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Definition of surface-exposed and trans-membranous regions of the (Ca^{2+}–Mg^{2+})-ATPase of sarcoplasmic reticulum using anti-peptide antibodies

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Peptides have been synthesized representing parts of the transduction, phosphorylation, nucleotide-binding and hinge domains of the (Ca^{2+}–Mg^{2+})-ATPase of skeletal muscle sarcoplasmic reticulum (SR), and corresponding to segments of all of the postulated short inter-membranous loops of the (Ca^{2+}–Mg^{2+})-ATPase (residues 77–88, 277–287, 780–791, 808–818, 915–924 and 949–958). A number of antibodies raised to these peptides have been shown to bind to the ATPase, defining surface-exposed regions. Many of these are concentrated in the phosphorylation and nucleotide-binding domains, suggesting that these domains could be exposed on the top surface of the ATPase. The cytoplasmic location of the loop containing residues 808–818 was confirmed by the finding that proteinase K treatment of intact SR vesicles enhanced the binding of antibodies against this segment. These findings support the 10-α-helix model of the ATPase. These results also suggest that only inter-membranous loops larger than about 20 residues are likely to be detected by immunological methods in transmembranous proteins. Binding of anti-peptide antibodies to proteolytic fragments of the ATPase has been used to define the domain structure of the enzyme. Some of the anti-peptide antibodies have been characterized by studying their binding to sets of hexameric peptides synthesized on plastic pegs. A wide pattern of responses is observed, with a restricted range of epitopes being recognized by each anti-peptide antibody.

INTRODUCTION

Electron microscopy has shown that the bulk of the extra-membranous region of the (Ca^{2+}–Mg^{2+})-ATPase of skeletal muscle sarcoplasmic reticulum (SR) is on the cytoplasmic side of the membrane and consists of a pear-shaped lobe connected to the membrane by a narrow stalk (Stokes & Green, 1990*a*). A particularly important feature of the ATPase is the number of transmembranous α-helices, particularly since the Ca^{2+}-binding sites on the ATPase are believed to be located in this region (Clarke *et al.*, 1989). Despite much sequence similarity between the P-type ATPases, Ovchinnikov *et al.* (1986) predicted 11 transmembranous α-helices for the (Na^+–K^+)-ATPase from hydropathy plots, but reduced this to seven based on immunological and proteolytic studies (Ovchinnikov *et al.*, 1987, 1988), compared with the 10 predicted for the (Ca^{2+}–Mg^{2+})-ATPase (Brandl *et al.*, 1986). We have used anti-peptide antibodies to demonstrate that both the *N*- and *C*-termini of the (Ca^{2+}–Mg^{2+})-ATPase are located on the same side of the membrane, indicating an even number of transmembranous α-helices (Matthews *et al.*, 1989). More recent immunological studies on the (Na^+–K^+)-ATPase also indicate an even number of transmembranous α-helices for the (Na^+–K^+)-ATPase (Antolovic *et al.*, 1991). It is more difficult to distinguish between the two possibilities of eight or 10 transmembranous α-helices for the (Ca^{2+}–Mg^{2+})-ATPase. Studies with anti-peptide antibodies (Matthews *et al.*, 1990) and monoclonal antibodies (mAbs) (Clarke *et al.*, 1990*a*) have shown that a loop containing residues 877–888 is lumenal, as predicted by the 10-helix model (Fig. 1). The 10-helix model would also suggest that the region of the ATPase from the fifth postulated transmembrane helix to the *C*-terminus is very largely buried in the membrane (see Fig. 1), consistent with the observation of a 30 kDa fragment of the ATPase, stable against proteolytic degradation, including both the *C*-terminus and residues 877–888 (Matthews *et al.*, 1990). Cryo-electron microscopy has been used to study the intramembranous region of the ATPase, which has been shown to be consistent with the presence of 10 α-helices arranged in two rows of five, bent in a crescent shape (Stokes & Green, 1990*b*). Further information has come from fluorescence techniques. Fluorescence quenching experiments have located 11 of the 13 Trp residues of the ATPase within the transmembranous region, many being close to the membrane–water interface (Froud *et al.*, 1986), consistent with the 10-helix model proposed by Brandl *et al.* (1986).

Fluorescence energy transfer experiments have suggested that the binding sites for Ca^{2+} and ATP on the ATPase are widely separated (Scott, 1985), with the ATP-binding site being on the uppermost surface (Gutierrez Merino *et al.*, 1987). The relative positions of the Cys residues 344, 364, 670 and 674 have also been determined, with one of Cys residues 344 or 364 being towards the top surface of the ATPase, and the other being approx. 3.5–4.0 nm (35–40 Å) from the lipid bilayer surface, with Cys residues 670 and 674 also being at a similar height above the bilayer surface (Bigelow & Inesi, 1991). Site-directed mutagenesis has located six amino acid residues thought to be components of the Ca^{2+}-binding site, all in transmembranous regions of the ATPase (Clarke *et al.*, 1989), and these same residues have been found to be conserved in all (Ca^{2+}–Mg^{2+})-ATPases, including that from the crustacean *Artemia* (Palmero & Sastre, 1989), confirming their importance. Fluorescence labelling studies with carboxyl-directed reagents have located sites involved in Ca^{2+} binding in the cytoplasmic region of the

Abbreviations used: $C_{12}E_8$, octa(ethylene glycol) dodecyl monoether; FITC, fluorescein isothiocyanate; KLH, keyhole limpet haemocyanin; mAb, monoclonal antibody; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide; PBS, phosphate-buffered saline; SR, sarcoplasmic reticulum.

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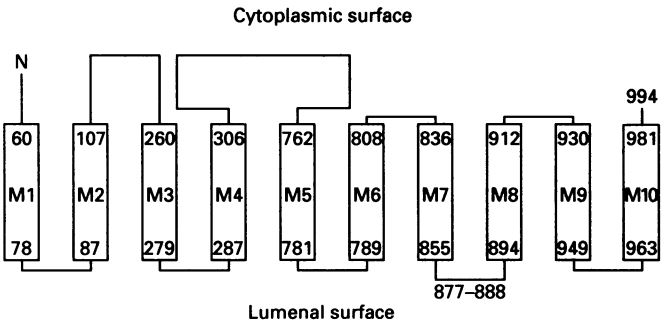

Fig. 1. Model of the transmembranous organization of the (Ca^{2+}–Mg^{2+})-ATPase

The model is taken from Matthews *et al.* (1990).

ATPase, about 2 nm (20 Å) above the lipid bilayer surface (Munkonge *et al.*, 1989). X-ray diffraction studies of La^{3+} bound to the ATPase show La^{3+} binding at sites about 1.2 nm (12 Å) above the phospholipid head group region of the bilayer (Asturias & Blasie, 1991). The similarity of these two estimates would suggest that this region of the ATPase could constitute part of a channel leading to the high-affinity Ca^{2+}-binding sites located in the transmembranous part of the ATPase.

Further information about the structure of the ATPase has come from molecular modelling (Brandl *et al.*, 1986; Green *et al.*, 1988; Taylor & Green, 1989; Green & MacLennan, 1989). It has been suggested that the stalk region consists of five α-helices connected to three large cytoplasmic domains: a nucleotide-binding domain where ATP binds, a phosphorylation domain containing the aspartyl residue (Asp-351) on the ATPase that is phosphorylated by ATP or P_i, and a β-strand or transduction domain which serves to link phosphorylation of the ATPase to the transport of Ca^{2+}. A small hinge or central domain links the nucleotide-binding domain to the fifth stalk region (MacLennan *et al.*, 1985; Brandl *et al.*, 1986) (Fig. 2).

We have been using immunological techniques to define regions of the ATPase exposed on the surface. For an antibody to bind to a native protein, the epitope for the antibody must be surface-exposed. Epitopes have been classified as either discontinuous or continuous. In discontinuous epitopes, a number of amino acids, widely separated in the primary structure, come together to make up the epitope, whereas continuous epitopes are made up of a continuous length of polypeptide chain. The majority of epitopes as defined by X-ray crystallography are discontinuous (Laver *et al.*, 1990). However, studies with synthetic peptides suggest that continuous epitopes also exist (Atassi, 1975; Geysen *et al.*, 1987; Van Regenmortel *et al.*, 1988; Novotny *et al.*, 1989; Van Regenmortel, 1989*a*), and it has been suggested that in many discontinuous epitopes only five to six residues, which may be continuous, contribute the bulk of the binding energy (Novotny *et al.*, 1989). This is in agreement with a recent crystal structure determination of a 19-residue peptide–antibody complex, show-

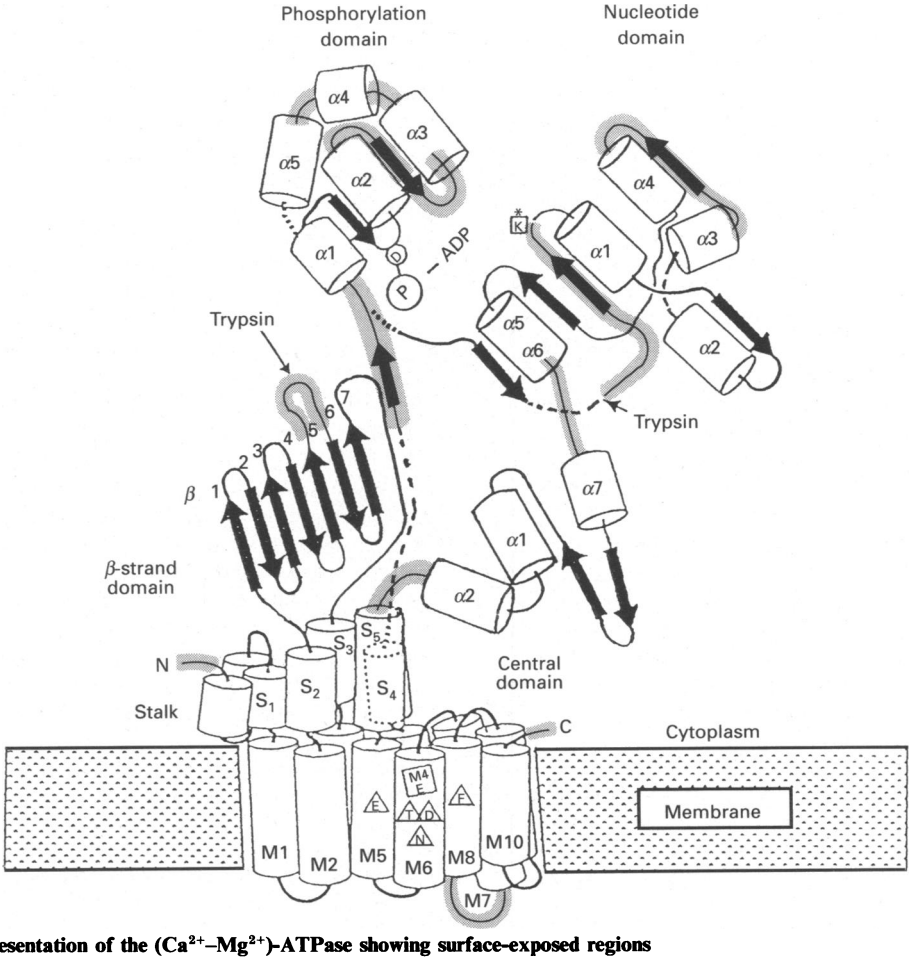

Fig. 2. Diagramatic representation of the (Ca^{2+}–Mg^{2+})-ATPase showing surface-exposed regions

Surface-exposed regions of the ATPase, as defined by binding of mAbs and anti-peptide antibodies, are shaded. Also shown are Lys-515 labelled by FITC (K*) and two trypsin cleavage sites. Based on a figure by N. M. Green.

ing that the peptide epitope consists of the first seven of the 19 residues (Stanfield *et al.*, 1990). It is believed that continuous epitopes generally correspond to mobile loops and ridges on the surface of the protein (Van Regenmortel *et al.*, 1988; Van Regenmortel, 1989*b*).

We have mapped a number of mAbs to four epitopes on the ATPase, studying binding to proteolytic fragments of the ATPase (Colyer *et al.*, 1989), to fusion proteins generated from cDNA fragment libraries of the ATPase (Tunwell *et al.*, 1991*b*), and to a series of overlapping hexameric peptides synthesized on plastic pegs (Tunwell *et al.*, 1991*a*). The assignment of these epitopes has been confirmed by studying the binding of anti-peptide antibodies to the ATPase (Matthews *et al.*, 1989; Tunwell *et al.*, 1991*a*). Here we report the use of an extended set of anti-peptide antibodies to define further features of the three-dimensional structure of the ATPase.

A survey of the use of peptides to produce antibodies that bind to native proteins showed that antibodies raised to peptides less than 10 residues in length were unlikely to bind, and that the optimal length of peptide was 10–15 residues (Palfreyman *et al.*, 1984). It has been shown that anti-peptide sera contain only a limited repertoire of paratopes, and that the response of the immune system to a relatively conformationally free molecule such as a peptide is rather limited (Geysen *et al.*, 1984). For example, Schoofs *et al.* (1988) have demonstrated an immunodominant site in a peptide, detected by binding of an anti-peptide antibody to a pentameric peptide. Thus, given that a typical continuous epitope is 5–7 residues in length, binding of an antibody raised against a 10–15-residue peptide to a native protein would not necessarily indicate that the whole of the corresponding peptide loop was exposed on the surface of the protein. The definition of the surface-exposed regions on a protein based on binding of anti-peptide antibodies could be further refined if it were possible to define the major antigenic determinants recognized by the antisera. Schoofs *et al.* (1988) have studied the binding of anti-peptide antibodies to peptides of between five and eight residues synthesized on plastic pegs, and showed that the major immunodominant region is detected by binding to sets of pentameric peptides. Here we use sets of overlapping hexameric peptides (Geysen *et al.*, 1984) to further characterize the sites recognized by our anti-peptide antibodies.

MATERIALS AND METHODS

Preparation of SR and (Ca^{2+}–Mg^{2+})-ATPase

SR and purified (Ca^{2+}–Mg^{2+})-ATPase were prepared from female rabbit (New Zealand White) skeletal muscle as described previously (Matthews *et al.*, 1989). The purified ATPase gave a single band on polyacrylamide gels stained with Coomassie Blue (Gould *et al.*, 1987). ATPase was labelled with fluorescein isothiocyanate (FITC) by incubation of the ATPase (9.0 nmol) with FITC (18 nmol) in buffer (75 μl; 100 mM-KCl, 20 mM-Hepes, pH 7.4) for 1 h. Unreacted FITC was then removed by passage through Sephadex G50 as described previously (Froud & Lee, 1986), to give a final labelling ratio of 1 mol of FITC/mol of ATPase.

Peptide synthesis

Peptides are named according to the residues to which they correspond in the fast-twitch muscle (Ca^{2+}–Mg^{2+})-ATPase (MacLennan *et al.*, 1985). Peptides were synthesized by the method of Merrifield (1986) and checked for purity by h.p.l.c. on a reversed-phase C8 column. All the peptides were synthesized with a *C*-terminal cysteine to allow coupling to keyhole limpet haemocyanin (KLH) by the method of Green *et al.* (1982) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) as cross-linker. KLH (8 mg) in 10 mM-sodium phosphate buffer (pH 7.8; 0.25 ml) was reacted with MBS (1.4 mg in 20 μl of dimethylformamide) for 30 min at room temperature. The KLH–MBS conjugate (5 mg), separated by gel-exclusion chromatography on a 10 ml Sephadex G-10 column which had been equilibrated with buffer (10 mM-sodium phosphate, pH 6.5), was then mixed with peptide (5 mg) dissolved in buffer (0.5 ml; 10 mM-sodium phosphate, pH 6.5) and the coupling reaction was allowed to proceed overnight. Some peptides were insoluble at pH 6.5, and for these coupling had to be performed at pH 8–9. The product was assayed for free-SH groups by the Ellman reaction (Ellman, 1959) to check for complete coupling.

Hexapeptides were synthesized on blocks of polyethylene pins designed to fit into 96-well microtitre plates, using kits supplied by Cambridge Research Biochemicals, as described previously (Tunwell *et al.*, 1991*a*). Overlapping sets of hexapeptides were synthesized corresponding to amino acids 1–208, 277–381 and 486–751 of the (Ca^{2+}–Mg^{2+})-ATPase (Tunwell *et al.*, 1991*a*).

Immunization

Anti-peptide antibodies were raised in rabbits. Primary immunizations were carried out with approx. 0.5 mg of peptide–KLH in Freund's complete adjuvent, injected by the intramuscular route into New Zealand white rabbits on day 1. A booster injection of 0.25 mg of peptide–KLH was given in Freund's incomplete adjuvant by the same route after 28 days. Several booster injections were given every 2 weeks using 0.3 mg of peptide–KLH or peptide alone in Freund's incomplete adjuvant, and blood was taken for the production of antisera 5–10 days later. Antisera were stored at −70 °C. Antibodies were purified from antisera by precipitation with 40% ammonium sulphate followed by dialysis against 2 × 1 litre of phosphate-buffered saline (PBS; 137 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4, pH 7.2). In some cases, antibodies were further purified on a Protein A–Sepharose column followed by dialysis against 1 litre of PBS.

E.l.i.s.a.

E.l.i.s.a.s were performed using the method outlined in Hudson & Hay (1983). Polystyrene e.l.i.s.a. plates (Dynatech; Immulon 1) were coated with peptide at 1 μg per well in 50 mM-sodium carbonate/bicarbonate buffer, pH 9.6, overnight at 3 °C. Incubations with antisera or purified antibody were carried out in PBS containing 0.05% Tween-20 (PBS/Tween) for 1 h at 37 °C, and following extensive washing with PBS/Tween the plate was incubated for 1 h with sheep anti-rabbit IgGAM conjugated with horseradish peroxidase (Sera-Tec) diluted 1:5000 with PBS-Tween (200 μl/well). After further washing, the plate was incubated for 30 min at room temperature with 200 μl of *o*-phenylenediamine (0.34 mg/ml) and 0.003% H_2O_2 in 0.15 M-citrate/phosphate buffer, pH 5.0. The reaction was stopped by addition of 50 μl of 12.5% H_2SO_4 and the absorbance was measured at 495 nm. SDS dilution e.l.i.s.a.s were carried out by incubating coated plates with antibody in the presence of various concentrations of SDS in PBS, following the same protocol as above.

A modified e.l.i.s.a. technique was used to demonstrate that the anti-peptide antibodies bound to native ATPase (Colyer *et al.*, 1989). Antibody was incubated with native or denatured ATPase and the level of unbound antibody was detected by binding to denatured ATPase coated on to e.l.i.s.a. plates. ATPase was denatured by heating at 100 °C for 3 min in buffer in the presence of 0.5% SDS. E.l.i.s.a. plates were coated with denatured ATPase by first diluting the denatured ATPase with carbonate/bicarbonate buffer, giving an SDS concentration of 0.00015%. Aliquots (200 μl) were then added to each well of the

Table 1. Immunogenic properties of peptides

Binding of anti-peptide antibodies to peptide, native ATPase or denatured ATPase was detected by e.l.i.s.a. +, binding detected; w, weak binding to Western blots; −, no detectable binding; nd, not determined.

Peptide	Peptide sequence	Binding to:			Recognition in Western blots	
		Peptide	Denatured ATPase	Native ATPase	Intact ATPase	Trypsinized ATPase
1–12	MEAAHSKSTEEC[a]	+	+	+	+	+
78–88	FEEGEETITAF-Cys	−	−	−	−	−
177–189	QSILTGESVSVIK-Cys	+	[c]	−	w	+
191–205	TEPVPDPRAVNQDKK-Cys[b]	+	+	+	+	+
277–287	GHFNDPVHGGS-Cys	+	[c]	−	+	+
303–314	AVAAIPEGLPAV-Cys[b]	+	[c]	−	w	+
324–339	RRMAKKNAIVRSLPSV-Cys[b]	+	+	+	+	+
366–377	MFIIDKVDGDFC	+	+	+[d]	+	+
381–400	EFSITGSTYAPEGEVLKNDK[a]	+	+	+	+	+
403–417	RSGQFDGLVELATIC	+	+	+	+	+
422–432	DSSLDFNETKG-Cys	+	+	+[d]	+	+
454–466	VFNTEVRNLSKVE-Cys	+	+	+	+	+
493–503	SMSVYCSPAKS-Cys	+	+	−	w	+
504–511	SRAAVGNK-Cys[a]	+	[e]	[e]	nd	nd
508–517	VGNKMFVKGA-Cys[b]	+	+	+	+	+
539–553	GPVKEKILSVIKEWG-Cys	+	+	−	w	+
567–582	RDTPPKREEMVLDDSS[a]	+	−	−	−	−
577–587	VLDDSSRFMEY-Cys[b]	+	+	+	+	+
601–614	DPPRKEVMGSIQLC	+	+	+	+	+
618–628	GIRVIMITGDN-Cys	+	−	−	nd	nd
643–654	GENEEVADRAYT-Cys[b]	+	+	+	+	+
659–668	DDLPLAEQRE-Cys[b]	+	+	+	+	+
671–682	RRACCFARVEPS-Cys	+	−	−	w	w
693–704	SYDEITAMTGDG-Cys	+	−	−	w	w
731–749	SEMVLADDNFSTIVAAVEE-Cys	+	+	+	+	+
780–791	ALGLPEALIPVQ-Cys	+	[c]	−	+	+
808–818	GFNPPDLDIMD-Cys	+	+	−	+	+
877–888	TEDHPHFEGLDC	+	+	+	+	+
915–924	SLSENQSLMR-Cys	+	+	−	+	+
949–958	YVDPLPMIFK-Cys	+	[c]	−	+	+
985–994	Cys-KFIARNYLED[a]	+	+	+	+	+

[a] From Matthews *et al.* (1989).
[b] From Tunwell *et al.* (1991*a*).
[c] Only binds at high concentrations of SDS (see text).
[d] Only partial depletion of antibody by native ATPase.
[e] Cross reacts with peptide 381–400[a].
[f] From Matthews *et al.* (1990).

plate, equivalent to 1 μg of ATPase per well. Following overnight incubation at 4 °C, the plates were washed with PBS/Tween. Antibody (1–10 μg) was incubated for 2 h at room temperature in PBS in 96-well plates with serial dilutions of the ATPase (2–160 μg/ml) in the native or denatured form. For the incubations with denatured ATPase, SDS was present from the denaturation medium. So that this would not vary with serial dilution of the denatured ATPase, additional SDS was added as necessary to maintain a constant concentration of 0.006% SDS in all incubations with denatured ATPase. Samples were then directly transferred to e.l.i.s.a. plates coated with the denatured ATPase (1 μg/well) and e.l.i.s.a. was performed as described above. Where appropriate, SR was disrupted initially by adding 1 mg of $C_{12}E_8$ [octa(ethylene glycol) dodecyl monoether Calbiochem]/mg of SR protein and carrying out the incubation with antibody in the presence of 0.05% $C_{12}E_8$. This concentration of detergent was shown to be without effect on the e.l.i.s.a. The unbound antibody was then detected by transferring the incubates to the SDS-denatured ATPase-coated e.l.i.s.a. plates.

Assays of antibody binding to hexameric peptides synthesized on plastic pegs were carried out by first precoating the pins for 1 h by insertion into wells of e.l.i.s.a. plates containing buffer (1% ovalbumin, 1% BSA, 0.1% Tween 20 and 0.05% NaN_3 in PBS). Pins were then incubated overnight at 4 °C in e.l.i.s.a. plates containing anti-peptide antibody at a dilution of 1:500 in PBS/Tween. The pins were then washed three times for 10 min with PBS/Tween, with shaking. Bound antibody was detected by incubation for 1 h at 25 °C in e.l.i.s.a. plates containing sheep anti-rabbit IgG/A/M conjugated to horseradish peroxidase. The pins were washed and incubated in substrate solution [50 mg of azino-di-3-ethylbenzthiazodinosulphate, 35 μl of 100 vol. H_2O_2 in 100 ml of citrate/phosphate buffer (80 mM), pH 4.0], in the wells of an e.l.i.s.a. plate. The colour was allowed to develop in the dark for 30 min and then the plate was read at 410 nm on a Dynatech MR588 Microelisa Auto Reader.

Immunoblotting

Proteins (300 μg per gel) were separated on 14% polyacrylamide gels (Laemmli, 1970) and transferred electrophoretically to nitrocellulose paper (0.45 μm; Schleicher and Schuell) as described by Towbin *et al.* (1979). The nitrocellulose was blocked with PBS/0.5% Tween, cut into strips and exposed

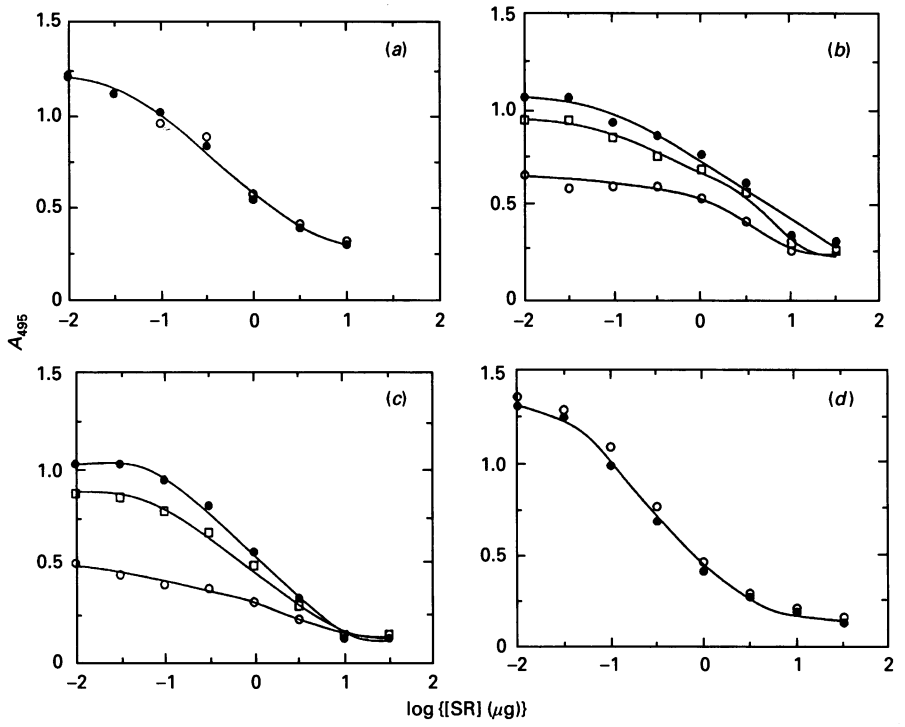

Fig. 3. Anti-peptide antibody binding to both native and denatured ATPase as demonstrated by competitive e.l.i.s.a.

Anti-peptide antibodies raised to peptides (*a*) 403–417, (*b*) 454–466, (*c*) 601–614 and (*d*) 731–749 were incubated with the given amount of native (●) or denatured (○) ATPase. The incubation mixture was transferred to e.l.i.s.a. plates coated with denatured ATPase (1 μg) to determine the amount of free antibody by e.l.i.s.a.. Incubations with denatured ATPase contained 0.006% SDS (○). Anti-peptide antibodies 454–466 (*b*) and 601–614 (*c*) were also assayed in the presence of 0.002% SDS (□).

sequentially in PBS/0.05% Tween to anti-peptide antibodies (1:100 dilution; 1 h), horseradish peroxidase conjugated to sheep anti-rabbit IgGAM (diluted 1:3000, 1 h), and substrate (Bio-Rad; 4-chloro-1-naphthol plus 0.15% H_2O_2), washing extensively between each step with PBS/0.05% Tween.

Preparation and analysis of proteolytic fragments of (Ca^{2+}–Mg^{2+})-ATPase

Trypsin digestion of SR was performed as described in Colyer *et al.* (1989). SR (300 μg) was digested with 5 μg of trypsin (type I, Sigma) for 40 min in 100 μl of 10 mM-Tris/HCl buffer, pH 7.4, or with 12.5 μg of trypsin for 30 min in 100 μl of 20 mM-Mops, (pH 6.8)/800 mM-KCl, at room temperature. The reaction was terminated by adding soybean trypsin inhibitor (Sigma) (inhibitor/trypsin molar ratio 2:1). Samples were solubilized in Laemmli sample buffer (Laemmli, 1970) containing 2% SDS and 10% β-mercaptoethanol and processed by SDS/PAGE (Laemmli, 1970). Proteinase K digestion of SR was carried out as described previously (Matthews *et al.*, 1990). SR (300 μg) was digested with 10 μg of proteinase K (Sigma) for 30 min in 100 μl of PBS at room temperature. The reaction was terminated by the addition of phenylmethanesulphonyl fluoride (2 mM final concentration) and the sample was prepared for PAGE as outlined above.

RESULTS

Anti-peptide antibodies

Synthetic peptides of 8–19 residues with a *C*-terminal Cys were synthesized (Table 1). These peptides represent portions of the transduction or β-strand domain (177–189 and 191–205), the fourth stalk region (303–314 and 324–339), the phosphorylation domain (366–377, 381–400, 403–417, 422–432 and 454–466), the nucleotide-binding domain (493–503, 504–511, 508–517, 539–553, 567–582, 601–614 and 618–628), the hinge or central domain (671–682, 693–704 and 731–749), and the inter-membranous segments between transmembranous regions M1–M2, M3–M4, M5–M6, M6–M7, M8–M9 and M9–M10 (Figs. 1 and 2; Table 1). All peptides showed one major peak on reverse-phase h.p.l.c., eluting at 20–30% acetonitrile, with very minor peaks (< 5% in total) corresponding to deletion and termination products.

Antibody production was detected using the respective peptides in e.l.i.s.a., and all peptides produced antibodies that bound the respective peptide except for peptide 78–88, where the antiserum taken from three rabbits exhaustively immunized with the peptide failed to recognize either the peptide or the denatured ATPase (Table 1). Half-maximal binding was observed at antibody dilutions of between 1:500 and 1:10000. In no case was any significant binding observed of preimmune serum to the peptides.

Binding to the (Ca^{2+}–Mg^{2+})-ATPase

Competitive e.l.i.s.a.s were performed to investigate the binding of anti-peptide antibodies to native and denatured ATPase. In these assays, anti-peptide antibody was incubated with serial dilutions of either native ATPase or ATPase denatured by heating in the presence of 0.5% SDS. At the highest concentrations of denatured ATPase used, this resulted in a final concentration of 0.006% SDS in the incubation medium. For the more dilute concentrations of denatured ATPase, additional SDS was added to maintain an SDS concentration of 0.006%, unless otherwise stated. Native and denatured ATPases were incubated for 2 h at room temperature with the anti-peptide antibodies, and the incubations were then transferred to e.l.i.s.a. plates coated with denatured ATPase to determine the amount of

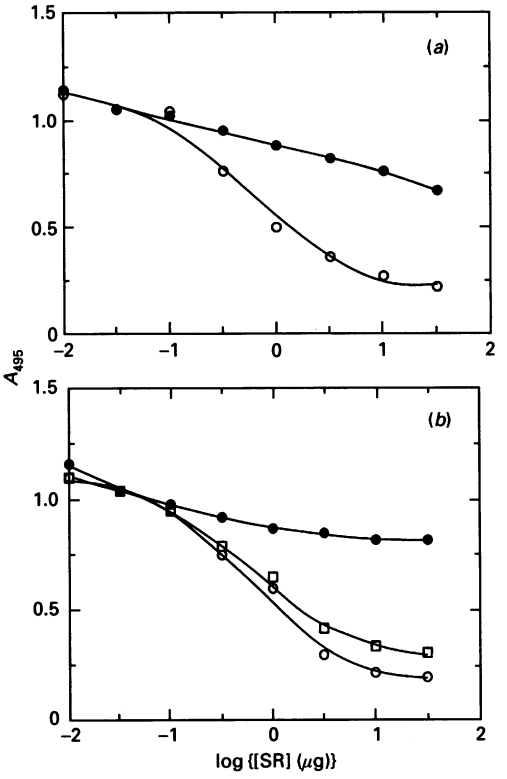

Fig. 4. Anti-peptide antibody binding to denatured ATPase but not to native ATPase, as demonstrated by competitive e.l.i.s.a.

Anti-peptide antibodies raised to peptides (*a*) 493–503 and (*b*) 539–553 were incubated overnight with the given amount of native (●) or SDS-denatured (○) ATPase, and the incubation mixture was transferred to e.l.i.s.a. plates coated with denatured ATPase (1 μg) to determine the amount of free antibody by e.l.i.s.a.. Anti-539–553 antibody was also assayed in the presence of 0.002% SDS (□).

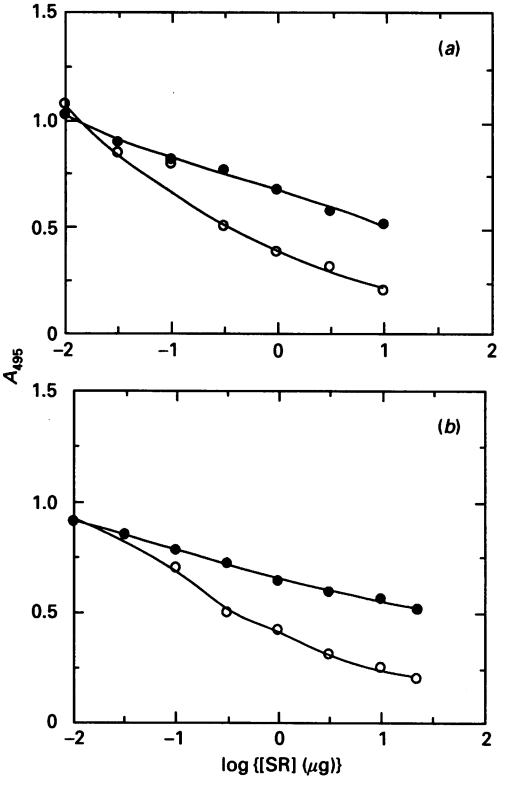

Fig. 5. Binding of anti-366–377 and -422–432 antibodies to native and denatured ATPase

Anti-peptide antibodies raised to peptides (*a*) 366–377 and (*b*) 422–432 were incubated overnight with the given amount of native (●) or SDS-denatured (○) ATPase, and the incubation mixture was transferred to e.l.i.s.a. plates coated with denatured ATPase (1 μg) to determine the amount of free antibody by e.l.i.s.a.

free antibody remaining in the incubations. As shown in Table 1, all anti-peptide antibodies except those against 277–287, 567–582, 618–628, 671–682 and 693–704 bound to denatured ATPase in a competitive e.l.i.s.a.. Anti-peptide antibody against 567–582 also failed to recognize the ATPase in immunoblots. Of the remaining new anti-peptide antibodies produced here, four bound to both native and denatured ATPase (Fig. 3; Table 1). Two of these anti-peptide antibodies (anti-454–466 and anti-601–614) showed less binding in these competitive e.l.i.s.a.s with denatured than with native ATPase (Fig. 3). This can, however, be attributed to effects of the SDS present in the incubations with denatured ATPase on the binding of antibody to the plate coated with denatured ATPase. Thus if the concentration of SDS in the e.l.i.s.a. was reduced from 0.006% to 0.002%, very similar binding was observed to both native and denatured ATPase (Fig. 3). The effect of SDS can probably be attributed to effects on the binding of antibody to the denatured ATPase rather than to any effect on the extent of denaturation of the ATPase. Thus for anti-539–553 antibody, which binds preferentially to denatured ATPase (Fig. 4), decreasing the SDS concentration from 0.006% to 0.002% had no significant effect on binding; if lowering the concentrations of SDS resulted in some refolding of the ATPase, then reduced binding of this anti-peptide antibody would have been observed at the lower concentration of SDS.

Two anti-peptide antibodies (against peptides 366–377 and 422–432) showed some depletion on incubation with native ATPase (Fig. 5), but less than that observed for the anti-peptide antibodies that bound equally to native and denatured ATPase. Since the observed response was reduced by a factor of 2 from that observed for those antibodies giving full depletion (Fig. 3), a possible explanation would be the presence of two main anti-peptide antibodies in the antibody mixture, one binding to an epitope exposed on the surface of the native ATPase, and one buried and thus inaccessible to antibody. The remaining anti-peptide antibodies (Table 1) showed no binding to the native ATPase in competitive e.l.i.s.a. (Fig. 4).

The effect of SDS on the binding of some of the antibodies raised against peptides corresponding to inter-membranous loops is shown in Fig. 6(*a*). Anti-915–924 antibody bound to the denatured ATPase in the absence of SDS, and binding was unaffected by the presence of SDS except when the SDS concentration exceeded 0.02%. The decrease in binding at high concentrations of SDS is probably attributable to denaturation of the antibody, since a similar fall in binding was seen when identical experiments were performed with e.l.i.s.a. plates coated with peptide 915–924 (results not shown). Surprisingly, anti-277–287 antibody only weakly recognized denatured ATPase in e.l.i.s.a. (Fig. 6*a*), although it recognized the ATPase in Western blots (Table 1). However, enhanced binding was observed on addition of SDS to the e.l.i.s.a. up to 0.008%, beyond which binding was observed to decrease. Reduced binding at high concentrations of SDS can presumably be attributed to denaturation of the antibody, as observed for anti-915–924. The requirement for low concentrations of SDS for binding to denatured ATPase would suggest some refolding of the SDS-denatured ATPase on dilution of the SDS during the coating procedure, and also suggests that anti-277–287 antibody binds to

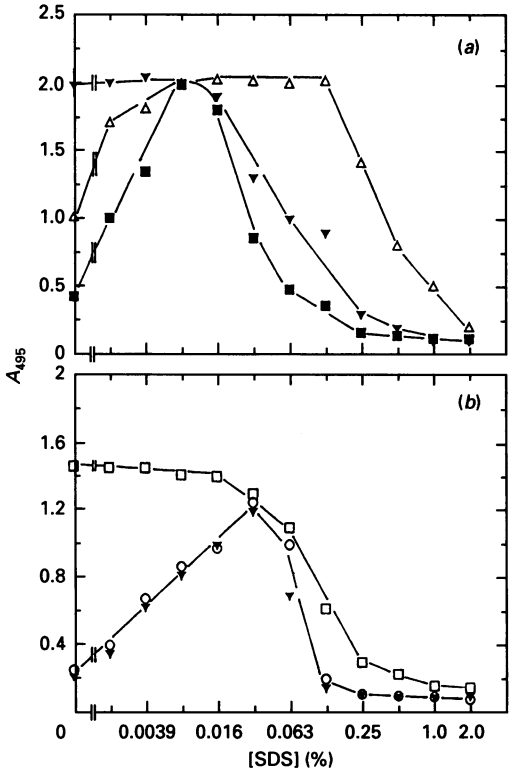

Fig. 6. Antipeptide antibody binding to SDS-denatured (Ca^{2+}–Mg^{2+})-ATPase in the presence of various SDS concentrations

E.l.i.s.a. plates coated with denatured ATPase were incubated with anti-peptide antibodies against (*a*) peptides 277–287 (■), 780–791 (△) and 915–924 (▼), and (*b*) peptides 177–189 (▼), 303–314 (○) and 324–339 (□) in the presence of the given concentration of SDS. The amount of bound antibody was determined by e.l.i.s.a. Anti-peptide antibodies directed against 949–958 gave a similar profile to that seen for anti-780–791, and anti-808–818 gave a similar profile to that seen for anti-915–924; these have been omitted for clarity.

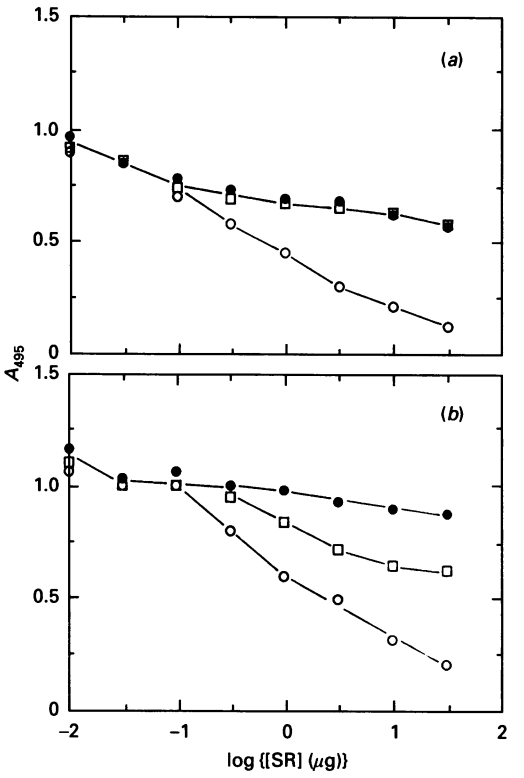

Fig. 7. Characterization of anti-peptide antibody binding to native, denatured and $C_{12}E_8$-solubilized SR

Antibodies raised against peptides 808–818 (*a*) and 915–924 (*b*), diluted 1:2000, were incubated with the given amount of native (●), denatured (○) or $C_{12}E_8$-solubilized (□) SR in PBS. The incubate was transferred to e.l.i.s.a. plates coated with denatured SR to determine the amount of free antibody by e.l.i.s.a..

a relatively stable region in the structure of the ATPase. Results with anti-780–791 and -949–958 antibodies were broadly similar to those with antibody against peptide 277–288 (Fig. 6*a*), although the higher level of binding observed to the denatured ATPase at low concentrations of SDS would suggest less refolding of the SDS-denatured ATPase.

Of the anti-peptide antibodies raised to possible inter-membranous loops, only those against the two peptides 808–818 and 915–924 bound sufficiently well to the ATPase in the absence of SDS to allow them to be used in competitive e.l.i.s.a. to probe the structure of the native ATPase. Fig. 7 shows the results of competitive e.l.i.s.a. in which the antibodies were incubated with up to 30 μg of native, SDS-denatured or $C_{12}E_8$-solubilized SR. It is clear that antibodies against both 808–818 and 915–924 bind more strongly to denatured SR than to native SR (Figs. 7*a* and 7*b*). A hidden epitope in native SR could indicate either a buried epitope in the ATPase or an epitope on the ATPase exposed on the lumenal face of the SR. These two possibilities can be distinguished by solubilizing the SR in $C_{12}E_8$, which will expose any available epitopes on the lumenal side of the SR. An experiment of this type is illustrated for anti-877–888, as this epitope has been shown to be exposed on the lumenal side of the membrane, in Fig. 8(*c*) (Matthews *et al.* 1990; Clarke *et al.* 1990*a*). As shown in Fig. 7, treatment with $C_{12}E_8$ had relatively little effect on the binding of antibodies against peptides 808–818 and 915–924, suggesting that the epitopes are buried in the native ATPase.

Binding to proteolytic fragments

Fig. 9 shows the results of epitope mapping by limited proteolysis of SR followed by Western blotting. Trypsin cleaves the ATPase initially at the *C*-terminal side of Arg-505 (T_1 site) to give A and B fragments, with further cleavage of the A fragment at the *C*-terminal side of Arg-198 (T_2 site) giving A_1 and A_2 fragments (Thorley-Lawson & Green, 1973; Imamura & Kawakita, 1989).

Binding of anti-peptide antibodies to intact and cleaved ATPase in Western blots is tabulated in Table 1. Anti-177–189 and -303–314 antibodies, which bind only weakly to intact ATPase in Western blots, showed strong binding to trypsinized ATPase. Surprisingly, these antibodies showed no significant binding to denatured ATPase in competitive e.l.i.s.a. in the presence of 0.006% SDS (Table 1). However, binding was observed to denatured ATPase in e.l.i.s.a. at higher concentrations of SDS (Fig. 6*b*). As shown, significant binding was only observed for SDS concentrations higher than approx. 0.01%. This suggests that, at lower concentrations, significant secondary structure is retained by the denatured ATPase on the plates. Anti-493–503 and -539–553 antibodies, which failed to bind to native ATPase on e.l.i.s.a. (Table 1), also showed only weak binding to the ATPase in Western blots (Fig. 9*a*), but did bind after proteolysis (Fig. 9*b*), consistent with buried epitopes for these anti-peptide antibodies. Binding of anti-539–553 antibody was also observed to an unidentified component of apparent molecular mass 50 kDa in intact SR (Fig. 9*a*). Antibodies against peptides 1–12, 454–466, 601–614, 731–749 and 985–994, which bound to the native ATPase in e.l.i.s.a., also bound to the intact

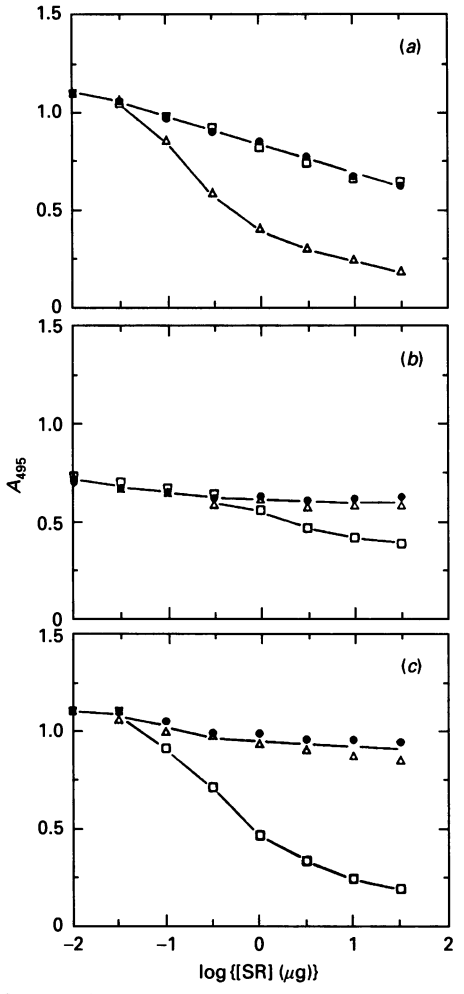

Fig. 8. Characterization of anti-peptide antibody binding to native and proteinase K-treated SR

Anti-peptide antibodies raised against peptides 808–818 (*a*), 915–924 (*b*) and 877–888 (*c*), all diluted 1:2000, were incubated for 2 h at room temperature with the given amount of native (●), proteinase K-treated (△), or $C_{12}E_8$-solubilized (□) SR in PBS. The incubate was transferred to e.l.i.s.a. plates coated with denatured SR to determine the amount of free antibody by e.l.i.s.a.

ATPase in Western blots (Fig. 9*a*). In the Western blots, the A_2 tryptic fragment was clearly identified by anti-1–12 antibody, the A_1 tryptic fragment at apparent molecular mass 32 kDa bound antibodies against peptides 454–466 and 493–503, and the B fragment running with the A fragment at apparent molecular mass 50–55 kDa bound anti-peptide antibodies against 601–614, 731–749 and 985–994. The fragment at apparent molecular mass 30 kDa bound both the *C*-terminal anti-peptide antibody (anti-985–994) and anti-731–749, but none of the others (Fig. 9*b*), and corresponds to the stable *C*-terminal fragment reported elsewhere (Matthews *et al.*, 1990). It also probably corresponds to cleavage at the T_4 site described by Imamura & Kawakita (1989).

Binding of anti-peptide antibodies against 508–517, 539–553, 577–587, 601–614, 643–654 and 659–668 to a fragment of apparent molecular mass 18 kDa (Fig. 9*c*) corresponds to the fragment of the nucleotide-binding domain from the T_1 cleavage site to Arg-667, giving a fragment of 17.8 kDa, or to Arg-671 or Arg-672, giving fragments of 18.3 or 18.4 kDa respectively. The smaller fragment of apparent molecular mass 16.5 kDa, which binds the same anti-peptide antibodies as the fragment of apparent molecular mass 18 kDa except for anti-659–668, would then correspond to a fragment from T_1 to Arg-656 (16.6 kDa) or Arg-651 (16.1 kDa). Binding of anti-peptide antibodies against peptides 366–377, 381–400, 403–417, 422–432, 454–466 and 493–503 to a fragment of apparent molecular mass 18–19 kDa corresponds to a fragment of the phosphorylation domain from Arg-334 to the T_1 cleavage site (18.8 kDa), and the smaller fragment of apparent molecular mass 16.5 kDa binding the same range of anti-peptide antibodies, faint in Fig. 9(*c*) but clearer on the blots shown in Fig. 9(*b*), could then correspond to a fragment from Lys-352 to the T_1 cleavage site (16.8 kDa).

As shown in Fig. 10, the anti-peptide antibody against 277–287 binds both the A fragment (55 kDa) and the A_1 fragment (32 kDa; residues 199–505) identified by the binding of anti-191–205 and -366–377 antibodies that have been previously characterized (Colyer *et al.*, 1989; Matthews *et al.*, 1990). Anti-1–12 antibody identified the A_2 fragment (23 kDa; residues 1–198). None of the anti-peptides generated here bound to this fragment, as expected, since peptide 78–88 was not immunogenic and serum from rabbits immunized with this peptide failed to recognize the ATPase or any of the fragments on the Western blot (Table 1). Anti-peptide antibodies against peptides 780–791, 808–818, 915–924 and 949–958 bound the B fragment (50 kDa; residues 506–994) and also a 30 kDa fragment, which is thought to be the largely membrane-associated *C*-terminal fragment (Matthews *et al.*, 1990) containing residues 729–994, identified by binding of anti-731–749 and anti-985–994.

We have shown that treatment of the ATPase with proteinase K produces a proteolytically stable fragment of apparent molecular mass 30 kDa extending from the *C*-terminus to Lys-728 (Matthews *et al.*, 1990). Fig. 8(*a*) shows that anti-808–818 binds strongly to proteinase K-treated SR, suggesting that the lack of binding to the native ATPase is due to overlaying cytoplasmic structures in the native protein. The experiment also shows that the epitope 808–818 must be on the cytoplasmic surface, since the proteinase K-treated SR vesicles are still intact, as shown by the lack of binding of anti-877–888 which recognizes the intralumenal loop (Fig. 8*c*). In contrast, binding of anti-915–924 to the SR was unaltered by proteinase K treatment (Fig. 8*b*).

Characterization of anti-peptide antibodies

A number of the anti-peptide antibodies were screened against sets of overlapping hexameric peptides corresponding to the sequence of the (Ca^{2+}–Mg^{2+})-ATPase, synthesized on polyethylene pins arranged to fit the wells of standard microtitre plates (Geysen *et al.*, 1987). All anti-peptide antibodies were found to bind to hexameric peptides whose sequences were part of the peptide used to raise the antibody. However, equal binding was not observed to the whole set of hexameric peptides spanning the sequence of the peptide. Thus clear binding of anti-643–654 antibody was only observed to hexapeptides EEVADR and EVADRA (Table 2). Studies of binding of mAbs to overlapping sets of hexameric peptides show similar clusters of binding [see Tunwell *et al.* (1991*a*) for the (Ca^{2+}–Mg^{2+})-ATPase] and allow the definition of a consenus epitope which includes the minimum number of residues essential for binding (Geysen *et al.*, 1984). It is likely, therefore, that antiserum to peptide 643–654 only contains antibodies binding to the consensus sequence EVADR (Table 2). Strong binding of anti-539–553 antibody was only observed to hexapeptide VKEKIL. Weak binding was also observed to hexapeptides IKEWGT, KEWGTG and EWGTGR. Since peptide 539–553 (GPVKEKILSVIKEWG) used to produce the anti-peptide antibody only includes the first three of the residues found in the last of the hexapeptides (EWG), it is likely that antibody is also present which binds to the consensus epitope EWG (Table 2).

Other anti-peptide antibodies showed equal binding to two

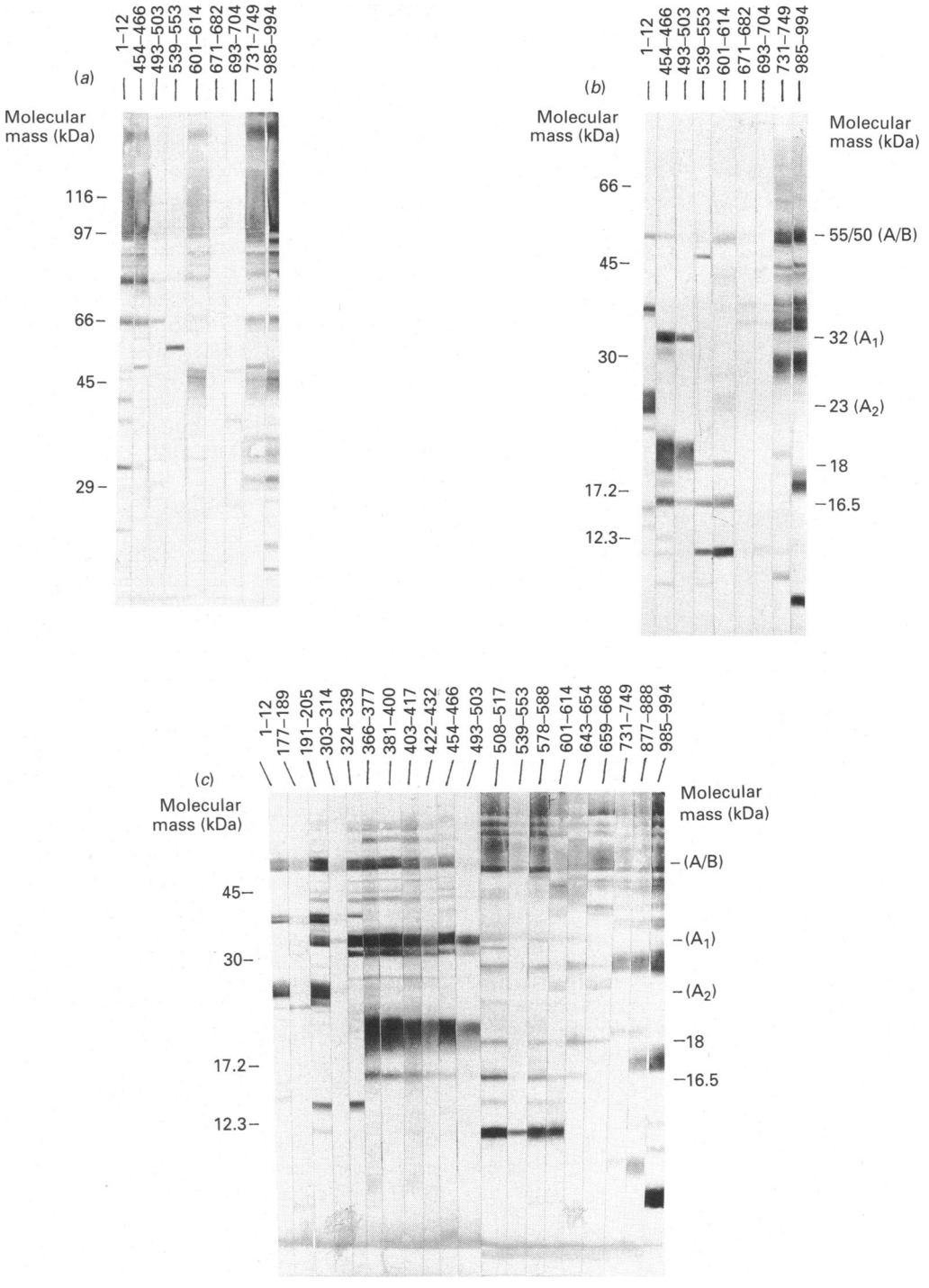

Fig. 9. Identification of epitopes on proteolytic fragments of (Ca^{2+}–Mg^{2+})-ATPase

SR (*a*) or SR following digestion with trypsin (*b* and *c*) was resolved on polyacrylamide gels and then transferred to nitrocellulose. Blots were divided into strips, blocked overnight and incubated with anti-peptide antibodies raised against the peptides indicated, followed by horseradish-peroxidase-conjugated second antibody, and substrate. A and B indicate tryptic fragments.

distinct groups of hexapeptides (Fig. 11). Thus anti-191–205 antibody shows binding to consensus epitopes PVPD and VNQDK (Fig. 11*a*). Antibody against peptide 601–614 (DPPRKEVMGSIQLC) bound to two regions, between hexameric peptides GMLDPP and PRKEVM and between MGSIQL and IQLCRD (Fig. 11*b*). Binding to the hexapeptide GMLDPP demonstrates the presence of antibody with the most important residues in the epitope being DPP, and binding to other hexapeptides up to PRKEVM, with no binding to the next hexapeptide RKEVMG, suggests the presence of other antibodies with a common minimal requirement of PR (Pro-Arg). Binding to hexapeptides between MGSIQL and IQLCRD would suggest the presence of antibody binding to the consensus epitope IQL. Antibody raised to peptide 508–517 (VGNKMFVKGA) bound to hexapeptide RAAVGN, which, together with the observed binding to hexapeptides up to VGNKMF, indicates an antibody with a consensus epitope VGN (Fig. 11*c*). Binding to hexapeptides GNKMFV to MFVKGA, with no binding to the

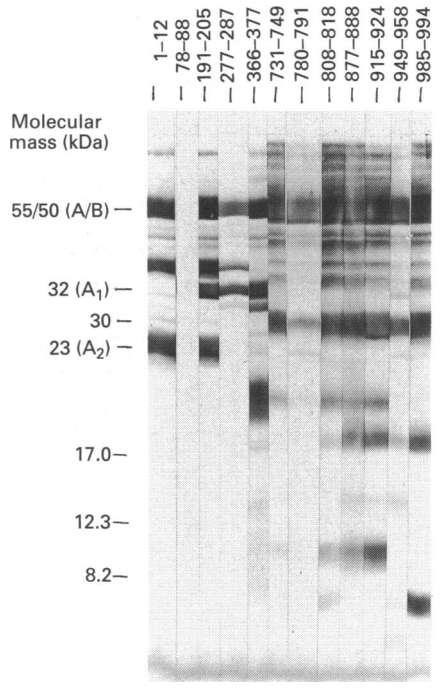

Fig. 10. Western blot of trypsin-digested SR with antibodies against the *N*- and *C*-termini and anti-peptide antibodies to possible intermembranous loop regions

SR digested with trypsin was resolved on polyacrylamide gels and then transferred to nitrocellulose. Blots were divided into strips, blocked overnight and incubated with anti-peptide antibodies against the peptides indicated, followed by horseradish-peroxidase-conjugated second antibody, and substrate. A, A_2 and B indicate tryptic fragments.

Table 2. Minimum consensus epitopes for anti-peptide antibodies

Epitopes were defined by binding of anti-peptide antibodies to overlapping sequences of hexameric peptides. Numbers indicate the corresponding residues in the sequence of the (Ca^{2+}–Mg^{2+})-ATPase and -Cys indicates where an additional cysteine residue has been added to the *C*-terminus of the peptide to allow coupling to a carrier protein. The underlined residues correspond to the minimum consensus epitopes required to explain the observed binding.

Peptide	Peptide sequence
1–12	MEAAHSKSTEEC
177–189	QSILTGESVSVIK-Cys
191–205	TEPVPDPRAVNQDKK-Cys
303–314	AVAAIPEGLPAV-Cys
324–339	RRMAKKNAIVRSLPSV-Cys
366–377	MFIIDKVDGDFC
493–503	SMSVYCSPAKS-Cys
508–517	VGNKMFVKGA-Cys
539–553	GPVKEKILSVIKEWG-Cys
567–582	RDTPPKREEMVLDDSS
577–587	VLDDSSRFMEY-Cys
601–614	DPPRKEVMGSIQLC
643–654	GENEEVADRAYT-Cys
659–668	DDLPLAEQRE-Cys

remaining hexapeptides, indicates a consensus epitope MF (Met-Phe).

For other anti-peptide antibodies, those with epitopes covering the whole range of the peptide appear to be present. Thus antibody raised to peptide 659–668 (DDLPLAEQRE) bound to hexapeptides FDDLPL to DLPLAE, consistent with a consensus epitope DLP, and to hexapeptides PLAEQR and LAEQRE, consistent with a consensus epitope LAEQR (Table 2). Similarly, binding of antibody raised to peptide 366–377 corresponds to two main consensus epitopes, FIIDK and KVDG (Table 2).

Fig. 12 illustrates the effects of labelling the ATPase with FITC at Lys-515 on the binding of anti-508–517 antibody and mAb Y/1F4, whose epitope is NKMFVK (residues 510–515) (Tunwell *et al.*, 1991 *a*). E.l.i.s.a. plates were coated with native or heat-denatured ATPase, either labelled with FITC or unlabelled, and then incubated with serial dilutions of conditioned medium containing mAb Y/1F4 or anti-508–517 antibody for 1 h at room temperature. Bound antibody was detected with second antibody conjugated to horseradish peroxidase. As shown in Fig. 12, labelling with FITC resulted in a marked reduction in the level of binding of mAb Y/1F4, for both denatured and native ATPase. The effects of labelling the ATPase with FITC on the binding of anti-508–517 antibody, however, were much smaller than those observed with mAb Y/1F4 (Fig. 12).

DISCUSSION

Immunological methods can be used to define surface-exposed regions on proteins, using either mAbs raised against the whole protein or anti-peptide antibodies. mAbs raised against intact proteins can be selected for binding to native protein. Characterization of the epitopes for such mAbs is, however, not trivial and a rather restricted range of mAbs is generally obtained following immunization. The advantage of anti-peptide antibodies is that they can be targeted to any region of the protein of interest. A disadvantage of anti-peptide antibodies for mapping studies is that they are of low spatial resolution. The optimal peptide length for antibody production is 10–15 residues (Palfreyman *et al.*, 1984; Van Regenmortel *et al.*, 1988), but continuous epitopes are typically 5–7 residues in length (Geysen *et al.*, 1987; Novotny *et al.*, 1989; Stanfield *et al.*, 1990; Novotny, 1991), often with a subset of these providing most of the binding specificity (Geysen *et al.*, 1984). Since anti-peptide sera generally contain only a limited repertoire of paratopes (Geysen *et al.*, 1984), binding of an anti-peptide antibody to a protein does not necessarily indicate that the whole of the loop corresponding to the peptide used to raise the anti-peptide antibody is surface-exposed. The problem is analogous to that of cross-reactivity observed with anti-peptide antibodies; binding of an anti-peptide antibody to a protein does not indicate that the protein contains the whole of the amino acid sequence corresponding to the peptide used for raising the antibodies. Thus Khachigian *et al.* (1991) reported an anti-peptide mAb for which the 2-amino-acid sequence Lys-Lys could serve as antigenic determinant, and which consequently showed binding to a wide range of proteins.

Geysen *et al.* (1987) have shown that epitopes can be mapped by studying the binding of antibodies to overlapping sets of peptides synthesized on plastic pegs. Schoofs *et al.* (1988) showed that the immunodominant region on a peptide could be mapped by studying the binding of anti-peptide antibody to sets of peptides of lengths between five and eight residues, but that no binding was observed to peptides shorter than four residues. We have used this technique to define the epitope specificity of some of our anti-peptide antibodies, using sets of hexameric peptides. Table 2 lists the anti-peptide antibodies studied, and shows the minimum consensus epitopes required to explain the observed binding to the hexapeptides. The pattern is different for all the peptides, although observed epitopes tend to cluster towards the centres of the peptides. For some peptides (e.g. 177–189), antigenicity appears to be restricted to a small part of the peptide, whereas for others (e.g. 659–668) the distribution of epitopes more nearly covers the whole length of the peptide.

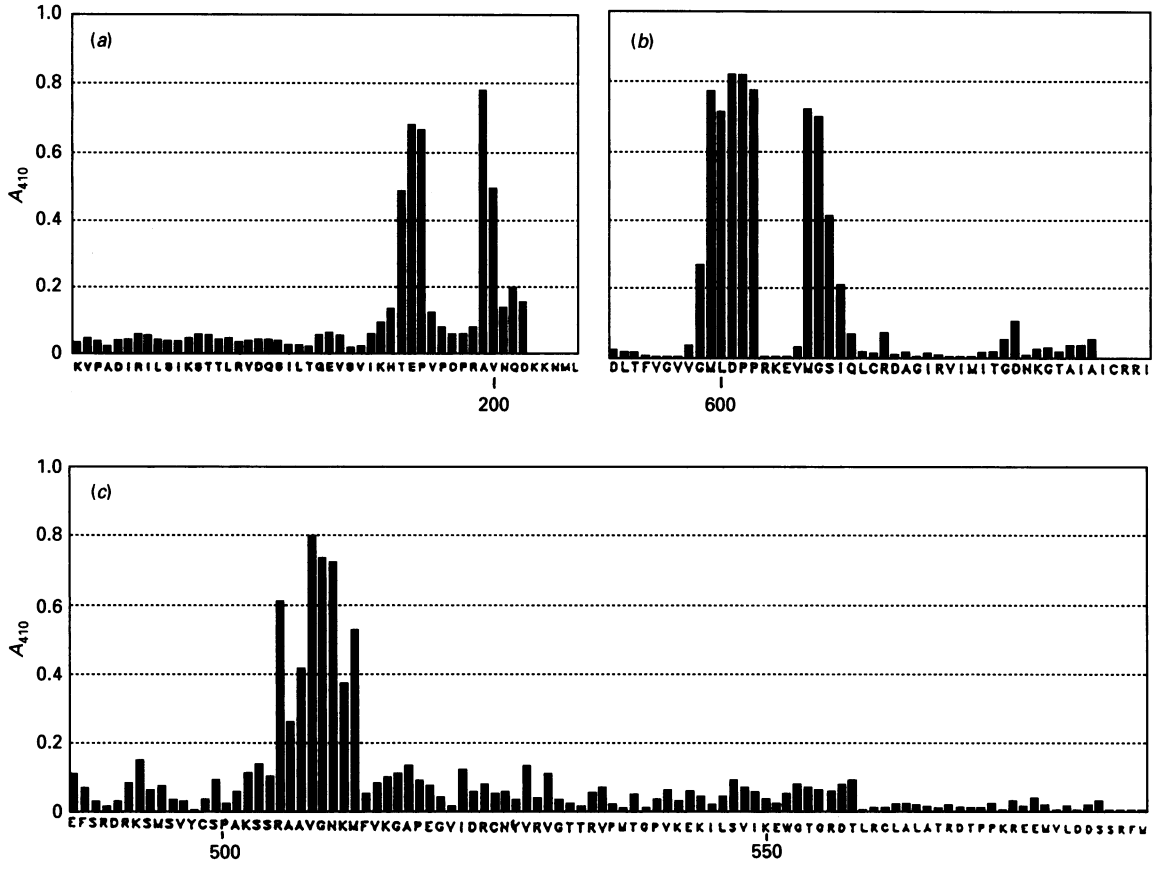

Fig. 11. Binding of anti-peptide antibodies against peptides 191–205, 601–614 and 508–517 to hexapeptides

Scans are shown of hexapeptides corresponding to residues 159–208 (*a*), 590–639 (*b*) and 486–526 (*c*) of the ATPase with anti-191–205 (*a*), anti-601–614 (*b*), and anti-508–517 (*c*) antibodies.

For those anti-peptide antibodies binding to native ATPase and binding to only one cluster of hexapeptides, we can only safely conclude that the consensus epitope is surface-exposed on the ATPase. In one case (anti-366–377) of an anti-peptide antibody binding to more than one cluster of hexapeptides, we observed evidence for partial binding of anti-peptide antibody to the native ATPase (Fig. 5*a*), suggesting that only one of the two consensus epitopes might be exposed. For the other anti-peptide antibodies in Table 2 binding to more than one cluster of hexapeptides, it would appear that most of the corresponding peptide on the ATPase must be surface-exposed.

The mapping results presented here provide an explanation for an otherwise unexpected observation on the effects of modifications of the ATPase at Lys-515 with FITC. As shown in Fig. 12(*a*), modification of the ATPase with FITC decreases the binding of mAb Y/1F4 to the ATPase, consistent with the epitope mapped for this antibody, NKMFVK (residues 510–515) (Tunwell *et al.*, 1991*a*). The effect of modification with FITC on the binding of anti-508–517 antibody is, however, relatively small (Fig. 12*b*). The epitope mapping experiments with this antibody give consensus epitopes that do not include Lys-515 (Table 2).

In previous papers we have used mAbs to define surface-exposed regions on the (Ca^{2+}–Mg^{2+})-ATPase of SR. We have defined four surface-exposed epitopes, corresponding to residues 333–338, 510–515, 580–588 and 662–666, and these assignments were confirmed by demonstration of the binding of anti-peptide antibodies raised against residues 324–339, 508–517, 578–588 and 643–654 of the ATPase (Colyer *et al.*, 1989; Matthews *et al.*, 1989; Tunwell *et al.*, 1991*a*,*b*). Mapping of epitopes for polyclonal antibodies on the ATPase also suggested a surface-exposed epitope at the T_2 trypsin cleavage site on the ATPase (Tunwell *et al.*, 1991*a*).

Structural predictions based on the amino acid sequence of the ATPase suggest the presence of two large cytoplasmic domains, a phosphorylation domain containing the Asp residue (351) phosphorylated by ATP, and a nucleotide-binding domain. It has been proposed that movement of the nucleotide-binding domain and phosphorylation domains is necessary to bring the γ-phosphate of bound ATP close to Asp-351 (MacLennan *et al.*, 1985; Petithory & Jencks, 1986). It has also been suggested that the ATPase contains 10 transmembrane α-helices, connected to a stalk region made up of five amphipathic helices. Linking the phosphorylation domain and the second stalk is a β-strand or transduction domain, and linking the nucleotide-binding domain to the fifth stalk is a central or hinge domain (Fig. 2) (MacLennan *et al.*, 1985; Brandl *et al.*, 1986). We have shown that both the *N*- and *C*-termini of the ATPase are located on the cytoplasmic side of the membrane, indicating an even number of transmembranous α-helices (Matthews *et al.*, 1989). Studies with anti-peptide antibodies (Matthews *et al.*, 1990) and mAbs (Clarke *et al.*, 1990*a*) have shown that a loop containing residues 877–888 is lumenal, as predicted by the 10-helix model (Fig. 1). The 10-helix model also predicts that the region of the ATPase from the fifth postulated transmembrane helix to the *C*-terminus is very largely buried in the membrane (see Fig. 1). This is consistent with our observation of a 30 kDa *C*-terminal fragment of the ATPase which is stable against proteolytic degradation (Matthews *et al.*, 1990).

Attempts to use anti-peptide antibodies to locate the small

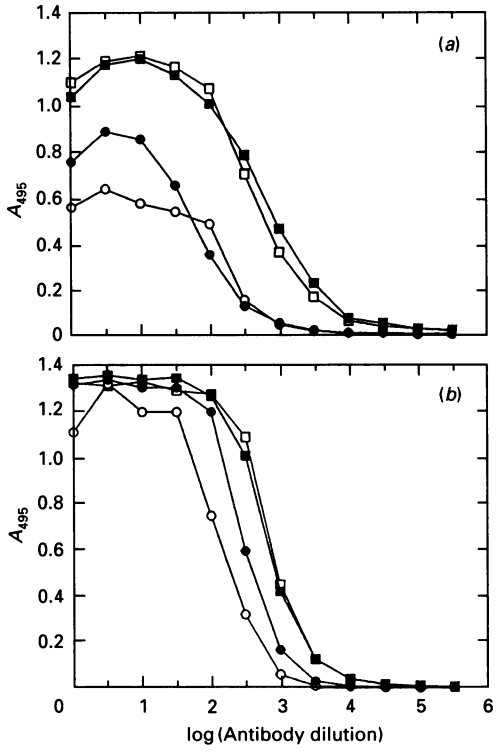

Fig. 12. Effect of labelling the ATPase with FITC on antibody binding

E.l.i.s.a. plates were coated with native ATPase (■), denatured ATPase (□), native FITC–ATPase (●) or denatured FITC–ATPase (○) at 1 μg per well and incubated with either mAb Y1/F4 (*a*) or anti-508–517 antibody at the given dilutions. Bound antibody was then detected using second antibody conjugated to horseradish peroxidase.

inter-membranous loops postulated between helices M1–M2, M3–M4, M5–M6, M6–M7, M8–M9 and M9–M10 were not successful, since in one case no anti-peptide antibody could be produced, and in the other cases no binding was observed to the native ATPase either in sealed SR vesicles or in vesicles made leaky to antibody with the detergent $C_{12}E_8$ (Table 1; Figs. 7 and 8). However, anti-808–818 antibody did bind to SR vesicles in which the ATPase had been treated with proteinase K under conditions where the vesicles remained intact (Fig. 8), suggesting both that the loop 808–836 is exposed on the cytoplasmic side of the membrane (as in the 10-helix model; Fig. 1) and that the lack of binding of anti-808–818 to the native ATPase is due to overlaying regions of the ATPase (see Fig. 2). Binding of anti-915–924 to SR was, however, unaltered by proteinase K treatment (Fig. 8), suggesting that in this case the failure of the anti-peptide antibody to bind is the result of an unfavourable conformation for this region of the ATPase in the membrane. It is perhaps pertinent that, in the proposed model for the ATPase (Fig. 1), the loop betwen transmembranous α-helices M8 and M9 (residues 912–930) is considerably shorter than that between helices M6 and M7 (residues 808–836). If these results can be extrapolated to other membrane proteins, then it would seem that only inter-membranous loops larger than about 20 residues are likely to be detectable by immunological methods.

Fig. 2 illustrates a structure for the ATPase modified from that of Green and Stokes (see Stokes, 1991) showing surface-exposed regions of the ATPase, as defined by mAb and anti-peptide antibody studies (Table 1). Since continuous epitopes on proteins generally correspond to mobile loops and ridges on the protein surface (Van Regenmortel *et al.*, 1988; Van Regenmortel, 1989*b*), these regions on the ATPase probably correspond to flexible, surface-exposed loops.

The relatively large number of anti-peptide antibodies binding to the phosphorylation and nucleotide-binding domains, and the concentration of mAb epitopes in this region (Tunwell *et al.*, 1991*a*), would suggest that these two domains are relatively surface-exposed on the top surface of the large pear-shaped lobe observed in electron micrographs (Stokes & Green, 1990*a*). FITC labels the ATPase at Lys-515 and, since labelling with FITC is competitive with binding of ATP, Lys-515 is thought to be part of the purine subsite of the ATP-binding site (Pick, 1981; Mitchinson *et al.*, 1982). Measurements using fluorescence energy transfer have located the fluorescein on the top surface of the ATPase (Gutierrez Merino *et al.*, 1987), as shown in Fig. 2. It has been shown that antibodies to fluorescein bind to denatured FITC-labelled ATPase, but not to native FITC-labelled ATPase, consistent with a location for the fluorescein in a hydrophobic cleft between the phosphorylation and nucleotide-binding domains (Mata *et al.*, 1989). Surface exposure of residues 510–515 would be inconsistent with their forming a β-sheet structure in the binding cleft for ATP, as suggested by Taylor & Green (1989). Site-directed mutagenesis has shown that many of the highly conserved amino acid residues in this region are critical for enzyme activity, but their possible role in binding ATP is unclear (Clarke *et al.*, 1990*c*).

Labelling the ATPase with adenosine triphosphopyridoxal in the presence of Ca^{2+} has identified Lys-684 (between α7 of the nucleotide domain and the central domain) as being part of the ATP-binding site (Yamamoto *et al.*, 1988), suggesting that this could be folded back behind the rest of the domain shown opened out in two dimensions in Fig. 2, to bring it close to Asp-351 in the phosphorylation domain. In the absence of Ca^{2+}, Lys-492 and Lys-684 are labelled with equal probability, suggesting that in this conformation of the ATPase, Lys-492 (in the region of the T_1 tryptic cleavage site) and Lys-684 are located close together (Yamamoto *et al.*, 1989). In yeast H^+-ATPase, a peptide is labelled by 2-azido-AMP which corresponds to the conserved region of the (Ca^{2+}–Mg^{2+})-ATPase from Asp-626 to Val-632 (Davis *et al.*, 1990). It has been suggested that Cys-670 and -674 are surface-exposed (Bishop *et al.*, 1988). Labelling of Cys-670 and Cys-674 with a variety of fluorescent groups has been shown not to inhibit phosphorylation by ATP, but fluorescence has been shown to be sensitive to conformational changes concurrent with or following binding of ATP (Suzuki *et al.*, 1987; Bigelow & Inesi, 1991); these sites have been estimated to be distant from Lys-515 (Squier *et al.*, 1987). Conformation-sensitive V8 proteinase cleavage sites have been identified at Glu-668 and Glu-715 (le Maire *et al.*, 1990). In the (Na^+–K^+)-ATPase, γ-(4-*N*-2-chloroethyl-*N*-methyamino)benzylamide-ATP has been observed to label Asp-710 (see Pedemonte & Kaplan, 1990), equivalent to the conserved Asp-704 in the (Ca^{2+}–Mg^{2+})-ATPase. Unfortunately we were unable to raise high-titre anti-peptide antibodies to peptides 671–682 or 693–704 (Table 1) to enable us to test the extent of surface exposure of this area. Anti-731–749 antibody was, however, demonstrated to bind to native ATPase, indicating surface exposure for the region between α2 of the central domain and the fifth stalk (Fig. 2).

On the basis of our failure to detect mAbs or polyclonal antibodies binding to the region of the ATPase from the *N*-terminus to the T_2 tryptic cleavage site, except at the *N*-terminus itself, we suggested that this whole region of the ATPase could be buried in the native structure, to form a central core for the protein (Colyer *et al.*, 1989). Site-directed mutagenesis has suggested that Thr-181, Gly-182 and Glu-183 play essential roles in the conformational change between $E1PCa_2$ and $E2PCa_2$ (Clarke *et al.*, 1990*b*). Anti-177–189 antibody failed to bind to

native ATPase and only bound to denatured ATPase in the presence of high concentrations of SDS (Table 1), consistent with a rather stable structure in this region of the ATPase, not exposed in the native structure. This would also be consistent with the weak binding observed to native ATPase in Western blots, with stronger binding observed to tryptic fragments of the ATPase.

Yamasaki *et al.* (1990) have reported that intermolecular cross-linking sites on the ATPase occur at Cys-377 and Cys-614, with cross-linking from the latter sites being prevented by nucleotide binding. Both these Cys residues occur close to surface-exposed regions of the ATPase, the former between α2 and α3 in the phosphorylation domain and the latter in the region of α4 in the nucleotide-binding domain (Fig. 2). These regions of the ATPase could then be involved in the protein–protein contacts observed in electron micrographs of the ATPase in the membrane (Stokes & Green, 1990*a*).

Binding of anti-peptide antibodies to proteolytic fragments of the ATPase in Western blots confirms our previous report of a proteolytically stable ATPase fragment with apparent molecular mass of 30 kDa (Matthews *et al.*, 1990) extending from the *C*-terminus probably to the T_4 tryptic cleavage site of Imamura & Kawakita (1989) at the *C*-terminal side of Lys-728. This would be consistent with the observed binding of anti-731–749 antibody to this fragment (Fig. 9*b*). A second interesting result of these experiments is the observation of a fragment of apparent molecular mass 18 kDa, binding anti-peptide antibodies against peptides 508–517 to 659–668, corresponding to a fragment from the T_1 cleavage site to Arg-667, Arg-671 or Arg-672. This fragment would include the whole of the postulated nucleotide-binding domain from the link to the phosphorylation domain to the central or hinge domain (Fig. 2). The observed fragment of apparent molecular mass 18–19 kDa binding anti-peptide antibodies against peptides 366–377 to 493–503 would correspond to the whole of the postulated phosphorylation domain from Arg-334, close to α1 of the phosphorylation domain, to the T_1 cleavage site. Although it cannot be assumed that fragments obtained by limited proteolysis necessarily correspond to structural domains, they often do (Wilson, 1991), and the observation of these fragments would then argue for the correctness of the assignment of the phosphorylation and nucleotide-binding domains made by MacLennan *et al.* (1985).

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