ELSEVIER

Biochimica et Biophysica Acta 1233 (1995) 175–184

BBA Biochimica et Biophysica Acta

Determination of the sidedness of the carboxy-terminus of the Na^+/K^+-ATPase α-subunit using lactoperoxidase iodination

Natalia M. Vladimirova [a], Natalia A. Potapenko [a], George Sachs [b,*], Nikolai N. Modyanov [a,c,1]

[a] *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117871, Moscow, Russian Federation*
[b] *UCLA and the Wadsworth VA Medical Center, Building 113, Room 326, Los Angeles, CA 90073, USA*
[c] *Department of Pharmacology, Medical College of Ohio, Toledo, OH, USA*

Received 23 June 1994; accepted 4 October 1994

Abstract

The orientation of the carboxy-terminal pair of tyrosines of the Na^+/K^+-ATPase α-subunit with respect to the plane of the plasma membrane was determined. The approach was based on lactoperoxidase-catalysed radioiodination of the tyrosine residues accessible on the surface of the enzyme molecule in intact cells of a pig kidney embryonic cell line and those accessible in a broken plasma membrane fraction and in isolated membrane-bound Na^+/K^+-ATPase. The labeled α-subunit was isolated by SDS gel electrophoresis followed by electroblotting. Then the COOH-terminal amino acids were hydrolyzed by carboxypeptidases B and Y. Radioactivity and quantitative analysis of the protein and released amino acids showed that the COOH-terminal tyrosine residues of the α-subunit were only accessible to modification only when lactoperoxidase had access to the inner side of the plasma membrane. Therefore, the COOH-terminus of the Na^+/K^+-ATPase α-subunit is located on the cytoplasmic surface of the pump molecule and its polypeptide chain must have an even number of transmembrane segments.

Keywords: ATPase, Na^+/K^+-; Membrane topology; Iodination

1. Introduction

The Na^+/K^+-ATPase is the enzyme responsible for active transport of sodium and potassium ions across the plasma membrane of animal cells. The enzyme molecule consists of two subunits found in equimolar ratio. The catalytic α-subunit (ca. 112 kDa) contains all known functional sites of the ion pump, such as binding sites for ATP, cations and specific inhibitors. The glycosylated β-subunit (the protein moiety ca. 35 kDa) is required for assembling the mature Na^+/K^+-ATPase. Current ideas on basic processes underlying the active ion transport are deficient in molecular detail, in part due to the lack of reliable data on the spatial aspects of protein structure (for recent reviews, see [1,2]).

One step towards describing the spatial organization of the Na^+/K^+-ATPase is to elucidate the transmembrane arrangement of the subunits. It is generally accepted that the β-subunit spans the membrane only once, and that its large COOH-terminal part forms the single extracellular domain [2,3]. The description of the transmembrane organization of the catalytic α-subunit has been difficult. There is much evidence that most of protein mass including the N-terminus is exposed to cytoplasm and that the N-terminal half of its polypeptide chain crosses the membrane four times [3,4]. In contrast, the transmembrane folding of the C-terminal half has been the subject of considerable controversy. The prediction of the α-subunit transmembrane organization by hydropathy analysis is ambiguous for the C-terminal half of the protein. Several topological models with six [5], seven [6], eight [7], or ten [8] transmembrane segments have been proposed, based on such analyses. They differ in the number (two, three, four or six) and location of the membrane-spanning segments within the

Abbreviations: Na^+/K^+-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.37); cp A, cp B, cp Y, carboxypeptidase A, carboxypeptidase B, carboxypeptidase Y; DNS-, dansyl-, 5-dimethylaminonaphthalene-1-sulfonyl-; LPO, lactoperoxidase; PBS, phosphate-buffered saline; PKE cells, pig kidney embryonic cells; SDS, sodium dodecyl sulfate; Immobilon membranes, polyvinylidene difluoride (PVDF) membranes; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

* Corresponding author. Fax: +1 (310) 3129478.

[1] Present address: Department of Pharmacology, School of Medicine, University of Ohio, Toledo, OH, USA.

SSDI 0005-2736(94)00247-9

C-terminal half of the polypeptide chain and therefore some differ in the predicted orientation of the COOH-terminus.

Independent techniques to determine chain folding have often given different results for the C-terminal half of the α-subunit. The existence of three transmembrane segments in the C-terminal part of the α-subunit was postulated based on mapping of the exposed domains by sequencing the peptides released from the membrane by trypsinolysis of the native Na^+/K^+-ATPase [9]. Photolabeling of the membrane-bound Na^+/K^+-ATPase with hydrophobic reagents gave five regions of modification within that part of the α-subunit sequence, four of which were designated as intramembranous [3,10,11]. Attempts to probe the sidedness of the COOH-terminal fragment of the α-subunit by peptide-directed antibodies led to varying conclusions in different laboratories, both extracellular and cytoplasmic [12–15] orientations being proposed. For example, probing the sidedness of the COOH-terminus of the α-subunit by sequence-specific antibodies against synthetic peptides produced conflicting results. This region in mammalian enzymes has the following sequence: (991)IFVY-DEVRKLIIRRRPGGWVEKETYY(1016) [6,7]. The COOH-terminus of the α-subunit was thought to be detected on the outer surface of viable cells using antibodies against peptide 999–1008 [12]. On the other hand, the interaction of right-side-out vesicles with antibodies to peptides 991–1005 and 1005–1016 suggested the localization of the α-subunit COOH-terminus to the cytoplasmic side of the enzyme [14,15]. Although apparently providing different results, these antibody studies do show that the C-terminal region of the molecule is accessible in the membrane-bound form. This suggested that iodination of the exposed C-terminal tyrosines would be successful as a method for resolving the location of the C-terminal amino acids with respect to the plane of the membrane. The transmembrane topology of the α-subunit COOH-terminal part, crucial for the formation of the cation binding sites [8,16], is still controversial, and more than one technique needs to be applied to this problem.

We describe here a biochemical approach to the spatial location of the COOH-terminus of the Na^+/K^+-ATPase α-subunit. This was used successfully for analysis of the orientation of the C-terminal tyrosines of the gastric H^+/K^+-ATPase obtained as intact inside out vesicles [17]. The method compares the extent of the LPO-catalyzed radioiodination of the two C-terminal tyrosine residues exposed on the surface of the enzyme molecule in intact cells or exposed in broken membrane preparations or in purified, membrane bound enzyme, using iodination and carboxypeptidase digestion [20]. This method can, in principle, be applied to any integral membrane protein provided there are exposed C-terminal tyrosines. It is shown that the COOH-terminal tyrosine residues of the α-subunit are accessible to modification only when LPO has access to the inner side of the plasma membrane as is the case in the membrane preparations but not in intact cells. Thus, this method confirms that the COOH-terminus of the α-subunit is located on the cytoplasmic surface of the pump molecule, and the polypeptide chain must have an even number of transmembrane segments.

2. Experimental procedures

2.1. Materials and reagents

PBS, Trypan blue were purchased from Gibco, UK. Polyvinylpyrrolidone-40, lactoperoxidase from bovine milk (activity: 80–100 units per mg protein), DNS-amino acids, 3-iodo-L-tyrosine, CAPS, α-D(+)-glucose were from Sigma, USA. Polyvinylidene difluoride (PVDF) membranes (immobilon transfer), 0.45 μm pore size, were obtained from Millipore, USA. Hybridoma isotyping Kit (mouse), carboxypeptidase A from bovine pancreas (activity 55 units per mg protein), carboxypeptidase Y from yeast (activity 132 units per mg protein), carboxypeptidase B from porcine pancreas (activity 460 units per mg protein), and glucose oxidase (250 units per mg dry weight) were products of Calbiochem, USA. Tween-20 was from Ferak, Germany. 5-Dimethylaminonaphthalene-1-sulfonyl chloride was from Fluka, Switzerland. Nitrocellulose membrane, 0.45 μm pore size, and reagents for electrophoresis were from Bio-Rad, USA. SDS was obtained from Bio-Rad Laboratories and twice recrystallized from 95% methanol. Hyperfilm-βmax was from Amersham, UK. Low molecular weight (LMW) calibration Kit was from Pharmacia, Sweden. Pig kidney embryonic (PKE) cell line was donated by the Institute of Experimental Veterinary, Moscow, Russian Federation. $Na^{125}I$ (1 mCi/3 μl 0.1 M NaOH) was from Izotop, Russian Federation. Other reagents were of the highest available purity.

Pig kidney embryonic (PKE) cells

Viable cells were removed from the surface of culture flasks by sterile Versene solution (0.02% EDTA, 0.15 M NaCl, pH 7.4). Soluble proteins were removed from the cells by washing in isotonic PBS buffer (40–50 fold volume) with the following centrifugation (300 × g, 7 min, 40°C). The cells were washed three to four times, while the supernatant absorption decreased up to 0.1 at 280 nm. Before the last centrifugation the cell suspension was divided into two equal parts. The viability of cells was determined by Trypan blue exclusion.

2.2. Isolation of the plasma membrane fraction

The cells ($(1–1.2) \cdot 10^7$) were suspended in 0.5 ml of PBS, or in 0.5 ml H_2O, containing 3 mM diisopropyl fluorophosphate. The cell disruption was performed by addition of Triton X-100 into PBS up to 0.5% [18], with following keeping the mixture at room temperature for 30

min or by hypotonic shock and three freezing-thawing cycles. Large debris and nuclei were removed from the cell lysate by centrifugation ($1000 \times g$, 10 min, 40°C). The supernatant was diluted up to 13 ml by cold PBS and centrifuged ($230\,000 \times g$, 60 min, 40°C) and this wash procedure was repeated twice. The final pellet enriched with the plasma membrane was immediately used for iodination.

2.3. The membrane-bound Na^+/K^+-ATPase

The enzyme from pig kidney outer medulla was isolated according to the modified Jorgensen procedure, with centrifugation in a glycerol density gradient [19]. The specific activity of the enzyme preparation was about 25–30 μmol ATP hydrolysed per mg protein per min at pH 7.5 (37°C). The protein concentration was estimated by quantitative amino acid analysis.

2.4. Lactoperoxidase-catalysed iodination

In a typical experiment the samples of PKE cells ($(1–1.2) \cdot 10^7$), plasma membrane fraction (obtained from $(1–1.2) \cdot 10^7$ cells), or membrane-bound Na^+/K^+-ATPase (150 μg, 1 nmol) were suspended in 150 μl of PBS, pH 7.2. LPO (5 μg in 5 μl of PBS) and $Na^{125}I$ (1 mCi, 0.46 nmol in 3 μl of 0.1 M NaOH) were successively added. In analytical experiments the labeling reaction was carried out in two ways. (a) The reaction was initiated by addition of 2.5 μl 0.03% H_2O_2 in the dark at room temperature (25°C) upon stirring [20]. The peroxide solution (30%) was diluted by cold PBS just prior to use. The concentration was calculated using the coefficient of millimolar extinction of 72 at 230 nm. After 5 min the second equal pulse of H_2O_2 was added up to the final concentration of 0.001%. Following 5 min incubation the labeling reaction was terminated by dilution with ice-cold PBS (100-fold volume). (b) For initiation of the reaction we used also the peroxide generating system, employing glucose and glucose oxidase [21]. Glucose oxidase (1.8 μg in 10 μl of PBS) and α-D(+)-glucose (1.07 μg in 10 μl of PBS) were successively added to the reaction mixture. The reaction was carried out in the dark at room temperature (25°C) upon stirring. Following 20 min incubation the labeling reaction was terminated by dilution with ice-cold PBS (100-fold volume). In preparative experiments we used the technique described in (a).

The labeled cells were then spun down at $300 \times g$ for 5 min (4°C) and washed twice with 100-fold volume of cold PBS. The supernatants were discarded. The cell viability after iodination and washing was determined by Trypan blue exclusion. The labeled cells were immediately used for obtaining a plasma membrane fraction as described above.

In all other experiments the excess of radioactive label was removed from iodinated preparations by three washes in cold PBS (13 ml) and centrifugation ($230\,000 \times g$, 60 min, 4°C). After careful removal of supernatants the final pellets were homogenized in 200 μl of 0.1 M sodium phosphate buffer (pH 7.2), containing diisopropyl fluorophosphate. Then SDS, β-mercaptoethanol and EDTA were added up to concentrations of 5%, 2.5% and 1 mM, respectively.

The experiments on labeling of the membrane-bound Na^+/K^+-ATPase with excess iodide were performed as follows. The protein samples (50 μg, 335 pmol and 100 μg, 670 pmol) were suspended in 85 ml and 170 μl of PBS, respectively. LP0 (5 μg in 5 μl of PBS), KI (15 nmol in 5 μl of PBS) and $Na^{125}I$ (0.08 mCi, 37 pmol in 5 μl of 0.008 M NaOH) were successively added to each sample. The labeling reaction was initiated by addition of 1.5 μl of 0.03% H_2O_2 in the dark at room temperature (25°C) upon stirring. The reaction proceeded by adding two equal pulses of H_2O_2 with 5-min intervals. After the last 5-min incubation the labeling reaction was terminated and the excess of label was removed as described above. The labeling reaction was carried out also with glucose/glucose oxidase peroxide generating system as described above. The control experiments were performed under the same conditions, but LPO, or peroxide, or glucose were excluded from the reaction mixture. Incorporation of the label into the samples was determined using a 1282 Compugamma counter (LKB, Sweden).

2.5. Electrophoresis and electroblotting

Electrophoresis was performed as previously described [22,23]. Gradient slab gels (4–15% acrylamide, 2.7 mm thickness) were run for 16 h at 25 mA (constant current). We found the following conditions to be important: (a) diisopropyl fluorophosphate was used to prevent unspecific proteolysis; (b) the sample was heated at 37°C for 15 min before electrophoresis; (c) electrophoresis was performed at 15°C. To locate the protein bands, the gel was stained with Coomassie brilliant blue in 12.5% trichloroacetic acid and subjected to autoradiography (using an intensifier screen, −70°C).

Electroblotting on to immobilon or nitrocellulose membranes was done in a box-type transfer apparatus filled with 10 mM CAPS buffer (pH 11), containing 10% methanol for 2 h at 15°C and 200 mA (constant current). The gel was placed onto 7 layers of Whatman 3 mm paper prewetted with transfer buffer and covered sequentially by immobilon, nitrocellulose and 7 layers of Whatman paper all equilibrated with transfer buffer. After transfer the immobilon membrane was thoroughly washed with methanol, several times with milli-Q water, finally with 30% methanol and air-dried.

The protein bands on immobilon and gel were detected by staining with Coomassie brilliant blue and by autoradiography of control strips. For precise identification on immobilon of the adjacent protein bands upon separation

of the complex mixture of plasma membrane proteins, the visualization procedure without staining [24] was also used. It was found that the protein bands dried more slowly, while the immobilon membrane was being air-dried after 20–30% methanol washing. They became visible with the naked eye as wet bands on the dry background in reflected day light or as bands darker than the background in UV-light at 366 nm. Bands will disappear with drying but can be revisualized at any time by rewetting in 20–30% methanol. Narrow sections of 1–1.5 mm wide, corresponding to visible protein bands were cut out from each strip and counted.

To identify the α-subunit band, the nitrocellulose membrane was washed with water, then was blocked with an 1% ovalbumin solution in PBS (1 h at room temperature or overnight at 4°C) and then was incubated (16 h at 4°C) with monoclonal antibodies α-p999 (which were raised against the synthetic peptide, corresponding to the 999–1008 fragment of the pig kidney Na^+/K^+-ATPase α-subunit) [12], dissolved in PBS, containing 0.02% Tween and 1% ovalbumin. After six 10-min washes with 0.02% Tween in PBS, bound antibodies were detected with goat anti-mouse IgG-peroxidase conjugate in the presence of 0.049% 4-chloro-1-naphthol and 0.03% H_2O_2.

2.6. N-terminal sequence analysis of isolated samples

5 Cycles of Edman degradation were performed using a 470 gas-phase sequencer with an on-line 120A phenylthiohydantoin analyzer (Applied Biosystems) as previously detailed [25].

2.7. Determination of COOH-terminal amino acids by carboxypeptidases B and Y

Treatment of the immobilon membrane with polyvinylpyrrolidone (PVP-40) was used to prevent unspecific adsorption of the carboxypeptidases. The immobilon strips containing the protein were crushed to powder and wetted with 20 μl of methanol. The excess of methanol was removed with a capillary pipette. The powder was incubated in 100 ml of 0.5% PVP-40 dissolved in 100 mM acetic acid for 30 min at 37°C. The excess of PVP-40 was removed by extensive washing with milli-Q water (at least five changes).

COOH-terminal amino acids were cleaved by step-by-step hydrolysis by carboxypeptidases B and Y. Sequential digestion using the two carboxypeptidases, optimises C-terminal cleavage since the two enzymes have differing amino acid selectivity and is based on the C-terminal sequence of the enzyme and the final protocol was worked out using various enzymes and conditions of digestion (see Results).

The immobilon powder was twice washed with 0.1 M NH_4HCO_3 (pH 7.5), then cp B (0.5 μg in 200 μl of the same buffer) was added to initiate C-terminal amino acid hydrolysis. After 1 h incubation at 37°C, the second equal portion of cp B and 1 μg of cp Y in 5 μl of the same buffer were added. Following 1 h, the pH of the reaction mixture was decreased to 5.7 by 50 μl 100 mM CH_3COOH. Then 1 μg of cp Y in 5 μl 0.1 M NH_4HCO_3 (pH 5.7) was introduced. The mixture was incubated for 30 min at 37°C. The supernatant was collected by centrifugation. Residual amounts of released amino acids were eluted from the immobilon with 100 μl 0.1 M NH_4HCO_3 (pH 5.7), 100 μl water, and finally with 100 μl methanol, to make the recovery as quantitative as possible.

The amount of protein on immobilon before and after treatment with carboxypeptidases was determined by quantitative amino acid analysis. After counting, the rest of immobilon powder was used for N-terminal sequencing. The eluates from the immobilon powder were combined with the supernatant produced during digestion, dried by a vacuum pump, evaporated twice with milli-Q water and counted. Quantitative analysis of the released amino acids as phenylthiocarbamoyl-derivatives was done on an amino acid analyzer (Millipore-Waters, 680 (USA); column Pico-Tag 3.9 × 150 mm (Pico-Tag manual).

Qualitative analysis of released amino acids as DNS-derivatives was performed by two-dimensional thin-layer chromatography. The dried samples of released amino acids were treated by 10 μl of 0.1 M $NaHCO_3$ (pH 9.5) and 10 μl of DNS-Cl in acetone (2 mg/ml) for 60 min at 45°C, then the solution was evaporated. For identification of the dansylated amino acids we have used the scheme described in [26] and [27], using two-dimensional thin-layer chromatography on silica gel plates (6 × 6 cm) [27]. The composition of the chromatographic systems is given in the legend to Fig. 2. The plates were examined under an UV-lamp at 366 nm. The identification of released amino acids as dansyl-derivatives was done by comparing their mobilities with marker mixtures of dansyl amino acids [27]. These were prepared either from the solid compounds, or by labeling mixtures of amino acids as described before [28]. Samples of the single labeled derivatives of histidine, lysine, and tyrosine were prepared separately and added to the mixture. These samples were made by labeling a 2-fold excess of amino acid with dansyl chloride [28].

The iodinated tyrosine residues were identified as DNS derivatives by autoradiography. Silica gel from the spots of di- and mono-dansyl-^{125}I-tyrosine was collected and counted.

3. Results and discussion

3.1. Validation of labeling method

These experiments were designed to determine whether the C-terminal tyrosines were accessible to LPO catalysed iodination. Purified membrane-bound Na^+/K^+-ATPase

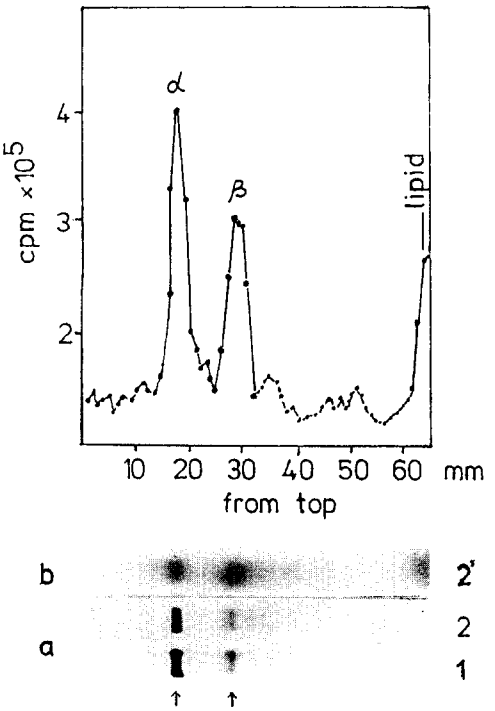

Fig. 1. Labeling of the purified membrane bound Na^+/K^+-ATPase with lactoperoxidase/^{125}I. (a) SDS-gel electrophoretograms of the Na^+/K^+-ATPase before (1) and after (2, 2') iodination. Lanes 1 and 2 were visualized by Coomassie brilliant blue staining, lane 2' by autoradiography (15 min exposure). (b) Representative graph of the amount of radioactivity in 1 mm slices from gradient gel of Na^+/K^+-ATPase labeled with ^{125}I. The positions of the α- and β-subunits are indicated.

was used in order to work out conditions for surface-specific labeling. In the first set of experiments the extent of modification was limited to a small part of the iodinatable sites [20]. Hence 0.5 nmol NaI per nmol of protein was added for labeling the purified Na^+/K^+-ATPase. The final concentration of iodide in the reaction mixture was $2.7 \cdot 10^{-6}$ M. Various temperature and time parameters of iodination were tested and the conditions described in the experimental part were found to provide the highest incorporation of the label into proteins (up to 24% of $^{125}I^-$ added). The use of either peroxide or the glucose/glucose oxidase peroxide generating system was equally effective. The absence of unspecific labeling was demonstrated in control experiments, where LPO or peroxide (or glucose) were omitted from the reaction mixture. In these control experiments incorporation of the label into the Na^+/K^+-ATPase did not exceed the background level (data not shown).

The results of iodination of the purified membrane bound enzyme are shown in Fig. 1. Iodination did not modify the mobility of the two subunits of the enzyme as shown in Fig. 1a. Measurement of radioactivity of 1 mm slices from gel showed that the α-subunit contained about 38% of covalent-bound label, the β-subunit about 32% (Fig. 1c). The rest was detected in low molecular weight fraction (contaminating proteins and lipids). When the protein bands were transferred from gel onto the immobilon membrane by electroblotting and autoradiography carried out, the location of the bands corresponded well to the data obtained by slicing the gel. The purity of the iodinated α-subunit was verified by NH_2-terminal sequencing. The label incorporation was on the average 0.14 pmol per 100 pmol of the protein determined by amino acid analysis.

3.2. Identification and location of C-terminal label

The COOH-terminal sequence of the pig kidney α-subunit includes the following amino acid sequence: (1010) Val-Glu-Lys-Glu-Thr-Tyr-Tyr (1016) [29]. It is known that carboxypeptidase A (at pH 8.5) rapidly releases Tyr, Thr, Val, slowly releases Lys, very slowly releases Glu, does not release Arg and Pro [30]; carboxypeptidase B (at pH 8.5) most rapidly releases Arg and Lys, but also removes nonbasic amino acid residues [30], sometimes at a rather high rate [31]; carboxypeptidase Y releases most amino acid residues, including Pro and acts more strongly on proteins than does carboxypeptidase A, the optimum for the release of the C-terminal basic and neutral amino acids is around 7, and that for the release of the C-terminal acidic amino acids is at, or below, pH 5.5 [32].

Carboxypeptidases A, B and Y were then tested for release of the C-terminal amino acids from the α-subunit immobilized on immobilon. Appreciable cleavage of the protein was found only after preliminary treatment of the immobilon membrane with polyvinylpyrrolidone-40 that prevented unspecific adsorption of the carboxypeptidase [33]. However, the extent of digestion of the immobilized protein was not satisfactory, when the carboxypeptidases were used individually.

Better cleavage of the α-subunit on immobilon was achieved by the step-by-step hydrolysis with cp B and cp Y with a change in pH of the reaction mixture from 7.5 to 5.7 (see Experimental procedures). Under these conditions about 23% of the initial label was moved from the membrane to the supernatant. The rest of the immobilized protein was then subjected to N-terminal sequence analysis. No additional amino acids in the amount exceeding the level of the background were detected. This indicates an absence of unspecific cleavage of the inner regions of the α-subunit chain by carboxypeptidase digestion, therefore the released amino acids derive from the C-terminal region of the peptide.

The following COOH-terminal amino acids were found by quantitative analysis of phenylthiocarbamoyl-derivatives: Tyr, 80–105 pmol (cp B, 1 h, pH 7.5); Tyr, 115–157 pmol, Thr, 42–65 pmol (cp B, 2 h, and cp Y, 1 h, pH 7.5); Tyr, 124–168 pmol, Thr, 60–75 pmol and Glu, 30–37 pmol (cp B, 2 h, cp Y, 1 h, pH 7.5 and cp Y, 30 min, pH 5.7) per 100 pmol of the protein. The set of released amino acids and kinetics of their hydrolysis corresponds to the COOH-terminal sequence of the α-subunit [6].

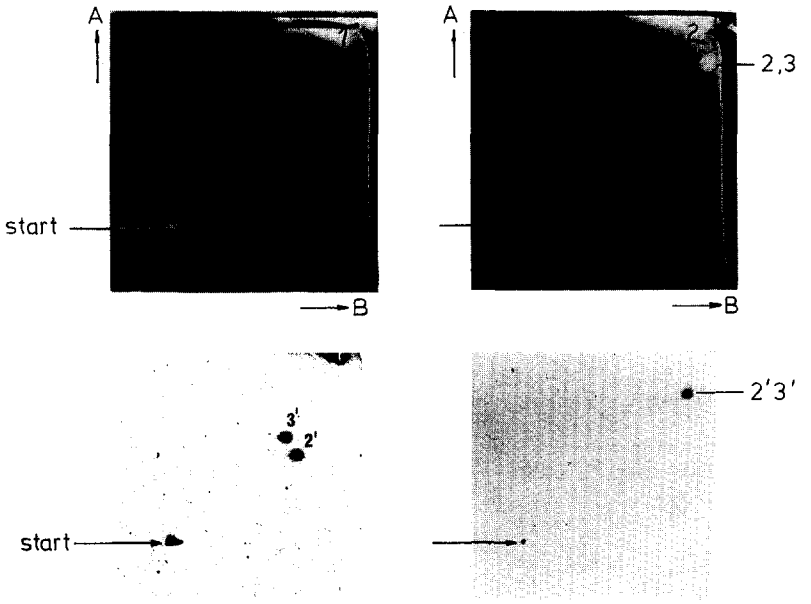

Fig. 2. Analysis of amino acids released from the Na^+/K^+-ATPase α-subunit by carboxypeptidases B and Y. Two-dimensional chromatography of dansylated derivatives on silica gel plates. Left plates: 2 chromatography systems: acetone/isopropanol/25% NH_4OH (9:7:0.5, v/v) and (9:7:0.7, v/v) (direction A); then chloroform/benzyl alcohol/ethyl acetate/acetic acid (6:4:5:0.2, v/v) (direction B). Right plates, 2 chromatography systems: acetone/isopropanol/25% NH_4OH (9:7:2, v/v) and (9:7:3, v/v) (direction A); chloroform/benzyl alcohol/ethyl acetate/acetic acid (5:4:1:1, v/v) (and then direction B). The fluorescence of the dansylated samples under ultraviolet light (at 366 nm) was determined (upper plates). Spots on the plates correspond to: 1, DNS-Thr; 2, mono-(N)-DNS-Tyr; 3, di-DNS-Tyr; 4, DNS-Glu. The iodinated amino acids were detected by 3-day exposure autoradiography (lower plates). Spots 2′ and 3′ correspond to mono-(N)-DNS-^{125}I-Tyr and di-(N,O)-DNS-^{125}I-Tyr, respectively.

That the radioactivity removed from the protein corresponded to iodinated tyrosine was shown by analysis of dansyl-derivatives of cleaved amino acids by two-dimensional thin-layer chromatography as shown in Fig. 2. The spots of DNS-Thr, DNS-Glu, N-DNS-Tyr and N,O-di-DNS-Tyr were detected in UV-light. The autoradiograpy revealed two spots which coincided with the location of mono- and di-dansyl-tyrosine. Practically all the radioactivity in the chromatogram was found in these spots by counting (data not shown). The total incorporation of the radioactive label into COOH-terminal tyrosines comprised about 30% of the total radioactivity incorporated into the α-subunit.

Table 1a presents data from four independent experiments on this limited iodination of the membrane-bound Na^+/K^+-ATPase followed by selective digestion of the isolated α-subunit by carboxypeptidases B and Y. In this set of experiments only a very small fraction of tyrosines in the α-subunit was labeled, and this raises the possibility that an unrepresentative population consisting of denatured

Table 1
Labeling of the α-subunit within the membrane-bound Na^+/K^+-ATPase with lactoperoxidase/^{125}I at molar ratio iodide to protein: (a) 0.5:1, (b) 22.5:1, (c) 45:1

α-subunit band on PVDF membrane (cpm) [a]		Iodine incorporation (pmol) [a]	cp B + cp Y treatment			COOH-terminus label (%)
			released label (cpm) [a]	PVDF membrane (cpm) [a]	Released tyrosine (pmol) [a]	
(a)	$6.72 \cdot 10^5$	0.129	$1.61 \cdot 10^5$	$5.05 \cdot 10^5$	152	32
	$8.01 \cdot 10^5$	0.154	$1.51 \cdot 10^5$	$6.44 \cdot 10^5$	136	28
	$7.30 \cdot 10^5$	0.140	$1.90 \cdot 10^5$	$5.33 \cdot 10^5$	168	31
	$7.55 \cdot 10^5$	0.145	$1.88 \cdot 10^5$	$5.70 \cdot 10^5$	164	30
(b)	$5.46 \cdot 10^5$	52	$1.32 \cdot 10^5$	$3.36 \cdot 10^5$	146	33
	$4.92 \cdot 10^5$	46	$1.08 \cdot 10^5$	$3.80 \cdot 10^5$	150	29
	$4.77 \cdot 10^5$	44	$1.14 \cdot 10^5$	$3.61 \cdot 10^5$	143	34
(c)	$1.93 \cdot 10^6$	183	$0.40 \cdot 10^6$	$1.50 \cdot 10^6$	132	32
	$2.12 \cdot 10^6$	200	$0.36 \cdot 10^6$	$1.72 \cdot 10^6$	124	27
	$2.06 \cdot 10^6$	195	$0.47 \cdot 10^6$	$1.55 \cdot 10^6$	137	34

[a] Data given per 100 pmol of protein. The radioactivity of 1 pmol iodide: (a) $5.21 \cdot 10^6$ cpm, (b) and (c) $1.06 \cdot 10^4$ cpm. The amount of the released tyrosine was determined by amino acid analysis. Percentage of COOH-terminus label was calculated taking into account the yield of carboxypeptidase hydrolysis and is the fraction of total label found in the C-terminal tyrosines.

molecules of the Na^+/K^+-ATPase preparation might be a preferential target for LPO-mediated iodination. To answer this question two additional experiments on the labeling with the excess of iodide were performed (Table 1b and c).

It was shown that up to two atoms of iodine could be incorporated into the α-subunit molecule, when molar ratio of reagent/protein was increased to 45:1. Even in this case, unspecific labeling was not detected in control experiments without LPO or peroxide. About 30% of the label was again found in C-terminal tyrosine residues of the α-subunit. The extent of modification of the particular residues in these experiments was about 60%, if one tyrosine was iodinated, or 30% if both were labeled. It is improbable that this high specific activity preparation contains this level of denatured enzyme.

Incidentally it was found that the LPO-mediated iodination of tyrosine residues located on the surface of the Na^+/K^+-ATPase molecule led to substantial inhibition of ATP-hydrolysing activity (data not shown). The effect was not detected, when LPO or peroxide (or glucose) were omitted from the reaction mixture. The iodide concentration used here ($7.8 \cdot 10^{-5}$ M) is much higher than that recommended for vectorial labeling [20] and therefore cannot be used in experiments with viable cells.

Thus, the experiments with the membrane-bound Na^+/K^+-ATPase show clearly that one or both COOH-terminal tyrosine residues of the α-subunit are substrates for LPO-catalyzed iodination and further that the carboxypeptidase digestion releases only the C-terminal amino acids. Therefore this method can be used to determine their membrane orientation.

3.3. Relative labeling of intact cells and plasma membrane fraction

To establish orientation of the α-subunit C-terminal tyrosine residues relative to the plasma membrane, a series of experiments on vectorial labeling of intact cells of the pig kidney embryonic (PKE) cell line were performed along with modification of the plasma membrane fraction isolated from the same cells. If these residues are exposed on the cell outer surface, their level of labeling should then be almost the same in both types of experiments. Conversely, if incorporation of the label increases significantly with production of the open plasma membrane fragments, the COOH-terminus is cytoplasmic.

The viability of the cells being labeled must be maintained as close to 100% as possible, because labeling of dead cells will not be restricted to externally accessible sites. In our case, the viability of cells, as determined by Trypan blue exclusion, was about 94-95%, and did not change after limited iodination. Equal portions of cells were used for direct radioiodination or for preparation of the plasma membrane fraction using either hypotonic shock or treatment by Triton X-100.

Labeling of the intact cells and plasma membrane fraction was performed under the conditions developed in the first set of experiments for the isolated Na^+/K^+-ATPase. The labeled cells were immediately used for plasma membrane isolation. The incorporation of the label in Na^+/K^+-ATPase α-subunit within the samples prepared by both methods of cell membrane disruption was shown to be almost equal (Table 2). The incorporation of the label into proteins comprised about 14% and 0.4% of $^{125}I^-$ added for plasma membrane fraction and cells, respectively. Without LPO or peroxide, unspecific labeling did not exceed the level of the background.

To identify the labeled α-subunit, the plasma membrane proteins were separated by SDS gel electrophoresis and electroblotted. The isolated samples of the plasma membrane according to Coomassie brilliant blue staining had a very similar protein composition, but differed drastically in extent of iodination (Fig. 3).

The Na^+/K^+-ATPase α-subunit was identified, within the zone of 100 kDa proteins among the several bands resolved by Coomassie brilliant blue, by means of monoclonal antibody α-p999 against a synthetic peptide, corresponding to positions 999–1008 fragment of pig kidney

Table 2
Labeling of the Na^+/K^+-ATPase α-subunit within the plasma membrane fraction and viable cells with lactoperoxidase/^{125}I

	α-subunit band on PVDF membrane (cpm) [a]	cp B + cp Y treatment			COOH-terminus label (%)
		released label (cpm) [a]	PVDF membrane (cpm) [a]	released tyrosine (pmol) [a]	
Plasma membrane fraction	(1) $2.53 \cdot 10^5$	$0.40 \cdot 10^5$	$2.08 \cdot 10^5$	122	27
	(2) $2.27 \cdot 10^5$	$0.41 \cdot 10^5$	$1.80 \cdot 10^5$	130	28
	(3) $2.39 \cdot 10^5$	$0.48 \cdot 10^5$	$1.85 \cdot 10^5$	141	28
Whole cells	(4) $1.52 \cdot 10^4$	$0.38 \cdot 10^4$	$1.10 \cdot 10^4$	140	35
	(5) $1.40 \cdot 10^4$	$0.29 \cdot 10^4$	$1.06 \cdot 10^4$	135	33
	(6) $1.63 \cdot 10^4$	$0.33 \cdot 10^4$	$1.34 \cdot 10^4$	121	33

Plasma membranes were obtained: (1,4) by treatment with Triton X-100; (2,3,5,6) by hypotonic shock. [a] Data given per 100 pmol of protein. The amount of the released tyrosine was determined by amino acid analysis. Percentage of COOH-terminus label was calculated taking into account the yield of carboxypeptidase hydrolysis.

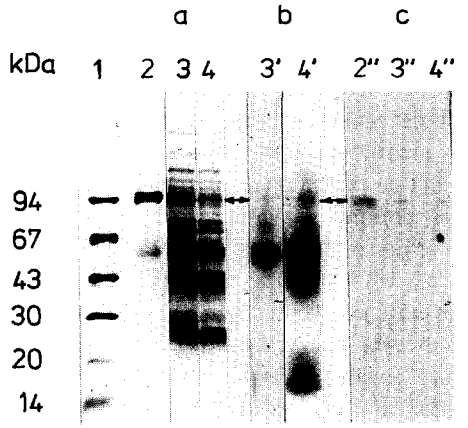

Fig. 3. Iodination of PKE-cell plasma membrane and isolated plasma membrane fraction with lactoperoxidase. SDS-gel electrophoretograms of the samples of plasma membrane iodinated before (lanes 3, 3′ and 3″) and after (lanes 4, 4′ and 4″) cell disruption. Lanes 2 and 2″, the control sample (Na^+/K^+-ATPase). Lanes 1 (Kit), 2, 3 and 4 were visualized by Coomassie brilliant blue staining. Lanes 3′ and 4′ by autoradiography (16 h and 15 min exposure, correspondingly). Lanes 2″, 3″ and 4″, by immunostaining with monoclonal antibodies α-p999. The position of the α-subunit band cut out from immobilon is indicated by arrows.

α-subunit [29] (Fig. 3c). It should be pointed that a satisfactory resolution of closely situated protein bands of this region on the immobilon membrane was achieved with the aid of the simple visualization procedure used previously for identification of Na^+/K^+-ATPase α-subunit isoforms [24,34]. As a result, narrow sections of width about 1 mm were cut out from each strip and analysed for an amino acid sequence. In one of these, as indicated in Fig. 3, only the N-terminal sequence of the α-subunit was detected.

Selective removal of COOH-terminal tyrosines from the samples of the individual α-subunit was performed by step-by-step hydrolysis by carboxipeptidases B and Y as described above. The absence of unspecific cleavage was verified by repeated N-terminal sequencing. The results of radioactive counting and quantitative determination of the protein and released amino acids obtained in three independent sets of experiments with viable cells and their plasma membrane fraction are shown in Table 2.

The comparison of these data demonstrates that the incorporation of the label into the Na^+/K^+-ATPase α-subunit in intact cells is only about 6% of that obtained upon iodination of plasma membrane fraction. With 95% of the cells excluding Trypan blue, the ratio of the α-subunit labeling within viable cells to that within plasma membrane fraction correlates well with the ratio of disrupted cells to intact ones in the cell samples used.

At the same time, the content of radioactive label in COOH-terminal tyrosines was on the average 28% and 34% of the total for plasma membrane fraction and cells, respectively. Thus, distribution of the label between COOH-terminus and other target residues of the α-subunit was found to be very similar in all experiments (Tables 1 and 2). This result can be obtained only if all the iodinatable tyrosines of the α-subunit are present on the same side of the plasma membrane.

Thus, disruption of the cells results in a drastic increase of the incorporation of the label into the Na^+/K^+-ATPase α-subunit, but does not change the actual amino acids modified. These findings lead us to conclude that upon limited enzymatic iodination of viable cells the Na^+/K^+-ATPase α-subunit can be labeled only within disrupted cells, i.e., modification of the α-subunit takes place only when lactoperoxidase has access to the inner side of the plasma membrane. Therefore, all targeted tyrosine residues (including the COOH-terminal ones) are exposed on the cytoplasmic surface of the pump molecule.

The orientation of the COOH-terminus of the Na^+/K^+-ATPase α-subunit determined in the present study is consistent with previous results [14] obtained by means of peptide-directed antibodies. A cytoplasmic location of the COOH-terminus of the α-subunit of the gastric H^+/K^+-ATPase, the most closely related to Na^+/K^+-ATPase in the family of cation pumps, was also deduced using iodination of intact, inside out vesicles [17].

3.4. Implication for topological models

The cytoplasmic orientation of the COOH-terminal end of the α-subunit indicates an even number of transmembrane segments in the polypeptide chain. The model for the α-subunit folding proposed earlier [9] assumed the existence of seven transmembrane segments. All of them were identified as lipid-contacting upon analysis of intramembrane moiety of the Na^+/K^+-ATPase by photolabeling with 3-(trifluoromethyl)-3(m-[^{125}I]iodophenyl)diazarine [3,11]. In addition the set of labeled peptides included also a fragment between Met-973 to Arg-999. Therefore, the hydrophobic portion of this peptide (Met-973 to Tyr-994) is the most probable candidate for an additional transmembrane segment. Taking this into account, a model with eight transmembrane segments for the α-subunit was proposed [11]. It is consistent with the data on affinity modification [3], hydrophobic labeling [3,11] and epitope mapping by specific antibodies [35,36].

On the other hand recent data on proteolytic digestion of tight membrane vesicles [37] and determination of sidedness of monoclonal antibody epitopes ([38,39] and Larin, Shakhparonov and Modyanov, unpublished observations) provides evidence for a more complex structural organization of the membrane domain of the Na^+/K^+-ATPase. Hydropathy plots would allow up to 12 segments in the aminoacid sequence of the Na^+/K^+-ATPase α-subunit (up to six within the COOH-terminal half) that could be considered as possible candidates for spanning the plasma membrane [8,9]. Different models for the Na^+/K^+-ATPase α-subunit folding with an even number of membrane spanning segments postulate 8 or 10 transmembrane segments [7,8,11,37]. Ten transmembrane α

helixes were proposed for the sarcoplasmic reticulum Ca^{2+}-ATPase [40,41]. Analysis of the membrane topology of the gastric H^+/K^+-ATPase by limited proteolysis and extracytoplasmic labeling with SH and K competitive photoaffinity reagents provided explicit evidence for 8 membrane spanning sequences in the α-subunit equivalent to the first 8 segments postulated for the Ca^{2+}-ATPase but does not exclude a C-terminal additional pair [42,43]. In vitro translation of putative transmembrane segments of the H^+/K^+-ATPase has shown that the two C-terminal hydrophobic sequences, H9 and H10, not detected by biochemical methods, were able to act as signal anchor and stop transfer sequences respectively, suggesting a 10 membrane segment model [44]. This model is consistent with that suggested for the Na^+/K^+-ATPase and the Ca^{2+}-ATPase of sarcoplasmic reticulum [38–40].

According to this 10 membrane segment model, of the 24 tyrosines present in the α-subunit, 12 are cytoplasmic, 9 in the membrane and 3 extra-cytoplasmic. If all targets were equally iodinated in our experiments, then only 3–7 cytoplasmic tyrosine residues (including the COOH-terminal ones) are accessible to modification. Similarly, the number of iodinatable tyrosines in the β-subunit, containing 21 such residues of which 19 are thought to be extracytoplasmic [9], is restricted to 2 to 5, since the label incorporation was about 84% of that found for the α-subunit. Determination of the location of these labeled tyrosine residues in these subunits could provide new information on the topology of the Na^+/K^+-ATPase molecule as a function of access of the iodination reaction. Of particular interest is the identification of tyrosine residue(s) involved in inhibition of the enzyme activity caused by LPO-mediated iodination.

Acknowledgements

This work was supported by Russian Foundation for Fundamental Investigations, grant 93-04-20327 and International Science Foundation and USVA SMI. We thank Elena A. Arystarkhova for participation in preliminary investigations, and Olga E. Lakhtina for preparation of the cell culture samples and monoclonal antibodies.

References

[1] Glynn, I.M. and Karlish, S.J.D. (1990) Annu. Rev. Biochem. 59, 171–205.
[2] Horisberger, J.D., Lemas, J.P., Kraehenbuhl, J.P. and Rossier, B.S. (1991) Annu. Rev. Physiol. 53, 565–584.
[3] Modyanov, N., Lutsenko, S., Chertova, E. and Efremov, R. (1991) in The Sodium Pump: Structure, Mechanism and Regulation (Kaplan, J.H. and De Weer, P., eds.), Vol. 46, pp. 99–115, Rockefeller University Press, New York.
[4] Jorgensen, P.L. and Andersen, J.P. (1988) J. Membr. Biol. 103, 95–120.
[5] Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, H., Nagano, K., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. and Numa, S. (1985) Nature 316, 733–736.
[6] Ovchinnikov, Yu., Arsenyan, S., Broude, N., Petrukhin, K., Grishin, A., Aldanova, N., Arzamazova, N., Arystarkhova, E., Melkov, A., Smirnov, Yu., Guryev, S., Monastyrskaya, G. and Modyanov, N. (1985) Proc. Acad. Sci. USSR 285, 1491–1495.
[7] Shull, G.E., Schwarts, A. and Lingrel, J.B. (1985) Nature 316, 691–695.
[8] Karlish, S.J.D., Goldshleger, R., Tal, D.M., Capasso, J.M., Hoving, S. and Stein W.D. (1992) Acta Physiol. Scand. 146, 69–76.
[9] Ovchinnikov, Yu., Arzamazov N.M., Arystarkhova E.A., Gevondyan, N.M., Aldanova, N.A. and Modyanov, N.N. (1987) FEBS Lett. 217, 269–274.
[10] Kyte, J., Xu, K. and Bayer, R. (1987) Biochemistry 26, 8350–8360.
[11] Modyanov, N., Lutsenko, S., Chertova, E., Efremov, R. and Gulyaev, D. (1992) Acta Physiol. Scand. 146, 49–58.
[12] Ovchinnikov, Yu.A., Modyanov, N.N., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Arzamazova, N.M., Aldanova, N.A., Monastyrskaya, G.S. and Sverdlov, E.D. (1986) FEBS Lett. 201, 237–245.
[13] Bayer, R. (1990) Biochemistry 29, 2251–2256.
[14] Antolovic, R., Bruller, H.-J., Bunk, S., Linder, D. and Schoner, W. (1991) Eur. J. Biochem. 199, 195–202.
[15] Thibault, D. (1993) Biochemistry 32, 2813–2821.
[16] Karlish, S.J.D., Goldshleger, R. and Stein, W.D. (1990) Proc. Natl. Acad. Sci. USA 87, 4566–4570.
[17] Scott, T., Munson, K., Modyanov, N. and Sachs, G. (1992) Biochim. Biophys. Acta 1112, 246–250.
[18] Cone, R.E. (1987) Methods Enzymol. 150, 388–399.
[19] Dzhandzhugazyan, K.N., Modyanov, N.N. and Ovchinnikov, Yu.A. (1981) Bioorganicheskaya Khimiya 7, 847–857 (in Russian).
[20] Morrison, M. (1980) Methods Enzymol. 70, 214–220.
[21] Warr, G.W. and Marchalonis, J.J. (1987) Methods Enzymol. 150, 399–418.
[22] Weber, W. and Osborn, M. (1969) J. Biol. Chem. 344, 4406–4412.
[23] Arzamazova, N.M., Arystarkhova, E.A., Shafieva, G.I., Nazimov, I.V., Aldanova, N.A. and Modyanov, N.N. (1985) Bioorganicheskaya Khimiya 11, 1598–1606 (in Russian).
[24] Reig, J. and Klein, D.C. (1988) Appl. Theor. Electrophoresis 1, 59.
[25] Levina, N.B., Slepak, V.Z., Kiselev, O.G., Shemyakin, V.V. and Chochlachev, A.A. (1989) Bioorganicheskaya Khimiya 15, 24–31 (in Russian).
[26] Gray, W.R. (1967) Methods Enzymol. 11, 139–151.
[27] Belenky, B.G., Gankina, E.S. and Nesterov, V.V. (1967) Proc. Acad. Sci. USSR 172, 91–93.
[28] Gray, W.R. (1972) Methods Enzymol. 25, 121–138.
[29] Ovchinnikov, Yu., Luneva, N., Arystarkhova, E., Gevondyan, N., Arzamazova, N., Kozhich, A., Nesmeyanov, V. and Modyanov, N. (1988) FEBS Lett. 227, 230–234.
[30] Ambler, R.P. (1967) Methods Enzymol. 11, 436–445.
[31] Guidotti, G., Hill, R.J. and Konigsberg, W. (1962) J. Biol. Chem. 237, 2184–2195.
[32] Hayashi, R. (1976) Methods Enzymol. 45, 568–587.
[33] Zvaritch, E., James, P., Vorherr, T., Falchetto, R., Modyanov, N. and Carafoli, E. (1990) Biochemistry 29, 8070–8076.
[34] Vladimirova, N.M., Potapenko, N.A., Levina, N.B. and Modyanov N.N. (1991) Biomed. Sci. 2, 68–78.
[35] Kano I., Satoh. K., Nagai. F., Ushiyama, K., Naka, T., Hara, Y. and Kano, K. (1990) Biochem. Cell. Biol. 68, 1262–1267.
[36] Arystarkhova, E., Gasparian, M., Modyanov, N. and Sweadner, K.J. (1992) J. Biol. Chem. 267, 13694–13701.
[37] Karlish, S.J.D., Goldshleger, R. and Jorgensen, P.L. (1993) J. Biol. Chem. 268, 3471–3478.
[38] Mohraz, M., Arystarchova, E. and Sweadner, K.J. (1993) J. Biol. Chem. 269, 2929–2936.

[39] Dzhandzhugazyan, K.N. and Modyanov, N.N. (1993) Biol. Chem. Hoppe-Seyler 374, 580–581.
[40] Green, N.M. and Stokes, D.L. (1992) Acta Physiol. Scand. 146, 59–68.
[41] MacLennan, D.H., Clarke, D.M., Loo, T.W. and Skerjanc, I.S. (1992) Acta Physiol. Scand. 146, 141–150.
[42] Besancon, M., Shin, J.M., Mercier, F., Munson, K., Miller M., Hersey, S. and Sachs, G. (1993) Biochemistry 32, 2345–2355.
[43] Munson, K.B., Gutierrez, C., Balaji, V.N., Ramnarayan, K. and Sachs, G. (1991) J. Biol. Chem. 266, 18976–18988.
[44] Bamberg, K. and Sachs G. (1994) J. Biol. Chem. 269, 16909–16919.