ELSEVIER

Biochimica et Biophysica Acta 1233 (1995) 1–6

BBA Biochimica et Biophysica Acta

Photoaffinity labelling of the ATP-binding sites of two Ca^{2+},Mg-ATPase isoforms in pancreatic endoplasmic reticulum

Richard Webb, Robert L. Dormer *

Department of Medical Biochemistry, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, UK

Received 6 July 1994; accepted 9 September 1994

Abstract

Pancreatic rough ER ATP-binding proteins, including two isoforms of SERCA-2b Ca^{2+},Mg-ATPase, were identified using specific photoaffinity labelling with 8-azido-ATP. 8-Azido-ATP irreversibly inhibited Ca^{2+},Mg-ATPase activity only after UV irradiation and the inhibition was prevented by inclusion of 5 mM ATP in the labelling reaction. Rough ER proteins of apparent molecular masses 141, 111, 100, 84, 69, 55 and 47 kDa were detected following photoaffinity-labelling with 8-azido-[α-^{32}P]ATP. The two bands at 111 kDa and 100 kDa corresponded in molecular mass to the two SERCA-2b Ca^{2+},Mg-ATPase isoforms previously demonstrated immunologically [1]. Immunoprecipitation of rough ER proteins by a SERCA-2b-specific antibody showed that the two ATPase bands were photoaffinity-labelled. Photoaffinity labelling of the 111 and 100 kDa proteins was: (a) abolished when Ca^{2+},Mg-ATPase activity was inactivated by EDTA-treatment of rough ER membranes; (b) inhibited by the Ca^{2+},Mg-ATPase inhibitor vanadate; (c) not affected by thapsigargin. The data demonstrate that pancreatic rough ER contains two isoforms of the SERCA-2b Ca^{2+},Mg-ATPase whose ATP-binding properties are susceptible to inhibition by vanadate but not thapsigargin.

Keywords: Endoplasmic reticulum; ATPase, (Ca^{2+} + Mg^{2+})-; Photoaffinity labeling; 8-Azido-ATP; (Pancreas)

1. Introduction

The initial phase of pancreatic enzyme secretion is stimulated by the release of Ca^{2+} from intracellular stores and may involve both Ins(1,4,5)P_3 -dependent and -independent mechanisms [2–6]. Maintenance of Ca^{2+} in rough ER stores and reuptake after release is dependent on active Ca^{2+} transport, mediated by Ca^{2+},Mg-ATPases [7,8].

It has been shown that pancreatic rough ER contains the SERCA-2b form of Ca^{2+},Mg-ATPase on the basis of Northern blot analysis [9] and immunological characterization using an antibody against the unique C-terminal amino acid sequence of the SERCA-2b Ca^{2+},Mg-ATPase [1]. The immunological data [1] further demonstrated the presence of two isoforms of different molecular weight whose Ca^{2+},Mg-ATPase activity was susceptible to inhibition by vanadate and thapsigargin. To test the hypothesis that the two isoforms are responsible for accumulating Ca^{2+} in different intracellular stores [1], as has been suggested for adrenal chromaffin cells and for platelets [10,11], requires their purification and characterization. Purification of the pancreatic rough ER Ca^{2+},Mg-ATPase has proved difficult due to lability of solubilized enzyme activity [12] and because single-step purification using Reactive Dye-Agarose, as previously used for the SR Ca^{2+},Mg-ATPase [13], was complicated by activation of enzyme activity [1]. The present study demonstrates that both Ca^{2+},Mg-ATPase isoforms can be specifically photoaffinity-labelled using 8-azido-ATP and provides a means of detecting the Ca^{2+},Mg-ATPase without relying on measurement of enzyme activity.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ER, endoplasmic reticulum; SERCA, sarco-, endoplasmic reticulum calcium ATPase; SR, sarcoplasmic reticulum; 8-azido-ATP, 8-azido-adenosine triphosphate.

* Corresponding author. Fax: +44 222766276.

2. Materials and methods

ATP, 8-azido-ATP, CHAPS, Insoluble Staph A protein (lyophilised crude extract from *Staphylococcus aureus*,

SSDI 0005-2736(94)00215-0

Cowan I strain), Aprotinin, PMSF and thapsigargin were from Sigma. 8-Azido-[α-^{32}P]ATP (2–10 Ci/mmol) was from ICN Biochemicals. All other reagents were of the highest quality available from BDH.

2.1. Preparation of pancreatic rough ER

Rough ER membranes from the pancreas of overnight-fasted Wistar rats were purified as previously described [1,7] and resuspended in sucrose buffer (0.25 M sucrose; 100 mM KCl; 2 mM Na azide; 5 mM $MgCl_2$; 100 mM imidazole-HCl (pH 6.8)) at a protein concentration of approx. 3 mg/ml.

In experiments to determine the effect of removal of ribosomes and luminal proteins, rough ER membranes were treated with EDTA as described by Jackson and Blobel [14]. Thus, rough ER membranes were incubated with an equal volume of 20 mM NaEDTA, 50 mM triethanolamine (pH 7.5) at 0°C for 2 min, underlayed with 0.5 M sucrose, 50 mM triethanolamine (pH 7.5), centrifuged at 100 000 × g for 60 min and the resulting pellet resuspended in 0.25 M sucrose, 50 mM triethanolamine (pH 7.5).

2.2. Assays

Ca^{2+},Mg-ATPase activity was assayed as previously described [1,7] and is defined as the difference in ATPase activity in the presence or absence of 0.7 μM free Ca^{2+} (1 mM EGTA/0.5 mM $CaCl_2$ at pH 6.8). Protein was estimated using the Bio-Rad protein assay kit and BSA as standard. RNA was assayed by absorbance at 260 nm of free nucleotides following precipitation with 0.3 M perchloric acid in the presence of 0.3 mM $MgCl_2$, 0.02% (w/v) BSA, hydrolysis with 0.3 M KOH for 1 h at 37°C and reprecipitation of DNA and protein with 0.6 M perchloric acid. Amylase was assayed by the method of Bernfeld [15].

2.3. Production of anti-peptide polyclonal antibodies

The procedure was based on that of Wuytack et al. [16]. The synthesis and coupling of a peptide consisting of the 12 C-terminal amino acids of the SERCA 2b Ca^{2+},Mg-ATPase to BSA or thyroglobulin, production and characterization of polyclonal antibodies in white New Zealand rabbits was as previously described [1,17]. Ca^{2+},Mg-ATPase content of rough ER membranes was estimated by ELISA using peroxidase-linked second antibody, colour development with 1,2-phenylenediamine dihydrochloride and detection at 492 nm. Membrane proteins (100 ng/well) were coupled to plastic microtitre plates by overnight incubation at 4°C in 50 mM carbonate/bicarbonate (pH 9.6). Results were expressed as absorbance units per μl of membrane suspension.

2.4. Photoaffinity labelling

Rough ER membranes (50 μg) or immunoprecipitates (see below) were resuspended in sucrose buffer to a final volume of 1 ml. In experiments where the effects of ATPase inhibitors were investigated, membranes were incubated with inhibitor for 10 min on ice before addition of 8-azido-ATP. After incubation in the presence of 8-azido-ATP for 30 min on ice in the dark (in order to allow the photoaffinity reagent to equilibrate with the membranes), samples were transferred to 1 ml quartz cuvettes and suspended in front of an Allen Type A-409/M 220–240 V fluorescence tube ultraviolet lamp at a distance of 5 cm (at shorter distances partial enzymic inactivation was seen in the presence or absence of 8-azido-ATP). The temperature of the sample was monitored throughout by the use of a Ni-Al, Ni-Cr thermocouple connected to a digital thermometer; at no point did the temperature exceed 37°C. After irradiation, samples were centrifuged (10 min in an Eppendorf microfuge) and pellets resuspended in either ATPase assay medium (see [7]), or electrophoresis solubilization buffer (0.1 M Tris-HCl (pH 6.8) containing 2% w/v SDS, 10% v/v glycerol). For detection of proteins radiolabelled following irradiation in the presence of 8-azido-[α-^{32}P]ATP (5 μCi/ml), samples were resuspended in 100 μl electrophoresis gel loading buffer and 90 μl subjected to electrophoresis on 7.5% polyacrylamide gels. Gels were stained with Coomassie brilliant blue R250, dried and autoradiographed using Amersham MP Hyperfilm and incubation at −70°C.

2.5. Immunoprecipitation

The procedure, based on the method of Doolittle et al. [18], was as previously described [1]. Briefly, solubilised rough ER membranes (1.8 mg protein/ml) were incubated with antiserum (1:56 final concentration) and immune complexes precipitated by addition of Protein A slurry. After washing, immunoprecipitates were diluted into sucrose buffer in the presence of 8-azido-ATP and subjected to UV irradiation as described above. Following irradiation, immune complexes were dissociated from Protein A by heating at 37°C for 5 min in polyacrylamide gel electrophoresis gel loading buffer and centrifuging for 5 min at 5000 × g. Supernatants were subjected to polyacrylamide gel electrophoresis and autoradiography as described above.

3. Results

Optimization of photoaffinity labelling

The formation of an irreversible bond between photoaffinity reagent and ATPase will deny access to the active site of the enzyme to subsequently added substrate; thus efficiency of photoaffinity labelling was monitored by its ability to inhibit Ca^{2+},Mg-ATPase activity. Fig. 1 shows

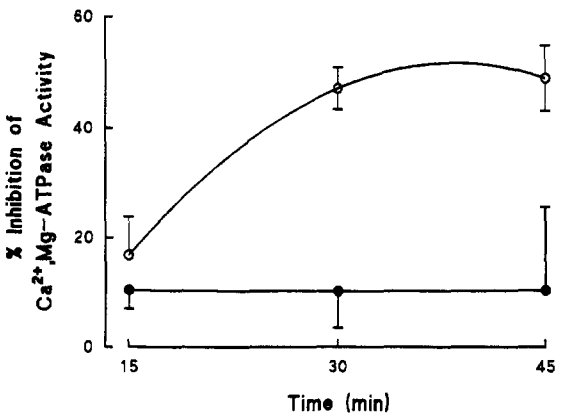

Fig. 1. Time-course of inhibition of rough ER Ca^{2+},Mg-ATPase by irradiation in the presence of 8-azido-ATP. 50 μg of rough ER membranes were subjected to UV irradiation in the presence (○) or absence (●) of 8-azido ATP as described in Materials and methods for the times indicated. Inhibition of Ca^{2+},Mg-ATPase activity of irradiated samples is expressed as percentage reduction of the activity of unirradiated membranes, kept on ice. Data are means ± S.E. from at least three different membrane preparations at each time point.

the time-course of inhibition of Ca^{2+},Mg-ATPase activity during UV irradiation in the presence or absence of 8-azido-ATP. UV irradiation alone resulted in inhibition of activity by approx. 10% which was unchanged with time. Significant inhibition of activity (53% of the activity of native rough ER) in the presence of 8-azido-ATP was optimal after 30 min irradiation (Fig. 1). Control experiments showed that in the absence of UV irradiation 8-azido-ATP did not significantly inhibit Ca^{2+},Mg-ATPase activity (82.75 ± 3.38% of control; $n = 3$) during 30 min incubation.

Fig. 2 shows that inhibition of Ca^{2+},Mg-ATPase activity during UV irradiation increased with increasing concentration of 8-azido-ATP. Assuming that 100% inhibition can be attained at an 8-azido-ATP concentration greater than 150 μM, the concentration causing half-maximal inhibition was calculated to be 97.1 ± 17.2 μM (mean ± S.E. for three determinations with different membrane preparations). Under optimal conditions (irradiation for 30 min at 5 cm from the UV source in the presence of 75 μM 8-azido-ATP), addition of excess (5 mM) ATP during irradiation prevented significant inhibition of Ca^{2+},Mg-ATPase activity (93.4 ± 15.5% of control; $n = 4$).

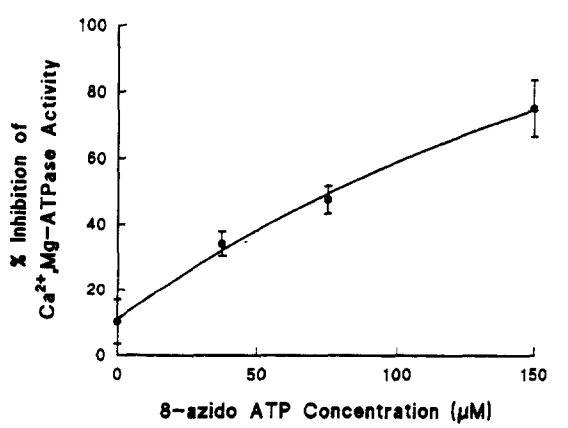

Fig. 2. Dependence of inhibition of rough ER Ca^{2+},Mg-ATPase during UV Irradiation on 8-azido-ATP concentration. 50 μg of rough ER membranes were subjected to UV irradiation in the presence of the 8-azido-ATP concentrations shown, for 30 min, as described in Materials and methods. Inhibition of Ca^{2+},Mg-ATPase activity of irradiated samples is expressed as percentage reduction of the activity of unirradiated membranes, kept on ice. Data are means ± S.E. from at least three different membrane preparations at each concentration.

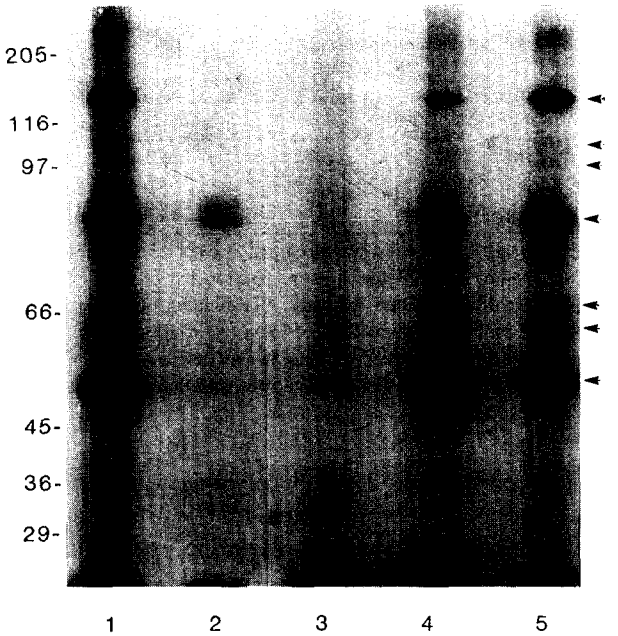

Fig. 3. Autoradiograph of rough ER proteins photoaffinity-labelled by 8-azido-ATP. Lane 1: 50 μg of rough ER membranes were subjected to UV irradiation in the presence of 5 μCi 8-azido-[α-^{32}P]ATP for 30 min and irradiated samples fractionated on 7.5% SDS-polyacrylamide gels and autoradiographed, as described in Materials and methods. Lane 2: as Lane 1 but without UV irradiation. Lane 3: as Lane 1 but in the presence of 5 mM ATP. Lane 4: as Lane 1 but in the presence of 0.1 mM vanadate. Lane 5: as Lane 1 but in the presence of 1 μM thapsigargin. The autoradiograph is representative of data obtained from at least seven different membrane preparations (Lanes 1–3) and for three additional preparations (Lanes 4 and 5). Values on left hand side show position of molecular mass markers (values in kDa). Arrows on right hand side show position of bands referred to in the text.

Identification of pancreatic rough ER ATP-binding proteins

Photoaffinity labelling was carried out under the optimum conditions described above, in the presence of 8-azido-[α-^{32}P]ATP. Several rough ER proteins were radiolabelled (Fig. 3, lane 1): bands of apparent molecular masses 140.7 ± 2.0, 110.9 ± 2.1, 100.3 ± 2.5, 84.1 ± 1.3, 69.3 ± 3.1, 54.8 ± 2.7 and 47.1 ± 0.6 kDa ($n = 7$ gels) were consistently observed. Two of these bands (111 and 100 kDa) had molecular masses similar to those of proteins previously shown by Western blotting and immunoprecipitation to be SERCA-2b Ca^{2+},Mg-ATPases [1]. As

Table 1
Effects of ATPase inhibitors on photoaffinity labelling of rough ER ATP-binding proteins

Inhibitor	ATP-binding (% control)						
Molecular mass (kDa):	141	111	100	84	69	54	47
Thapsigargin (1 μM)	107.2 ± 8.3	84.3 ± 17.9	82.0 ± 24.7	87.1 ± 6.4	168.7 ± 40.1	109.7 ± 7.9	98.8 ± 5.8
Vanadate (0.1 mM)	111.9 ± 15.1	33.6 ± 2.7	38.0 ± 4.6	113.5 ± 13.4	140.6 ± 9.0	78.9 ± 2.9	125.5 ± 21.0
Ouabain (0.1 mM)	113.3	125.3	136.4	78.5	122.9	96.3	63.5

50 μg of rough ER proteins was pre-incubated for 15 min at 4°C with ATPase inhibitors as indicated, subjected to UV irradiation in the presence of 5 μCi 8-azido-[α-^{32}P]ATP for 30 min, fractionated on 7.5% SDS-polyacrylamide gels, autoradiographed, and subjected to densitometric analysis as described in Materials and methods. ATP-binding of each protein, in the presence or absence of inhibitor, was first normalized by expression as: Intensity of individual band/Sum of intensity of all seven ATP-binding proteins in that lane. The data shown (% control) were calculated from the normalized values as: (ATP-binding in the presence of inhibitor/ATP-binding in the absence of inhibitor) × 100. Data are means ± S.E. for autoradiographs from experiments on three different membrane preparations (vanadate and thapsigargin) and on one preparation for ouabain.

can be seen in lanes 2 and 3, labelling was prevented if membranes were not subjected to UV irradiation or if excess (5 mM) ATP was present during irradiation.

Effects of Ca^{2+},Mg-ATPase inhibitors

Pancreatic rough ER Ca^{2+},Mg-ATPase activity was previously shown to be inhibited by both thapsigargin and vanadate [1]. To further determine which ATP-labelled protein bands correspond to Ca^{2+},Mg-ATPase isoforms, vanadate (0.1 mM), thapsigargin (1 μM) or ouabain (0.1 mM) were incubated with ER membranes prior to photoaffinity labelling. Representative autoradiographs showing the effects of vanadate and thapsigargin are shown in Fig. 3 (lanes 4 and 5, respectively). Densitometric analysis of three autoradiographs (Table 1) showed that vanadate (0.1 mM) caused a 60–70% reduction in labelling of the 111 and 100 kDa proteins but was without significant effect on the five other ATP-binding proteins. Neither the Na^+,K^+-ATPase inhibitor ouabain nor the Ca^{2+},Mg-ATPase inhibitor thapsigargin significantly inhibited ATP-binding to any protein, including the 111 and 100 kDa proteins. As expected, the addition of 0.1 mM ouabain had no inhibitory effect on Ca^{2+},Mg-ATPase activity (104.3 ± 15.6%; (n = 3) of that of native membranes).

In order to differentiate between the labelling of intrinsic membrane proteins and proteins associated with either ribosomes or the luminal contents, ER membranes were treated with EDTA as described in Materials and methods. As shown in Table 2, compared to native rough ER membranes, EDTA-treated membranes had lost 94%, 90% and 71% of their RNA, amylase and total protein content, respectively, demonstrating loss of ribosomes and luminal contents. However, it was shown by ELISA that EDTA-treatment resulted in no loss of SERCA-2b Ca^{2+},Mg-ATPase protein (Table 2). By contrast, 8-azido-[α-^{32}P]ATP photoaffinity labelling of the 111 and 100 kDa proteins was undetectable (representing > 90% inhibition of labelling) and Ca^{2+},Mg-ATPase activity was reduced by 81% (Table 2). Thus the data demonstrate that the Ca^{2+},Mg-ATPase isoforms are retained in an inactive form following EDTA treatment and that inactivation results in the loss of ATP-binding as measured by 8-azido-[α-^{32}P]ATP photoaffinity labelling.

Photoaffinity labelling of immunoprecipitates

In order to directly demonstrate that the 111 and 100 kDa proteins were both ATPases, solubilized ER proteins were immunoprecipitated by the previously described SERCA-2b Ca^{2+},Mg-ATPase antibody [1] and photoaffinity labelled with 8-azido-[α-^{32}P]ATP. Densitometric scans of autoradiographs showed two distinct peaks corresponding to molecular masses of 108 and 100 kDa (Fig. 4; peaks identified by arrows). A prominent peak at 135–150 kDa was present in precipitates carried out using SERCA-2b Ca^{2+},Mg-ATPase antibody or non-immune serum but not in those from which first antibody was omitted. This was due to the presence of large quantities of IgG in immunoprecipitates, as shown by Western blotting with anti-rabbit IgG antibodies (data not shown). ^{32}P-labelling of this IgG peak was probably due to non-specific interac-

Table 2
Effect of EDTA-treatment on ribosomal, luminal protein and Ca^{2+},Mg- ATPase content of pancreatic rough ER membranes

Fraction	Protein content (mg/ml)	RNA content (mg/ml)	Amylase activity (U/ml)	Ca^{2+},Mg-ATPase activity	
				enzymatic (U/ml)	immunological (U/μl)
Untreated rough ER	7.09 ± 2.20 (7)	4.83 ± 1.11 (5)	162.1 ± 35.7 (5)	18.9 ± 12.5 (3)	4.81 ± 0.57 (4)
EDTA-treated membranes	2.09 ± 0.52 (8)	0.25 ± 0.06 (6)	12.0 ± 2.8 (5)	4.5 ± 2.9 (3)	5.05 ± 0.82 (4)

Rough ER membranes were treated with EDTA as described in the text. Untreated membranes and pellets obtained after EDTA-treatment were assayed for RNA, amylase, Ca^{2+},Mg-ATPase enzymatic and immunological activity, as described in Materials and methods. Values are means ± S.E. for the number of different membrane preparations denoted in parentheses.

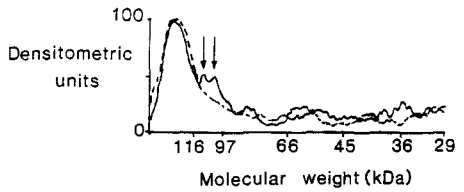

Fig. 4. Densitometric trace of photoaffinity-labelled proteins immunoprecipitated by anti-SERCA-2b Ca^{2+},Mg-ATPase antibody. Pancreatic rough ER membranes were solubilized, immunoprecipitated either with anti-SERCA-2b Ca^{2+},Mg-ATPase antibody or non-immune IgG and photoaffinity-labelled in the presence of 5μCi 8-azido-[α-^{32}P]ATP for 30 min, as described in the Materials and methods. Labelled samples were fractionated on 7.5% SDS-polyacrylamide gels, autoradiographed and subjected to densitometric analysis, as described in Materials and methods. The peaks indicated by arrows correspond to two ATP-binding proteins of molecular mass 108 and 100 kDa, respectively, present in the anti-SERCA-2b Ca^{2+},Mg-ATPase (solid line) but not the non-immune IgG (dashed line) immunoprecipitate. The traces are from an autoradiograph, representative of data from four different membrane preparations.

tion between IgG and 8-azido-ATP since abundant sample components have been previously reported to bind photoaffinity reagent non-specifically [19,20].

4. Discussion

In the present study, it has been shown that photoaffinity labelling with 8-azido-ATP can provide functional characterization of the Ca^{2+},Mg-ATPase and other ATP-binding proteins from pancreatic rough ER. Since Ca^{2+},Mg-ATPase activity is readily assayed in purified membranes, inhibition following covalent linkage of 8-azido-ATP has provided a simple monitor for optimization of labelling conditions (Figs. 1 and 2).

Specificity of photoaffinity labelling was demonstrated in accord with criteria outlined by Haley [19]. Thus, labelling of Ca^{2+},Mg-ATPase and inhibition of activity were shown to be: (1) dependent on UV irradiation in the presence of 8-azido-ATP; (2) prevented by inclusion of excess substrate (5 mM ATP) during photoaffinity labelling. In addition, rough ER membranes treated with EDTA retained immunological activity while > 80% of Ca^{2+},Mg-ATPase activity was abolished and photoaffinity labelling of the 111 and 100 kDa proteins was totally inhibited. Thus, the presence of inactive Ca^{2+},Mg-ATPase was insufficient for labelling to occur. Furthermore, the estimate of 97 μM as the 8-azido-ATP concentration required for half-maximal inhibition of Ca^{2+},Mg-ATPase activity is in the same range as reported for the low-affinity binding site for ATP and the K_m for activation of enzyme activity for ER Ca^{2+},Mg-ATPases [21]. The data suggest therefore that the low-affinity ATP-binding site is important for maximum activity of the pancreatic ER Ca^{2+},Mg-ATPase. The observation that inhibition of Ca^{2+},Mg-ATPase activity required up to 30 min of UV irradiation in the presence of 8-azido-ATP suggested that the mechanism for covalent binding, although specific, included an element of direct photoaffinity labelling involving free radical formation on aromatic amino acid residues [19].

Seven proteins were specifically radiolabelled by 8-azido-[α-^{32}P]ATP (Fig. 3). Removal of ribosomes and luminal proteins by EDTA treatment resulted in loss of 8-azido-[α-^{32}P]ATP labelling of the four proteins of lowest molecular mass which was comparable to the loss of total protein (data not shown). This suggested that the four proteins were not intrinsic membrane proteins and, by comparison with a series of ATP-binding proteins from ER [22], the proteins of molecular mass 84, 69 and 55 kDa can be tentatively identified as BiP, ERP72 and protein disulfide isomerase or calreticulin, respectively. The 47 kDa protein may correspond to an unidentified protein of similar molecular mass detected as an ATP-binding luminal protein in ER [22] and SR [23]. The 141 kDa protein labelled by 8-azido-[α-^{32}P]ATP is unlikely to be the plasma membrane Ca^{2+},Mg-ATPase since there is no detectable Na^+,K^+-ATPase in the rough ER preparation (see above and [24]) and because thapsigargin was shown to completely inhibit Ca^{2+},Mg-ATPase activity [1]. Also, the lack of effect of vanadate on ATP binding (Table 1) indicated that the 141 kDa protein is unlikely to be an ATPase and hence the SERCA-2b Ca^{2+},Mg-ATPase isoforms are the only ATPases in pancreatic rER membranes.

Previous immunological data [1] demonstrated that pancreatic rough ER Ca^{2+},Mg-ATPases consisted of two isoforms of SERCA-2b by virtue of possessing the C-terminal extension differentiating them from all other types [16,25,26]. The average molecular masses of the two isoforms identified immunologically were 111 and 97 kDa [1], which corresponded closely to the molecular masses of two of the ATP-binding proteins detected by specific photoaffinity labelling in the present study. The demonstration (Fig. 4) by photoaffinity labelling that ATP-binding proteins immunoprecipitated by the anti-SERCA-2b antibody had similar molecular masses (approx. 108 and 100 kDa) further confirmed the identification of these proteins as SERCA-2b Ca^{2+},Mg-ATPase isoforms.

In addition, the demonstration that the two rough ER SERCA-2b Ca^{2+},Mg-ATPase isoforms are susceptible to two inhibitors which act via different mechanisms further supports their identification as intact proteins which contain at least two of the domains known to be present in SERCA-2b ATPases. Thus, thapsigargin and vanadate inhibited photoaffinity labelling and enzymatic activity of the two ATPases to differing degrees while the Na^+,K^+-ATPase inhibitor ouabain had no effect on either property. Vanadate has been shown to act as a phosphate analogue causing competitive inhibition of ATP binding to the active site of ATP-binding proteins [27]. Vanadate inhibited photoaffinity labelling of both isoforms by 60–70% at the same concentration (0.1 mM) which inhibited ATPase activity by approx. 92% [1]. Thus, the data suggest that

inhibition of ATP-binding may contribute to the mechanism by which vanadate inhibits Ca^{2+},Mg-ATPase. The present data showed that thapsigargin had no effect on ATP-binding to the pancreatic SERCA-2b Ca^{2+},Mg-ATPase isoforms (Fig. 3; Table 1), at a concentration that maximally inhibited Ca^{2+},Mg-ATPase activity [1]. This is consistent with previous observations that ATP-binding regions of transport ATPases are highly conserved [9,28,29] yet thapsigargin is known to be a specific inhibitor of ER and SR Ca^{2+},Mg-ATPases [30–32]. Indeed, it has been suggested that thapsigargin produces a global effect on the enzymes, manifested by strong inhibition of Ca^{2+}-binding [33] and by the stabilization of the enzyme in a 'dead-end complex' [34,35], rather than by interaction with a specific domain. In the 'dead-end complex' conformation, the SR Ca^{2+},Mg-ATPase has a K_m for ATP of approx. 90 μM [36], which is similar to the concentration of photoaffinity reagent used in the present study (75 μM). This suggested that thapsigargin inhibits the pancreatic enzyme by a similar mechanism and stabilizes the enzyme such that only the 'dead-end complex' site is exposed.

Since, no differences in ATP-binding or immunological characteristics of the two Ca^{2+},Mg-ATPase isoforms have been detected in the present or previous studies [1], their physiological significance is not yet clear. The different isoforms may possess targeting information to direct them to distinct intracellular compartments. Thus, evidence that different Ca^{2+},Mg-ATPase isoforms may account for Ca^{2+} uptake into different Ca^{2+} stores has been obtained in adrenal chromaffin cells [10]. Further studies to elucidate differences in primary structure between the two isoforms will be facilitated by the ability to specifically label the ATP-binding sites and allow differences in function or intracellular location to be established.

Acknowledgements

We thank the MRC and Cystic Fibrosis Trust for financial support. R.W. is in receipt of a University of Wales Postgraduate Studentship. We are grateful to Dr. M.A. McPherson for much helpful discussion.

References

[1] Dormer, R.L., Capurro, D.E., Morris, R. and Webb, R. (1993) Biochim. Biophys. Acta 1152, 225–230.
[2] Dormer, R.L. (1989) in Molecular Basis of Membrane-Associated Diseases (Assi, A., Drahota, Z. and Papa, S., eds.), pp. 71–83, Springer-Verlag, Berlin, Heidelberg.
[3] Kasai, H. and Petersen, O.H. (1994) Trends NeuroSci. 17, 95–101.
[4] Wakui, M., Osipchuk, Y.V. and Petersen, O.H. (1990) Cell 63, 1025–1032.
[5] Thevenod, F., Dehlinger-Kremer, M., Kemmer, T.P., Christian, A.-L., Potter, B.V.L. and Schulz, I. (1989) J. Membr. Biol. 109, 173–186.
[6] Nathanson, M.H., Padfield, P.J., O'Sullivan, A.J., Burgstahler, A.D. and Jamieson, J.D. (1992) J. Biol. Chem. 267, 18118–18121.
[7] Brown, G.R., Richardson, A.E. and Dormer, R.L. (1987) Biochim. Biophys. Acta 902, 87–92.
[8] Bayerdorffer, E., Streb, H., Eckhardt, L., Haase, W. and Schulz, I. (1984) J. Membr. Biol. 81, 69–82.
[9] Burk, S.E., Lytton, J., MacLennan, D.H. and Shull, G.E. (1989) J. Biol. Chem. 264, 18561–18568.
[10] Burgoyne, R.D., Cheek, T.R., Morgan, A., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J., Mata, A.M., Colyer, J., Lee, A.G. and East, J.M. (1989) Nature 342, 72–74.
[11] Papp, B., Enyedi, A., Paszty, K., Kovacs, T., Sarkadi, B., Gardos, G., Magnier, C., Wuytack, F. and Enouf, J. (1992) Biochem. J. 288, 297–302.
[12] Dormer, R.L. and Brown, G.R. (1989) in Biochemical Approaches to Cellular Calcium (Methodological Surveys in Biochemistry and Analysis, Vol. 19) (Reid, E., Cook, G.M.W. and Luzio, J.P., eds.), pp. 321–325, The Royal Society of Chemistry.
[13] Coll, R.J. and Murphy, A.J. (1984) J. Biol. Chem. 259, 14249–14254.
[14] Jackson, R.C. and Blobel, G. (1977) Proc. Natl. Acad. Sci. USA 74, 5598–5602.
[15] Bernfeld, P. (1955) Methods Enzymol. 1, 149–158.
[16] Wuytack, F., Eggermont, J.A., Raeymaekers, L., Plessers, L. and Casteels, R. (1989) Biochem. J. 264, 765–769.
[17] Lloyd Mills, C., Pereira, M.M.C., Dormer, R.L. and McPherson, M.A. (1992) Biochem. Biophys. Res. Commun. 188, 1146–1152.
[18] Doolittle, M.H., Martin, D.C., Davis, R.C., Reuben, M.A. and Elovson, J. (1991) Anal. Biochem. 195, 364–368.
[19] Haley, B.E. (1991) Methods Enzymol. 200, 477–487.
[20] Ruoho, A.E., Kiefer, H., Roeder, P. and Singer, S.J. (1973) Proc. Natl. Acad. Sci. USA 70, 2567–71.
[21] Hack, N., Croset, M. and Crawford, N. (1986) Biochem. J. 233, 661–668.
[22] Nigam, S.K., Goldberg, A.L., Ho, S., Rohde, M.F., Bush, K.T., Sherman, M.Y. (1994) J. Biol. Chem. 269, 1744–1749.
[23] Zarka, A. and Shoshan-Barmatz, S. (1993) Eur. J. Biochem. 213, 147–154.
[24] Richardson, A.E. and Dormer, R.L. (1984) Biochem. J. 219, 679–685.
[25] Gunteski-Hamblin, A.-M., Greeb, J. and Shull, G.E. (1988) J. Biol. Chem. 263, 15032–15040.
[26] Lytton, J. and MacLennan, D.H. (1988) J. Biol. Chem. 263, 15024–15031.
[27] Pick, U. (1982) J. Biol. Chem. 257, 6111–6119.
[28] Ohta, T., Nagano, K. and Yoshida, M. (1986) Proc. Natl. Acad. Sci. USA 83, 2071–2075.
[29] Ovchinnikov, Y.A., Dzandzugazyan, K.N., Lutsenko, S.V., Mustayev, A.A. and Modyanov, N.N. (1987) FEBS Lett. 217, 111–116.
[30] Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R. and Dawson, A.P. (1990) Proc. Natl. Acad. Sci. USA 87, 2466–2470.
[31] Thastrup, O., Dawson, A.P., Scharff, O., Foder, B., Cullen, P.J., Drobak, B.K., Bjerrum, P.J., Christensen, S.B. and Hanley, M.R. (1989) Agents Actions 27, 17–23.
[32] Sagara, Y. and Inesi, G. (1991) J. Biol. Chem. 266, 13503–13506.
[33] Inesi, G. and Sagara, Y. (1992) Arch. Biochem. Biophys. 298, 313–317.
[34] Sagara, Y., Wade, J.B. and Inesi, G. (1992) J. Biol. Chem. 267, 1286–1292.
[35] Wictome, M., Henderson, I., Lee, A. and East, J.M. (1992) Biochem. J. 283, 525–529.
[36] DeJesus, F., Girardet, J.-L., Dupont, Y. (1993) FEBS Lett. 332, 229–232.