LABELLING OF THE HYDROPHOBIC DOMAIN OF THE Na^+,K^+-ATPase

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1. Introduction

The Na^+,K^+-ATPase is a ubiquitous plasma membrane protein which couples the transport of Na^+ out of the cell for K^+ into the cell with the hydrolysis of ATP [1–4]. The isolated active protein, which contains endogenous lipids, has been shown to be composed of a large polypeptide of M_r ~95 000 and of a sialoglycoprotein subunit of M_r ~45 000 [1–4]. Recently a third smaller component of M_r 10 000–12 000 has been proposed to be part of the ATPase complex on the basis of photolabelling studies performed with photoreactive derivatives of ouabain and strophantidin [5,6]. Little is known about the structure and localization of the ATPase subunits in relation to the lipid bilayer.

To gather more information on this point we have employed 2 photoreactive phosphatidylcholines and 2 amphipatic photoreactive derivatives of glucosamine and glycine. Their structures are depicted in fig.1. On illumination the photoactivatable nitroarylazido group is converted to a reactive nitrene intermediate which reacts with neighbouring molecules, thereby labelling them radioactively. These probes have been shown to label specifically those hydrophobic domains of several integral membrane proteins exposed to lipids [7–9,13,19]. We report here the labelling of the hydrophobic domain of the Na^+,K^+-ATPases from pig kidney and the electric organ of the eel *Electrophorus electricus.*

2. Materials and methods

[6-^{3}H]Glucosamine (38 Ci/mmol) and [2-^{3}H]-glycine (23 Ci/mmol) were purchased from the Radiochemical Centre, Amersham. Na^+,K^+-ATPase was purified from pig kidney according to [10]. Its activity at 37°C ranged from 12.6–18.1 μmol . min^{-1} . mg $protein^{-1}$ in different preparations and its lipid content was 0.8 mg/mg protein. The *Electrophorus electricus* ATPase was prepared according to [11]. Its activity, assayed as in [12], was 9.0 μmol . min^{-1} . mg $protein^{-1}$ at 25°C. Its lipid content was 0.3 mg lipid/mg protein. The preparation of 1-myristoyl, 2-[12-amino(4*N*-3-nitro-I-azidophenyl)]-dodecanoyl-*sn*-glycero-3-[^{14}C]phosphocholine (PL I) and of I-palmitoyl, 2-(2-azido-4-nitro)benzoyl-*sn*-glycero-3-[^{3}H]phosphocholine (PL II) was as in [7]. 12-Amino-*N*(2-nitro-4-azidophenyl)dodecanoyl-[6-^{3}H]glucosamine (Fa-G) was prepared according to [13] and 12-amino-*N*(2-nitro-4-azidophenyl)dodecanoyl-[2-^{3}H]glycine (Fa-gly) was prepared by scaling down the procedure in [14]. Their specific radioactivities were 38 Ci/mmol and 23 Ci/mmol, respectively. The structure of the photoreactive probes are shown in fig.1. The probes were mixed in chloroform: methanol (2:1) with egg lecithin (molar ratios from 1:400–1:10 000) and dried at the bottom of a glass tube under a gentle stream of nitrogen. 1.0–1.5 × 10^6 cpm of tritiated probes and 2–5 × 10^5 cpm of PL I were used. After 2 h under vacuum, 20 μl 0.25 M sucrose, 30 mM histidine, 1 mM EDTA, 0.15% cholate (pH 7.4) were added and the tube rotated for 1 h in the dark at room temperature. In some experiments 1% octyl-glucoside was used instead of cholate. 100 μg enzyme were added and incubated at 0°C for 2 h with occasional stirring. The sample was then diluted to 800 μl with buffer without cholate and illuminated in a glass cuvette with a glass–water filter with a 100 W long-wave UV lamp for 10 min at 0°C. Alternatively, liposomes of egg

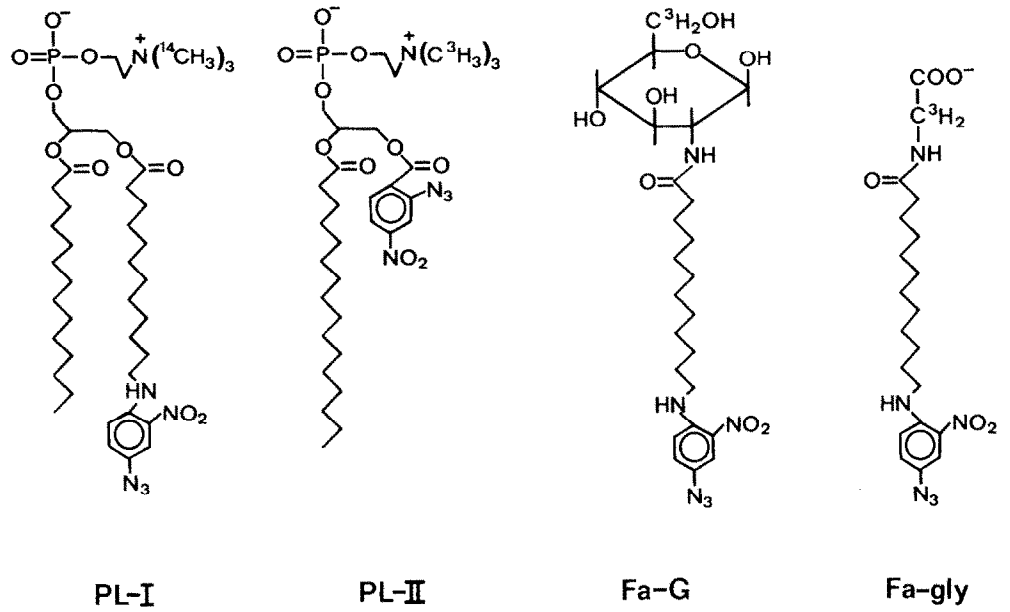

Fig.1. Structural formulae of the photoreactive phosphatidylcholines and lipid derivatives of glucosamine and glycine used.

lecithin tagged with the probes, prepared by sonication under nitrogen, were incubated with the protein for 4 h at 0°C and then illuminated as before. No change of enzymatic activity was found after illumination when oxygen was excluded. The sample was layered on 10% sucrose, 10 mM histidine, 1 mM EDTA (pH 7.4) and centrifuged for 2 h at 150 000 × *g*. The pellet was dissolved and analysed by SDS gel electrophoresis on a discontinuous system (5%–10% polyacrylamide) according to [15]. Gels were stained, sliced in 2 mm thick slices and counted as in [7].

3. Results and discussion

Fig.2 shows the patterns of labelling with PL I and PL II of the Na^+,K^+-ATPases from pig kidney and *Electrophorus electricus*. Both the α ($M_r \sim 95\,000$) and β ($M_r \sim 45\,000$) subunits are labelled. Only the radioactivity peak at the gel front, due to unbound probes, is found when the illumination step is omitted or when the protein is incubated with preilluminated lipids. In both ATPases PL I, which bears the photoreactive group near the centre of the lipid bilayer, labels the β subunit more effectively than PL II, which bears its photoreactive group near the lipid head groups. This difference in efficiency of labelling is most pronounced in the pig kidney enzyme were 33% of the radioactivity is associated with the β subunit using PL I compared to 17% in the case of PL II. This could reflect a difference between the subunits in the composition or in the number of those amino acid residues accessible to probes. It is clear, however, that the amount of radioactivity found associated with the β subunit is significant indicating that both the α and β subunits of the Na^+,K^+-ATPase expose part of their protein surface to lipids at the two different levels of the lipid bilayer probed by the photoreactive phosphatidylcholines used here.

Amphipatic molecules other than phospholipids are also able to interact directly with the Na^+,K^+-ATPase [16,17]. We have therefore performed the labelling of the Na^+,K^+-ATPases with the photoreactive derivatives Fa-G and Fa-gly, whose structures are shown in fig.1. Fa-G has been shown [18] to restrict its labelling to the hydrophobic sector of a well-characterized membrane protein such as the M13 coat protein. Fig.3 shows the pattern of labelling of the *Electrophorus electricus* ATPase with Fa-G and Fa-gly. Closely similar patterns were obtained for the pig kidney enzyme (not shown). The same pattern of labelling were obtained when other procedures, such as incubation with egg lecithin vesicles, were used to form lipid–protein complexes. Again both subunits

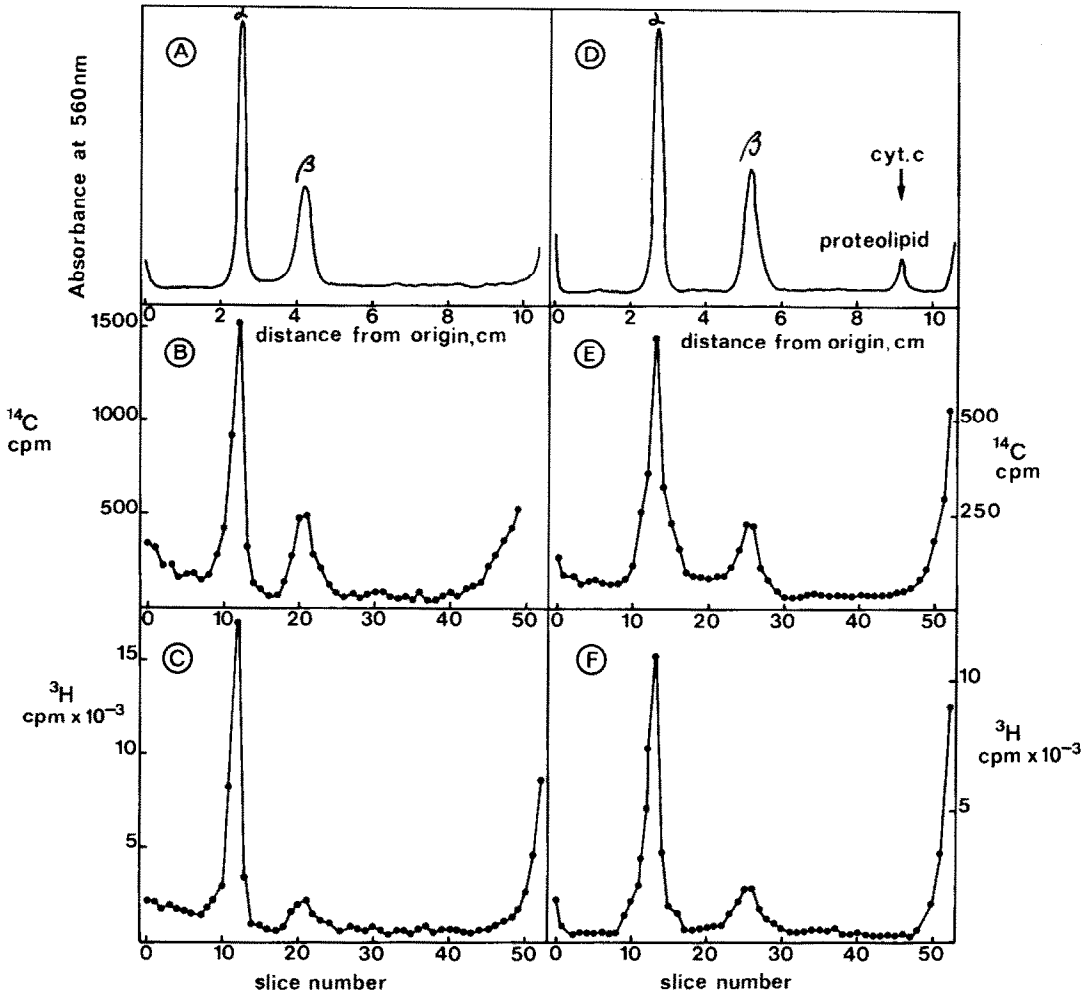

Fig.2. Labelling of Na^+,K^+-ATPases with PL I and PL II: (A,D) Coomassie blue staining profiles of SDS–polyacrylamide gels of pig kidney (A) and *Electrophorus electricus* (D) ATPases; (B,E) patterns of labelling with [^{14}C]PL I of pig kidney (B) and *Electrophorus electricus* (E) Na^+,K^+-ATPases; (C,F) patterns of labelling with [^{3}H]PL II of pig kidney (C) and *Electrophorus electricus* (F) Na^+,K^+-ATPases.

were labelled, confirming the results obtained with PL I and PL II. These labelling patterns differ from that obtained with [^{3}H]adamantane diazirine [20] which did not show a significant labelling of the β sialoglycoprotein subunit. Contradictory results were found with another small photoreactive probe, 5-[^{125}I]iodonapthyl-I-azide(INA) [19,24]. Several explanations may be advanced to explain the different results. Certain areas of the protein hydrophobic surface, where interaction with lipids is stronger, may not be accessible to small probes. Also steric hinderance may not favour the reaction with the protein surface. Moreover carbene precursors, such as adamantane diazirine, have been found to be less efficient than azido-derivatives in labelling membrane proteins [25]. The integral nature of the β subunit suggested here is consistent with its solubility in organic solvents and with the fact that when isolated in pure form it contains tightly bound phospholipids [21].

We have also found that Fa-gly binds to both subunits of the Na^+,K^+-ATPase from both sources 2.5–5-times more efficiently than Fa-G. The range of values results from the different methods used to form the lipid–protein complex and from the different

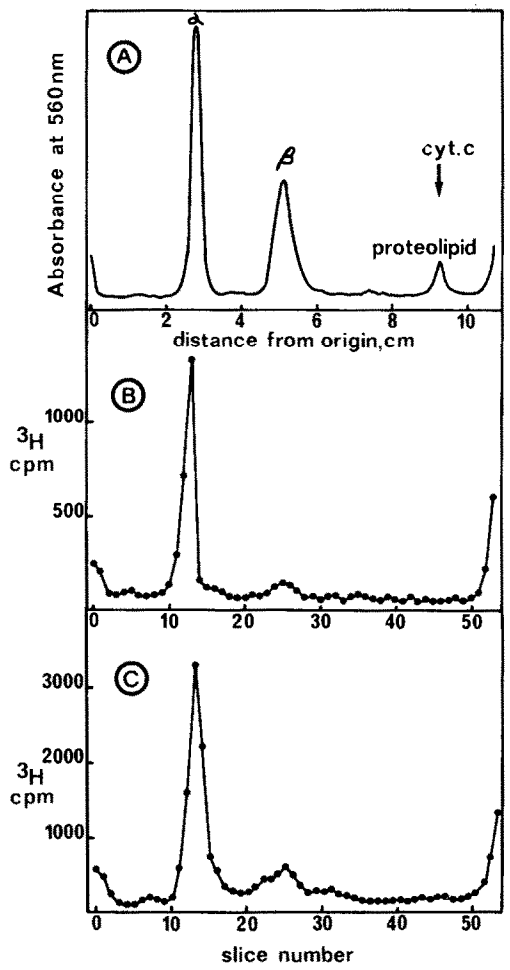

Fig.3. Labelling of *Electrophorus electricus* Na^+,K^+-ATPase with Fa-G and Fa-gly: (A) densitometric trace and distribution of radioactivity of Fa-gly (B) and Fa-G (C) of a SDS–polyacrylamide gel of the ATPase.

detergent used. The major difference between the 2 probes is represented by the negative charge associated with the head-group of Fa-gly. This result may indicate that the Na^+,K^+-ATPase interacts more strongly with negatively charged lipids and agrees with [17] using spin-labelled lipids and with the finding that negative lipids are required for maximal activation of this enzyme [22,23].

None of the labels used here was found associated with the proteolipid, which has been suggested to be part of the isolated Na^+,K^+-ATPase [5,6]. While little if any proteolipid appears, from the Coomassie blue staining pattern of the gels, to be present with our pig kidney preparations, this component is clearly present in the Na^+,K^+-ATPase isolated from the electric organ of *Electrophorus electricus*. Its electrophoretic mobility is very similar to that of cytochrome c (see fig.2D,3A). No labelling of this component was found in [19] while in [20] a peak of radioactivity was found on the gel at a position corresponding to M_r 16 000. One possible explanation for this lack of labelling is that the proteolipid, if part of the Na^+,K^+-ATPase, is shielded from contact with lipids by the other subunits.

This report indicates that both the α and β subunits of the Na^+,K^+-ATPase contribute to the protein surface of its hydrophobic sector. A larger portion seems to be contributed by the α subunit. Its interaction with negatively charged lipids appears to be stronger than that with neutral lipids. It will be interesting, when the amino acid sequence of this protein is available, to identify with these probes the amino acid stretches which are in contact with lipids.

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