply after 15 min. It is clear therefore that in the presence of 2-mercaptoethanol multiple Lys residues are labelled by OPA. The specificity of the reaction between the ATPase and OPA therefore follows from specific initial labelling of a single Cys residue in the ATPase.

SDS/polyacrylamide gels of SR reacted with OPA gave no indication of formation of dimers or higher-molecular-mass aggregates and no reduction in intensity of the band corresponding to monomeric ATPase (results not shown). Thus OPA does not cause intermolecular cross-linking of the ATPase. Intramolecular cross-linking of the ATPase with glutaraldehyde produces a species with an apparent molecular mass of 125 kDa on SDS/PAGE [8]. Reaction with OPA did not produce any change in position for the ATPase band in SDS/PAGE. The tryptic cleavage pattern of OPA-labelled and unlabelled ATPase were identical, and showed no evidence for a cross-linking of A₁ and B subfragments of the ATPase, as observed following cross-linking with glutaraldehyde [8].

Attempts to identify the labelled residues

The purified ATPase labelled with OPA was partially digested with thermolysin at a ratio of thermolysin:ATPase of 1:30 (w/w) for 1 h at pH 6.8, and soluble peptides were separated from membranous remnants by centrifugation. As estimated from measurements of absorbance at 335 nm, about 60% of the isoindole was recovered in the supernatant and 40% in the pellet. Attempts to purify labelled peptides in the supernatant by reverse-phase HPLC with a methanol gradient in 0.1% trifluoroacetic acid gave a number of fluorescent peaks none of which gave useable amino acid sequences. The isoindoles appear to be unstable under the conditions used for peptide purification (see [10]) and we are unaware of any report of the successful

Table 2 Effect of labelling Cys residues on reaction with OPA

The ATPase was first labelled with the given reagent as described in the Materials and methods section and then reacted with a 5-fold molar excess of OPA for 30 min at pH 7.2, followed by determination of the molar ratio of isoindole:ATPase.

Reagent	Cys residues labelled	Molar ratio of isoindole/ATPase
Br-DMC	344 [21]	1.1:1
Fmal	344,364 [24]	0.9:1
4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole	344 [25]	1.0:1
IAEDANS	670,674 [26]	1.1:1
NEM	344,364 [27]	1.0:1

identification of residues labelled by OPA using the protein sequencing approach.

The ATPase contains 24 Cys residues [23]. A number of these have been modified chemically. Labelling with a variety of sulphhydryl reagents was found to have no effect on the stoichiometry of labelling with OPA (Table 2). We also found that labelling the ATPase with fluorescein isothiocyanate at Lys-515 had no effect on the reaction with OPA (results not shown). The ATPase contains no Lys-Cys pairs, but does contain one Cys-Lys pair, at Cys-364. Since labelling with Fmal, which labels Cys-364 [24], had no effect on reaction with OPA (Table 2) we conclude that Cys-364 is not the Cys residue labelled by OPA. There are no Lys-Xaa-Cys or Cys-Xaa-Lys sequences in the ATPase. The sequence ³⁴⁹Cys-Ser-Asp-Lys occurs in the phosphorylation domain. Cross-linking of Cys and Lys residues separated in the primary sequence of the ATPase is consistent with the low rate of reaction with OPA observed here.

Conclusions

The Ca²⁺-ATPase reacts with OPA to form a single intramolecular link with no evidence of intermolecular cross-linking. The product shows zero ATPase activity, and inhibition of activity is probably attributable to the formation of the cross-linked product. The isoindole product of the reaction is fluorescent, making it easy to follow the course of the reaction. In the following paper we define the mechanism of inhibition.

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