

# Probing the topography of the intramembrane part of $\text{Na}^+, \text{K}^+$ -ATPase by photolabelling with 3-(trifluoromethyl)-3-(*m*-[ $^{125}\text{I}$ ]iodophenyl)diazirine

## Analysis of the hydrophobic domain of the $\beta$ -subunit

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The mutual disposition of the  $\alpha$ -helical intramembrane rods in  $\text{Na}^+, \text{K}^+$ -ATPase subunits was studied by photolabelling of the membrane-bound enzyme with 3-(trifluoromethyl)-3-(*m*-[ $^{125}\text{I}$ ]iodophenyl)diazirine ([ $^{125}\text{I}$ ]TID). Under the chosen conditions for modification, the ratio of label incorporated into the subunits was found to be  $\alpha/\beta = 2.2$ , demonstrating the peripheral location of the  $\beta$ -subunit in the oligomeric membrane complex. The [ $^{125}\text{I}$ ]TID-labelled  $\beta$ -subunit was subjected to tryptic hydrolysis and the modified fragment (Thr $^{27}$ -Arg $^{71}$ ) was isolated. The labelled amino acid residues are located predominantly on one side of the helix, which is helpful in unravelling the spatial orientation of the  $\beta$ -subunit relative to the  $\alpha$ -subunit.

ATPase,  $\text{Na}^+, \text{K}^+$ -; Hydrophobic photolabeling; Trifluoromethyliodophenyldiazirine

## 1. INTRODUCTION

Investigation into the topography of the membrane sector of functionally active membrane-bound  $\text{Na}^+, \text{K}^+$ -ATPase might elucidate the structural arrangement of its ion-conducting pathways. Analysis of the primary structure of both subunits, calculation of their hydrophobicity profiles and enzyme topography studies [1-3] identified the sites of the polypeptide chains forming the membrane-bound region; this information underlies a two-dimensional model of the  $\text{Na}^+, \text{K}^+$ -ATPase transmembrane organization [3]. The carbene-generating hydrophobic reagent TID was shown to be a highly selective marker of the membrane-embedded protein regions, modifying mainly the sites of contact with lipids [4-6]. [ $^{125}\text{I}$ ]TID labelling was used to determine the spatial organization of

the Na-pump hydrophobic sector, namely the mutual disposition of  $\alpha$ -helical rods and the identification of amino acid residues exposed to the lipid phase. The present work positions the  $\beta$ -subunit at the periphery of the oligomeric membrane complex and establishes the orientation of the  $\beta$ -subunit intramembrane segment relative to the  $\alpha$ -subunit in the membrane. The structural characteristics of the  $\alpha$ -subunit intramembrane fragments are currently being studied.

## 2. MATERIALS AND METHODS

$\text{Na}^+, \text{K}^+$ -ATPase from pig kidney outer medulla was isolated according to [7]. Membrane-bound enzyme was labelled with [ $^{125}\text{I}$ ]TID. Protein samples (10 mg, 1 mg/ml) in 10% sucrose, 30 mM histidine-HCl, 3 mM EDTA (pH 7.5) were flushed with a gentle stream of  $\text{N}_2$  for 15 min at 4°C. The reagent (25 nmol, 10 Ci/mmol, Amersham, England) was added to the membrane-bound enzyme. The mixture was equilibrated for 15 min at 4°C in the dark, then irradiated with a 365 nm lamp at a distance of 10 cm for 25 min. To remove unbound reagent, the sample was diluted with 1% bovine serum albumin (BSA) and pelleted by centrifugation (140 000  $\times$  g, 1.5 h, 4°C). The

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supernatant was removed and the pellet washed twice in 50 mM imidazole-HCl (pH 7.5). To isolate the  $\beta$ -subunit the  $\text{Na}^+, \text{K}^+$ -ATPase sample was solubilized and separated by HPLC on an UltroPac TSK-3000 SWG column (21.5  $\times$  600 mm, Beckman) in 0.1 M  $\text{CH}_3\text{COONa}$ , 0.2% SDS (pH 6.0). SDS was removed from the protein by dialysis at 15°C against 0.1 M  $\text{NH}_4\text{HCO}_3$  (pH 8.3) for 72 h. After ultrafiltration the  $\beta$ -subunit (0.5 mg/ml) was treated with trypsin (10%, 37°C, 24 h). The lyophilized trypsin digest was dissolved in 0.1 M  $\text{CH}_3\text{COONa}$ , 5% SDS, 5%  $\beta$ -mercaptoethanol and applied to a TSK-2000 SW column (7.5  $\times$  600 mm), equilibrated with 0.1 M  $\text{CH}_3\text{COONa}$ , 0.2% SDS. The distribution of  $^{125}\text{I}$  was determined by counting  $\gamma$ -radiation. The radioactive pools from gel filtration were lyophilized, dissolved in 10% acetonitrile/isopropanol (2:1), 0.1% TFA (solvent A) and fractionated on a Nucleosil 5C4-300 (4.6  $\times$  100 mm) column, equilibrated in the same solvent. Peptides were eluted with increasing concentrations of solvent B (acetonitrile/isopropanol, 2:1, 0.1% TFA); 0–25% B, 25 min; 25–50% B, 75 min; 50–100% B, 25 min; 100% B, 25 min at a flow rate of 0.5 ml/min. Edman degradation of peptides was performed on an Applied Biosystems model 470A sequencer. PTH-derivatives of amino acids were analyzed using an Applied Biosystems model 120A PTH-analyzer.

### 3. RESULTS AND DISCUSSION

Irradiation of membrane-bound  $\text{Na}^+, \text{K}^+$ -ATPase in the presence of [ $^{125}\text{I}$ ]TID led to incorporation of radioactivity into lipids (97% of label) and into both enzyme subunits. To determine the ratio of radiolabel incorporated into the subunits we separated solubilized  $\text{Na}^+, \text{K}^+$ -ATPase on an UltroPac TSK-3000 SWG column (fig.1). Incorporation was detected as part (%) of the total amount of eluted radioactivity. On average 2–3% of the original protein appeared to be labelled with the ratio of  $\alpha$ - and  $\beta$ -subunit labelling being about  $\alpha/\beta = 2.2$ . Jorgensen demonstrated an analogous ratio for [ $^{125}\text{I}$ ]TID incorporation into the subunits ( $\alpha/\beta = 2.8$ ) for enzyme solubilized in the  $\text{E}_1$  conformation [8], which is predominant in our experiments.

Calculation of the hydrophobicity profiles of the  $\alpha$ - and  $\beta$ -subunits [1] and investigation on the enzyme topography [2,3] gave a ratio of approx. 7:1 for the masses of the membrane-bound portions of the  $\alpha$ - and  $\beta$ -subunits. Thus, the specific activity of the  $\beta$ -subunit membrane portion is 3-fold greater than that of the corresponding  $\alpha$ -subunit region. This may seem paradoxical, however, it can be explained by a peripheral position for the  $\beta$ -subunit in the oligomeric membrane complex, and by the ability of the enzyme to form a crystal-like structure at low temperatures (4–6°C).

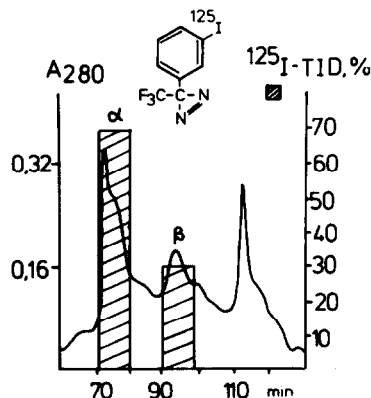


Fig.1. Separation of  $\text{Na}^+, \text{K}^+$ -ATPase subunits on a TSK G-3000 SWG column equilibrated with 0.1 M sodium acetate buffer (pH 7.0) containing 0.2% SDS. Hatched bars: radioactivity incorporated into enzyme subunit (% of total radioactivity in sample).

For identifying labelled fragments and positioning modification targets, isolated enzyme subunits were treated with trypsin. Gel-penetrating chromatography (TSK-2000 SW column) of the hydrolysate in both cases showed the total radiolabel to be bound to the fraction of high molecular mass fragments ( $\beta\text{II}$ ), whereas the low molecular

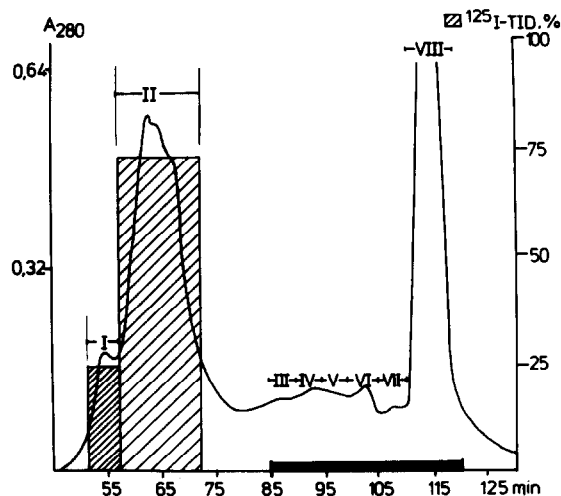


Fig.2. Separation of  $\beta$ -subunit tryptic hydrolysate on an UltroPac TSK-2000 SW column (7.5  $\times$  600 mm) equilibrated with 0.1 M sodium acetate buffer (pH 7.0) containing 0.2% SDS. Hatched bars: radioactivity in fraction (% of total radioactivity of hydrolysate).

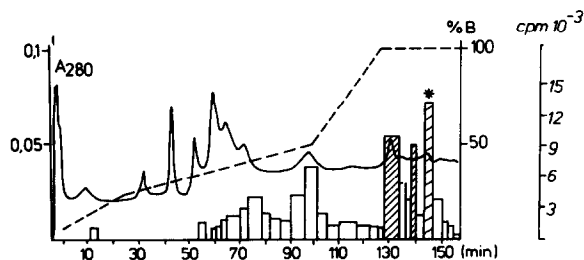


Fig.3. Separation of  $\beta$ -subunit tryptic peptides on a Nucleosil 300C<sub>4</sub> column (4.6  $\times$  100 mm) in a buffer B concentration gradient (acetonitrile/isopropanol, 1:1, in 0.1% TFA). Elution rate, 0.5 ml/min; broken line, change in buffer B (%) content. Unfilled bars: radioactivity content (cpm); hatched bars: fraction containing homogeneous peptide Thr<sup>27</sup>-Arg<sup>71</sup>.

mass products (III-VIII) resulting from exhaustive tryptic hydrolysis contain practically no label (fig.2). Apparently, fraction I contains the  $\beta$ -subunit.

Isolation of the modified fragments ( $\beta$ II) was carried out by reverse-phase chromatography (Nucleosil 5C<sub>4</sub>-300). Fig.3 shows that the radioac-

tivity yield does not coincide with absorption peaks and follows them directly. We propose that a broad radioactivity profile depends on differing degrees of modification of the limited number of fragments and that displacement of the yield of label relative to the absorption peak is explained by the small extent of labelling (2-3%) of the separated material, and TID modification increasing the hydrophobicity and, consequently, retention time. N-terminal analysis of the radioactive fractions supported our proposal: the fractions (broken line) in fig.3 containing the greatest amount of radioactivity appeared to be homogeneous (N-terminal amino acid Thr), and the rest are mixed but with a similar set of N-terminal amino acid residues, including Thr.

Determination of the amino acid sequence on a gas-phase sequencer (table 1) revealed modification of the  $\beta$ -subunit fragment Thr<sup>27</sup>-Arg<sup>71</sup>.

One third of the PTH-derivatives formed was used for identification of the amino acid residues, two thirds being employed in radioactivity assays. Several residues appeared to be labelled (table 1), the radioactivity being rather low (100-200 cpm), and the remainder determined on a filter after sequencing amounted to 2000 cpm or one fifth of the initial quantity. According to fig.3 and table 1 the isolated peptide is extremely hydrophobic, and therefore the low radioactivity yield can be explained as being the consequence of washing out of modified peptides with organic solvent during sequencing.

Table 1

Amino acid sequence of modified fragment Thr<sup>27</sup>-Arg<sup>71</sup>

Residue	No. of cycle	Yield (pmol)	Residue	No. of cycle	Yield (pmol)
Thr	1	400	Phe	23	-
Gly	2	400	Ile	24	26
Gly	3	500	Gly	25	-
Ser	4	300	Thr	26	-
Trp	5	-	Ile	27	10
Phe	6	60	Gln	28	-
Lys	7	67	Val*	29	-
Ile	8	99	Met	30	12
Leu	9	80	Leu	31	6
Leu	10	100	Leu	32	10
Phe	11	77	Thr*	33	-
Tyr	12	75	Ile	34	6
Val	13	70	Ser	35	-
Ile	14	50	Glu	36	+
Phe*	15	51	Phe	37	+
Tyr	16	38	Lys	38	+
Gly	17	30	Pro	39	-
Cys*	18	-	Thr	40	-
Leu	19	24	Tyr	41	-
Ala	20	30	Gln	42	-
Gly	21	10	Asp	43	+
Ile*	22	17	Arg	44	-

\*, amino acid residues preferentially labelled with [<sup>125</sup>I]TID; +, identified PTH-derivatives; -, nonidentified PTH-derivatives

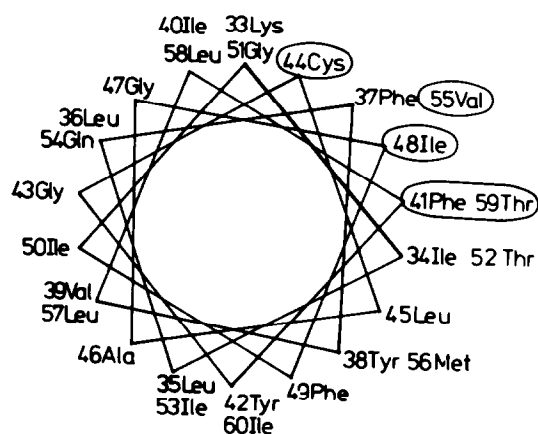


Fig.4. Axial projection of  $\alpha$ -helical fragment Lys<sup>33</sup>-Ile<sup>66</sup>. Amino acid residues preferentially labelled with [<sup>125</sup>I]TID are indicated.

The localization of predominantly labelled residues is not fortuitous. According to secondary structure computation [9], the Lys<sup>33</sup>-Ile<sup>61</sup> fragment – a part of the isolated modified peptide, containing the labelled residues – is an  $\alpha$ -helical region. An axial projection of the  $\alpha$ -helical chain (fig.4) clearly demonstrates the asymmetrical distribution of modified amino acids.

Thus, fragment Thr<sup>27</sup>-Arg<sup>71</sup> of the  $\beta$ -subunit is really bound to the membrane, one of the  $\alpha$ -helix sides formed by this fragment being exposed to the lipid bilayer, the other contacting the  $\alpha$ -subunit.

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