Biochimica et Biophysica Acta 817 (1985) 165–173
Elsevier

BBA 72664

Asymmetric orientation of amino groups in the α-subunit and the β-subunit of $(Na^+ + K^+)$-ATPase in tight right-side-out vesicles of basolateral membranes from outer medulla

Karine N. Dzhandzhugazyan * and Peter L. Jørgensen **

Institute of Physiology, Aarhus University, 8000 Aarhus C (Denmark)

(Received February 18th, 1985)

Key words: $(Na^+ + K^+)$-ATPase; Subunit orientation; Boldon-Hunter reagent

The orientation of amino groups in the membrane in the α- and β-subunits of $(Na^+ + K^+)$-ATPase was examined by labeling with Boldon-Hunter reagent, *N*-succinimidyl 3-(4-hydroxy,5-[^{125}I]iodophenyl)propionate), in right-side-out vesicles or in open membrane fragments from the thick ascending limbs of the Henles loop of pig kidney. Sealed right-side-out vesicles of basolateral membranes were separated from open membrane fragments by centrifugation in a linear metrizamide density gradient. After labeling, $(Na^+ + K^+)$-ATPase was purified using a micro-scale version of the ATP-SDS procedure. Distribution of label was analyzed after SDS-gel electrophoresis of α-subunit, β-subunit and proteolytic fragments of α-subunit. Both the α- and the β-subunit of $(Na^+ + K^+)$-ATPase are uniformly labeled, but the distribution of labeled residues on the two membrane surfaces differs markedly. All the labeled residues in the β-subunit are located on the extracellular surface. In the α-subunit, 65–80% of modified groups are localized to the cytoplasmic surface and 20–35% to the extracellular membrane surface. Proteolytic cleavage provides evidence for the random distribution of ^{125}I-labeling within the α-subunit. The preservation of $(Na^+ + K^+)$-ATPase activity and the observation of distinct proteolytic cleavage patterns of the E_1- and E_2-forms of the α-subunit show that the native enzyme structure is unaffected by labeling with Bolton-Hunter reagent. Bolton-Hunter reagent was shown not to permeate into sheep erythrocytes under the conditions of the labeling experiment. The data therefore allow the conclusion that the mass distribution is asymmetric, with all the labeled amino groups in the β-subunit being on the extracellular surface, while the α-subunit exposes 2.6-fold more amino groups on the cytoplasmic than on the extracellular surface.

Introduction

The organization of the protein of the Na^+, K^+-pump in the membrane can be studied in the native cell membrane if sealed vesicles with well-defined orientation of the pump molecules can be prepared. $(Na^+ + K^+)$-ATPase is found in high concentration in the basolateral cell membrane of the outer medulla of mammalian kidney where the thick ascending limb of Henle is endowed with a high capacity for NaCl transport [1]. In homogenates and subcellular fractions of this tissue a large fraction of the $(Na^+ + K^+)$-ATPase is latent [2]. Electron microscopic studies and labeling experiments show that the latent $(Na^+ + K^+)$-ATPase is localized in tightly sealed right-side-out vesicles [2–4]. This prevents access of ATP and cations to cytoplasmic aspects of the $(Na^+ + K^+)$-ATPase. The latent enzyme activity can be de-

* Permanent address: Shemyakin Institute of Bioorganic Chemistry, U.S.S.R. Academy of Sciences, Moscow, U.S.S.R.

** To whom correspondence should be addressed.

Abbreviations and trivial names: DABS, diazobenzenesulfonate; SDS, sodium dodecyl sulfate; metrizamide, 2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-β-glucose; Bolton-Hunter reagent, *N*-succidimidyl 3-(4-hydroxy, 5-[^{125}I]iodophenyl)propionate.

masked by detergents under controlled conditions [2].

In sucrose gradients, the kidney membranes could be separated into tight vesicles with 50–90% latent $(Na^+ + K^+)$-ATPase and a heavy fraction of open fragments without latent activity [2]. Density gradients of iodinated compounds were shown to provide a better medium for separation of sealed vesicles and open fragments [4]. In the present work tight right-side-out membrane vesicles with 98–100% latent $(Na^+ + K^+)$-ATPase could be separated from heavy open fragments on gradients of metrizamide, presumably because the membrane is impermeable to this compound.

The purpose of the present work has been to examine whether Bolton-Hunter reagent fulfills the requirements for determining the orientation of amino groups in α-subunit and β-subunit of $(Na^+ + K^+)$-ATPase. *N*-Succinimidyl 3-(4-hydroxy, 5-($[^{125}I]$iodophenyl)propionate) was introduced in chemical modifications of proteins by Bolton and Hunter [5] and has been widely employed in immunochemistry, including the iodination of membrane-bound antigens [6]. It is known to react peferentially with primary amino groups [5], but reaction with tyrosine and histidine residues is also observed [6]. To determine the proportion of amino groups on the membrane surfaces, the kidney membrane vesicles were labeled before and after demasking with detergent. After labeling, $(Na^+ + K^+)$-ATPase was purified from the vesicles by extraction with SDS in presence of ATP. As a control, labeling experiments with sheep erythrocytes or lysates were conducted to examine whether the reagent penetrates the cell membrane in the conditions of our experiments. Comparison of specific radioactivity in the α-subunit and the β-subunit shows distinct asymmetry in the distribution of amino groups between extracellular and cytoplasmic region. This result reflects a structural asymmetry in the spatial organization of α- and β-polypeptide chains in the membrane.

Methods

Preparation of right-side-out vesicles. Right-side-out, tightly sealed vesicles were isolated on metrizamide density gradients. Aliquots of the crude membrane fraction [2] containing 85 mg protein was resuspended in 3 ml 30 mM histidine/250 mM sucrose (pH 7.5). 1.5 ml of the sample was applied in each of two tubes on top of a linear 5–20% (w/v) density gradient of metrizamide in 250 mM sucrose/25 mM imidazole/1 mM EDTA buffer (pH 7.5) formed on a cushion of 0.7 ml 33% (w/v) metrizamide. After centrifugation at 20000 rpm for 18 h at 0°c in a Beckman rotor SW-27 using a Centricon 2070 centrifuge, 13 or 14 fractions each of 1 ml were collected.

Enzyme assay. For demasking of $(Na^+ + K^+)$-ATPase activity, two samples of 50 μl from each gradient fraction were incubated in the presence or absence of deoxycholate 0,65 mg/ml in 50 mM imidazole/2 mM EDTA (pH 7.0) in a total volume of 500 μl. After incubation for 30 min at 20°C, 25-μl aliquots were transferred to a test-tube containing 1 ml 130 mM NaCl/20 mM KCl/3 mM $MgCl_2$/3 mM ATP/25 mM imidazole (pH 7.5) with and without 1 mM ouabain. After incubation for 10 min at 37°C ATP hydrolysis was determined as before [7].

Protein determination. Protein was determined by the method of Lowry et al. [8] after precipitation with trichloroacetic acid and standardized with a membrane-bound $(Na^+ + K^+)$-ATPase preparation in which the protein concentration had been determined by quantitative amino acid analysis.

Proteolysis of $(Na^+ + K^+)$-ATPase. For graded tryptic digestion, 10μg $(Na^+ + K^+)$-ATPase was mixed with 0.04 μg TPCK-trypsin at 20°C in 100 μl 25 mM Tris-HCl (pH 7.5). At the indicated intervals (20 and 40 min), the reaction was stopped by adding soybean inhibitor to a weight ratio of inhibitor to trypsin of 3, and the tube was transferred to an ice-bath [9]. Chymotryptic digestion using 0.5 μg chymotrypsin per 10 μg $(Na^+ + K^+)$-ATPase was carried out in 100 μl 15 mM imidazole/10 mM NaCl (pH 7.5) at 37°C. At the indicated time (30, 45 and 60 min) 10 μl of 1 M NaCl was added to stop digestion [10]. The digested membranes were sedimented by centrifugation for 30 min at 30 lb/inch^2 or 160000 $\times g$ in the Beckman Airfuge and analysed by electrophoresis.

Labeling of the vesicles with Bolton-Hunter reagent. Four aliquots of vesicles, each containing 0.947 mg protein in 1 ml, were prepared for

iodination as follows: two aliquots were mixed with deoxycholate to a final concentration of 0.65 mg/ml. After incubation for 30 min at 20°C latent $(Na^+ + K^+)$-ATPase activity was determined to check that all activity had been demasked. The samples were diluted by adding 4 ml of 10% (w/v) sucrose in distilled water. The remaining two aliquots of the vesicles were diluted with 10% sucrose without deoxycholate treatment. All samples were sedimented at 30000 rpm for 90 min in the Beckman type 65 rotor. Untreated vesicles were resuspended in 0.2 M $NaBO_2$ or 0.2 M KBO_2 (pH 9.2). Deoxycholate-treated preparations were resuspended in the same buffer with addition of deoxycholate to 0.65 mg/ml. After equilibration of vesicles at 37°C for 10 min tubes were transferred to an ice-bath.

Bolton-Hunter reagent, 20 μl (Amersham, 5 mCi/ml, 2.5 nmol/ml), in benzene containing 0.2% dimethylformamide were transferred into each of four test tubes and solvent was evaporated under stream of nitrogen. After cooling the membrane preparations were transferred to the tubes. After shaking for 10 min at 0°C, the reaction was stopped by adding 0.2 ml 0.2 M glycine in 50 mM $NaBO_2$ or KBO_2, respectively, and shaking for an additional 5 min. Total and latent $(Na^+ + K^+)$-ATPase activities were determined after labeling. Samples were washed three times by dilution to 3 ml with 25 mM imidazole/1 mM EDTA (pH 7.5) application on the top of 1 ml of 10% sucrose in 25 mM imidazole/1 mM EDTA (pH 7.5) and centrifugation at 30000 rpm for 90 min. Pellets were resuspended in 25 mM imidazole/1 mM EDTA (pH 7.5) and washed by sedimentation in Beckman Airfuge at 30 lb/inch^2 for 30 min until radioactivity was constant in the sediment. Incorporation of the label was in the range of 30–70% of the total.

Purification of $(Na^+ + K^+)$-ATPase from labeled samples of sealed and open vesicles was done in a microscale modification of the previous procedure [7]. A sample of 75 μl containing 2 mg membrane protein per ml/0.65 mg SDS per ml/3 mM ATP-Na_2/25 mM imidazole/1 mM EDTA (pH 7.5) was incubated for 30 min at 20° and layered on discontinous gradients of 25 μl 15% (w/v) sucrose and 100 μl 25% (w/v) sucrose in 25 mM imidazole/1 mM EDTA (pH 7.5) in the 200 μl tubes of the Beckman Airfuge. After centrifugation for 45 min at 100000 rpm with cooling of the rotor to 10°C, the purified $(Na^+ + K^+)$-ATPase was collected in the pellet.

Iodination of intact erythrocytes with Bolton-Hunter reagent. Sheep erythrocytes from freshly drawn blood were washed three times in 0.15 M NaCl/0.05 M sodium phosphate/10% sucrose (pH 8.0) at 4°C and resuspended in the initial blood volume in 0.2 M $NaBO_2$/10% sucrose (pH 9.2). Three identical aliquots of 0.5 ml were modified with 10 μCi of Bolton-Hunter reagent for 5, 10 and 15 min, as described above. The fourth portion of erythrocytes was lysed by adding 2 ml distilled water to the sediment and dialyzed for 2 h at 4°C against 0.2 M $NaBO_2$ (pH 9.2). This portion was modified with Bolton-Hunter reagent for 10 min under the same conditions as first three aliquots. Reaction was stopped by glycine, as above, and intact cells were washed four times in phosphate-sucrose buffer. The membranes of the lysate (fourth sample) were sedimentated at 30000 rpm for 60 min (Beckman, SW-50). Each aliquot of washed iodinated erythrocytes were divided in two equal portions, one of which was lysed and membranes separated as described above. The second portion was resuspended in phosphate-sucrose buffer. After determination of the radioactivity of the membrane material and supernatants, trichloroacetic acid was added up to 5% to supernatants for precipiration of labeled cytoplasmic proteins and separation from free-Bolton-Hunter reagent and hydrolysis products. After repeated washing, the radioactivity of membranes and cytoplasmic proteins was determined. The degree of hemolysis during this procedure was controlled with absorption measurements at 412 nm. The amount of Bolton-Hunter reagent that had penetrated into intact erythrocytes was calculated as the difference in radioactivity between lysate and supernatant after washing of the cells.

Electrophoresis. Incorporation of the label into α- and β-subunits and proteolytic fragments of α-subunit was analysed after electrophoretic separation of components according to Laemmli in the presence of SDS [11]. Polyacrylamide gel slabs with gradients of 5–15% (T%) acrylamide were used. Pellets of membrane material after sedimentation in the Bechman Airfuge were dissolved in

2% SDS/1% β-mercaptoethanol and heated for 3 min at 100°C in a total volume of 50 μl. Glycerol was added to 10% and the samples were electrophoresed at a current of 3 mA/sample for 6–8 h. Gels were stained with Coomassie brilliant blue, destained and dissected in the direction of the electrophoresis, and separated columns, representing each sample, were scanned in a Beckman Acta spectrophotometer at 650 nm using a slit width of 0.1 mm. Two samples, containing 5 μg or 10 μg unlabeled purified $(Na^+ + K^+)$-ATPase protein, were run in the same slab and used for quantitation of the protein bands of the labeled enzyme after electrophoresis. After scanning the part of the gel containing the subunits and the tryptic fragments of the α-subunit, the gel was cut into slices of 1.5 mm and ^{125}I radioactivity was determined in a γ-spectrometer. Using bovine serum albumin as standard, it was shown that the Bolton-Hunter reagent exhibits the same reactivity to amino groups in Na^+ and K^+ media and that the labeled product is acid-stable under the conditions of staining and destaining of gels.

Results

Tightly sealed vesicles were isolated from crude membrane fractions of pig kidney outer medulla on metrizamide density gradients. The distribution in the gradient of protein and $(Na^+ + K^+)$-ATPase before and after demasking with deoxycholate is shown in Fig. 1. Tightly sealed vesicles containing 60–70% of $(Na^+ + K^+)$-ATPase in the sample were recovered in fractions 2–10 with very low apparent $(Na^+ + K^+)$-ATPase activity. A 50–70-fold increase of $(Na^+ + K^+)$-ATPase activity was seen after incubation with deoxycholate or SDS. After demasking of latent activity, the specific activity of $(Na^+ + K^+)$-ATPase in these fractions was 10–12 μmol P_i/min per mg protein. The data in Fig. 2 show that in fractions at the peak, less than 2% of the $(Na^+ + K^+)$-ATPase was apparent and that more than 98% of the activity was masked. According to electronmicroscopic analysis, the latent activity is located in right-side-out vesicles [3].

In fractions 13–14 of the gradient, the material from peak consisting predominantly of demasked $(Na^+ + K^+)$-ATPase was collected. In these fractions, the $(Na^+ + K^+)$-ATPase activity was only increased from 13.9 to 20 μmol P_i/min per fraction by incubation with deoxycholate. The distribution of vesicles and open fragments is similar to that observed earlier in sucrose gradients [2], but the separation of closed vesicles from open fragments is not as efficient in sucrose as in hypaque [4] or metrizamide.

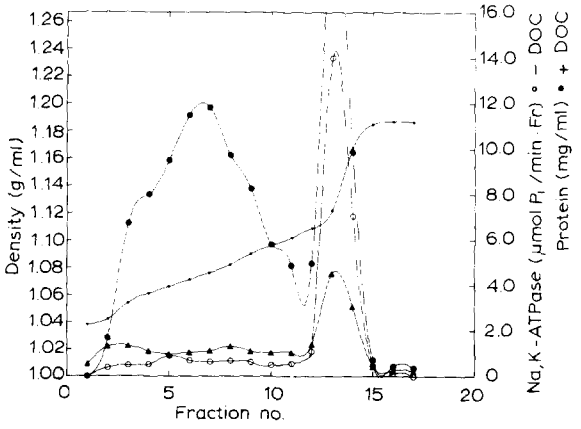

Fig. 1. Distribution of protein (▲), and $(Na^+ + K^+)$-ATPase activity before (○) and after (●) demasking with sodium deoxycholate after centrifugation in linear (5–20%) density gradients of metrizamide as described in Methods.

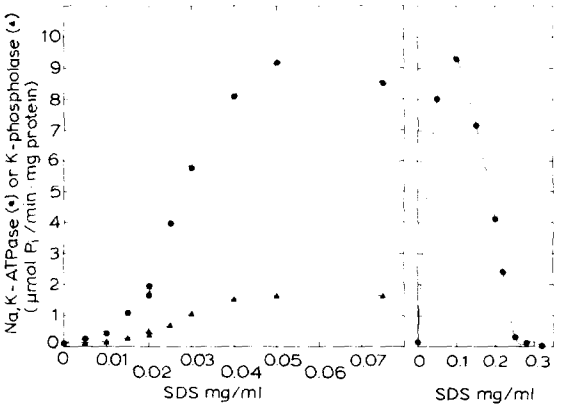

Fig. 2. Concentration-dependence of demasking (left) and inactivation (right) of $(Na^+ + K^+)$-ATPase (●) and potassium phosphatase (▲) activities by SDS. Incubation of membrane vesicles, 0.1 mg protein per ml, at 20°C with SDS at the concentrations shown on the abcissa in 25 mM imidazole/1 mM EDTA (pH 7.5) for 16 h at 20°C. In the experiment shown to the right, the composition of the medium was the same as above, but incubation was for 30 min at 20°C. For assay, 25-μl aliquots were transferred to test-tubes with and without ouabain.

Essential for any side-specific labeling experiment is the cell membrane's being impermeable to the modifying reagent. Erythrocytes were selected for the control studies, as they are devoid of cytoplasmic organelles and hemoglobin forms a sink of large capacity for the reagent [12]. Table I shows that 90–94% of the Bolton-Hunter reagent incorporated into sheep erythrocytes was bound to the membranes, while only 7–10% was released to the supernatant after lysis of the cells. The drastic change in the ratio of iodination of membranes to that of cytoplasmic proteins seen after lysis of the erythrocytes shows that the reagent reacts preferentially with the hemoglobin of the lysate. After incubation under conditions for optimum labeling, the penetration of Bolton-Hunter reagent is less than that after modification by lactoperoxidase iodination, 12% [13], or after modification with DABS, 17–20% [12]. The latter reagents are both considered to be nonpermeable. Our data therefore show that the Bolton-Hunter reagent is very suitable for side-specific modification of protein in sealed and open membrane preparations.

Due to the high reactivity of *N*-hydroxysuccinimide esters and the relatively low concentration of reagent in the medium, the efficiency of incorporation of the reagent into the membrane material was very high, 30–70%. After iodination of closed or demasked vesicles, $(Na^+ + K^+)$-ATPase was purified using a microscale version of the SDS-ATP procedure [7]. Typical distributions of the label on the α-subunit and β-subunit of the purified $(Na^+ + K^+)$-ATPase are illustrated in Fig. 3. Opening of the vesicles resulted in a 2.8-fold increase in the labeling of the α-subunit without a significant change in the label incorporated into the β-subunit (Table II). This experiment was done in Na^+ medium, but the distribution was the same in K^+ medium (data not shown). This shows that transition between the E_1Na form and E_2K form of the α-subunit does not involve a change in the total number of amino groups exposed at the surface to reaction with Bolton-Hunter reagent.

Only about 10% of the label attached to the membranes was incorporated into $(Na^+ + K^+)$-ATPase. Due to the high specific radioactivity of the Bolton-Hunter reagent, this amount of bound label is sufficient to trace the subunits of the enzyme. On the other hand, this labeling does not alter the general properties of the enzyme. The $(Na^+ + K^+)$-ATPase activity is retained after modification in both closed vesicles and open fragments and the proteolytic fragmentation pattern is similar to that of the native enzyme [9,10]. This points to random modification of the protein without specific modification of those amino groups which are important for enzymatic activity or conformational transitions of the α-subunit.

Fig. 4 shows the distribution of ^{125}I in the α-subunit, β-subunit and the 78 kDa fragment

TABLE I

REACTION OF BOLTON-HUNTER REAGENT WITH MEMBRANES AND CYTOPLASM OF SHEEP ERYTHROCYTES

Incubation conditions	Radioactivity (cpm) in different samples [a]				
	total	cell sediment	ghosts	supernatant after lysis	trichloroacetic acid precipitate of supernatant
Intact cells					
5 min	400123	180000	167084	14057 [b]	12472 (6.9) [c]
10 min	410087	205110	186002	18832	17204 (8.6)
15 min	395824	211087	189230	24076	22017(10.4)
Lysate					
10 min	398926		15214	380062	312042(95.3)

[a] Radioactivity is counted for one-fifth of the initially modified cells.

[b] Radioactivity in supernatant is calculated as difference between total radioactivity in the supernatant (30000 rpm, 60 min) after lysis and supernatant after washing of intact cells.

[c] The number in parentheses in the ratio of the radioactivity of the trichloroacetic acid precipitate to the sum of radioactivities in ghosts and trichloroacetic acid precipitate (as percentage).

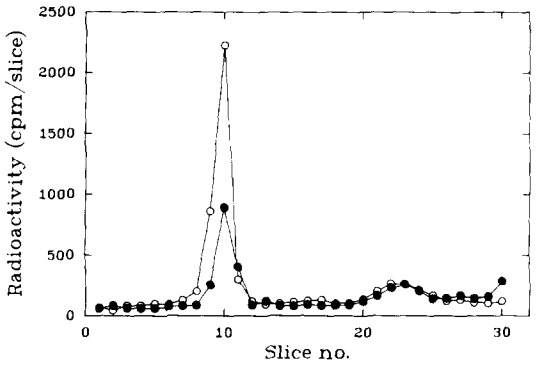

Fig. 3. Labeling of α- and β-subunits with Bolton-Hunter reagent in tightly sealed vesicles (●) or after treatment with deoxycholate (○). After labeling, $(Na^+ + K^+)$-ATPase was purified using the micro-scale version of the SDS-ATP procedure as described under Methods. Conditions of labeling, electrophoresis, slicing of gels and quantitation of applied protein amount as described in Methods.

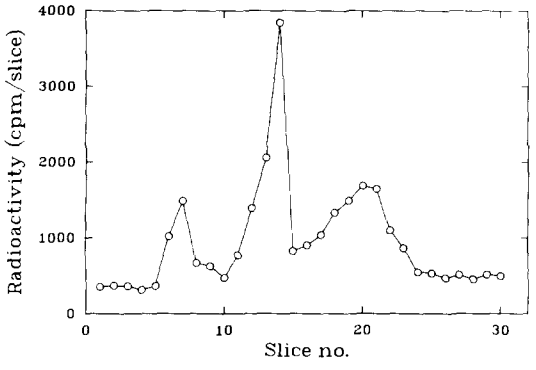

Fig. 4. Distribution of ^{125}I after digestion of $(Na^+ + K^+)$-ATPase for 45 min with chymotrypsin as described in Methods. Other experimental details as in Fig. 3.

appearing between the two subunits after chymotryptic cleavage of the $(Na^+ + K^+)$-ATPase which was iodinated in sealed vesicles. Comparison of specific labeling incorporated into α-subunit and its two proteolytic fragments – 78 kDa and 58 kDa – are shown in Table III. This shows that the α-subunit and its proteolytic fragments have very similar specific radioactivity per μg of protein. After modification of the enzyme in open vesicles, incorporation of the label increases proportionally 2.7-, 3.0- and 2.5-times, respectively, for α-subunits and 78 kDa and 58 kDa fragments. These values are in agreement with the 2.8-fold increase of labeling of α-subunit, shown in Table II. Data for 46 kDa fragment were not caculated due to a high background radioactivity in this part of the gradient polyacrylamide gel. In agreement with the model of the spatial organization of α-subunit in the membrane [14], the data show that both the α-subunit and its proteolytic fragments are accessible for modification from both sides of the membrane. The proportional increase in the labeling of α-subunit and its fragments upon opening of the vesicles shows that there is a random distribution of label along the polypeptide chain of the α-subunit. Since there are large amounts of lysine residues in α- and β-subunits [15,16], random iodination allows calculation of the masses of hydrophylic domains of α- and β-subunits that are located on the cytoplasmic and the extracellular surfaces of the membranes. The 2.8-fold increase of labeling of the α-subunit in open membrane fragments corresponds to ex-

TABLE II

^{125}I-LABELING OF α- AND β-SUBUNITS AFTER LABELING OF SEALED (−DOC) AND OPEN (+DOC) MEMBRANE VESICLES WITH BOLTON-HUNTER REAGENT

After labeling $(Na^+ + K^+)$-ATPase was purified using the microscale version of the SDS-ATP procedure as described in Methods. Values given as cpm/μg $(Na^+ + K^+)$-ATPase protein ± S.D.

	α-subunit	β-subunit
−DOC	1371 ± 122	693 ± 29 ($n = 4$)
+DOC	3850 ± 522	778 ± 38 ($n = 4$)

TABLE III

SPECIFIC RADIOACTIVITY OF α-SUBUNIT AND ITS PROTEOLYTIC FRAGMENTS, IODINATED IN SEALED (−DOC) AND OPEN (+DOC) VESICLES

Labeling, electrophresis, slicing and determination of protein amount in the band as described in Methods. Specific radioactivity (cpm) is calculated per μg protein in the corresponding band after gel electrophoresis in SDS.

	α-subunit	77 kDa fragment	58 kDa fragment
−DOC	2417	2279	2513
+DOC	6459	6745	6197

posure of 35% of its amino groups on the outer surface of the cell membrane. However, the enzyme assay after modification shows that one-fifth of the latent $(Na^+ + K^+)$-ATPase in sealed vesicles was demasked during modification. Taking this into account, the data show that not less than 20% and not more than 35% of amino groups in the α-subunit are exposed on the extracellular membrane surface. The average value, 28%, corresponds to exposition of 2.6-times larger mass of the α-subunit at the cytoplasmic surface than at the outer surface of the cell membrane.

Comparison of labeling of the β-subunit of the $(Na^+ + K^+)$-ATPase in sealed and open vesicles (Table II, Fig. 2) shows almost no difference in the iodination of this subunit. This points to a profound asymmetry in the organization in the membane of the β-subunit, which is thought to be an integral protein of the cell memrane [17]. This result is in agreement with data on the modification of the enzyme with fluorescamine in membrane fragments and inside-out proteoliposomes, where the β-subunit was shown to be inaccessible to modification [18].

According to the data in Table II, the mass of hydrophilic parts within the β-subunit that are exposed at the cytoplasmic surface must be less than 10% of the mass exposed on the extracellular surface. Thus the bulk of the hydrophilic domain of the β-subunit is extracellular.

Discussion

The distribution of the mass of the polypeptide chain on the two sides of the membrane provides important information of the tertiary structure of a membrane-spanning protein. Bolton-Hunter reagent labels the α-subunit and the β-subunit of $(Na^+ + K^+)$-ATPase uniformly, but the distribution of labeled residues on the two membrane surfaces differs markedly. In the α-subunit, 65–80% of the residues labeled by the reagent are localized on the cytoplasmic surface and 20–35% on the extracellular membrane surface. In contrast, the labeled residues of the β-subunit are exclusively located at the extracellular surface. These data are based on the method for isolation of tightly sealed (98–100%) right-side-out vesicles of basolateral membranes containing $(Na^+ + K^+)$-ATPase in the native state [2,4].

Bolton-Hunter reagent shows high reactivity with amino groups, it has a high specific radioactivity, a short life-time (9 min) of decomposition to inactive products and it reacts with protein under mild non-denaturating conditions (0°C, 10 min, pH 8.5–9.2) [5]. This ensures preferential modification of side-chains in hydrophilic environments that are represented in protein in high amount and consequently produces uniform distribution of the label along the polypeptide chains. The high specific radioactivity and random insertion into small amounts of potentially reactive groups should allow chemical modification without affecting the function of the molecule. Our data show that permeation of Bolton Hunter reagent through the red-cell membrane under the conditions of the experiment is lower than that observed for labeling with lactoperoxidase [13] and DABS [12]. Based on amino acid analysis data [15,16], α-subunit and β-subunit contain 45–46 and 15–21 lysine residues that are preferentially modified with Bolton-Hunter reagent. The subunits also contain tyrosine (23–26 and 12–15) and histine (14–18 and 5–6) that form stable covalent products with the reagent [6]. Incorporation of the reagent into the enzyme neither changes $(Na^+ + K^+)$-ATPase activity nor interferes with the transition between the two different patterns of tryptic and chymotryptic digestion that reflect conformational E_1-E_2 transitions occurring during enzyme turnover. Both subunits of the enzyme are modified and the labeled residues are evenly distributed on the proteolytic fragments of the α-subunit. Uniform labeling of α-subunit and β-subunit of $(Na^+ + K^+)$-ATPase without changing the native conformation of the molecule shows that the Bolton-Hunter reagent is adequate as a marker of polypeptide chain mass distribution.

For the α-subunit, the total amount of modified groups in the cytoplasmic domain is about 2.6-fold larger than the amount of groups modified on the extracellular surface. The cytoplasmic domain of Ca^{2+}-ATPase from sarcoplasmic reticulum contains 50% of the total sequence [19]. Neutron diffraction and X-ray scattering shows that 65% and 35% of the total mass of the Ca^{2+}-ATPase protein is localized in the inner and 35% in the outer monolayers of the membrane, respectively.

Based on homologies of the two proteins, one can assume that also the α-subunit of $(Na^+ + K^+)$-ATPase exposes 50% of its mass on the inner surface. With this assumption, our observed distribution of labeled residues corresponds to exposure of 19% of α-subunit of extracellular surface. This appears to be a resonable value, as the extracellular domain of the α-subunit forms the area for binding of almost all parts of the cardiac glycoside molecules, the steroid moiety and the carbohydrate chain to the position of the third digitoxose [21]. Our value for the mass of the extracellular domain of α-subunit is higher than that found by latoperoxidase iodination [13], but it is very similar to that observed with DABS [22]. The lower value seen after iodination can be due to steric limitations in the formation of the complex with lactoperoxidase, which is assumed to be necessary for iodination [12]. Coincidence of our results with those obtained for α-subunit with DABS could argue that both are correct. However, random distribution is essential for determining the mass distribution. While Bolton-Hunter reagent labels the α-subunit and the β-subunit equally, DABS does not insert into the β-subunit, although this subunit has the same percentage of reactive groups as the α-subunit [17,18] and exposes the N-terminal glycosylated part with reactive α-amino groups on the outer membrane surface [15]. In the experiments with DABS [22], the compound was supposed to be non-selective, reacting with histidine, tyrosine and lysine residues and expected to produce random labeling. But the available data point to rather selective modification – at 10-fold molar excess of the reagent to protein incorporation of the label is 0.002–0.08 mol reagent/mol α-subunit. This is at least 10^3-times lower than that observed under optimal conditions [23]. These observations point to rather selective modification of the α-subunit with DABS of some unusually reactive groups with low pK, apparently because modification was carried out at pH 7.0, which is far from optimal [23].

Our data show that the mass of hydrophilic residues of β-subunit at the extracellular surface is 10-fold larger than the mass of β-subunit material exposed at the cytoplasmic surface. Only a few data are available for comparison with this result. Results on the enzymatic iodination of β-subunit are not presented [13] and, as mentioned above, the β-subunit is not labeled with DABS [22]. Upon modification of $(Na^+ + K^+)$-ATPase in pure membrane enzyme and 'inside-out' proteoliposomes, it has been shown [18], that the α-subunit is modified from both sides of the membrane, whereas the β-subunit remains unlabeled in 'inside-out' proteoliposomes. As seen in Fig. 3, demasking of $(Na^+ + K^+)$-ATPase in right-side-out vesicles produced a considerable effect on the labeling of the α-subunit, but showed almost no effect on the labeling of the β-subunit. The same conclusion comes from data presented in Table II, where it is seen that opening of right-side-out vesicles with deoxycholate does not produce any significant change in the amount of accessible amino groups in the β-subunit.

The β-subunit is an integral membrane protein which is labeled from within the membrane bilayer [17], but there are some discrepancies concerning the existence of a hydrophilic region of the β-subunit on the cytoplasmic surface of the membrane. After immunoabsorption of the total pool of antibodies against β-subunit with erythrocytes, the remaining population of antibodies reacts with purified $(Na^+ + K^+)$-ATPase, presumably with cytoplasmic aspects of the β-subunit [24]. In contrast, other immunochemical methods did not demonstrate antigenic determinants of β-subunit on the inner surface of the membrane [25]. The latter result is in agreement with data on fluorescamine labeling [18], where amino groups of β-subunit were not detectable on the cytoplasmic surface of the membrane. These results agree well with our observation that the majority of the hydrophilic residues of the β-subunit are extracellular and that less than 10% of all the reactive side-chains of the β-subunit are exposed on the cytoplasmic surface.

Acknowledgements

We thank Janne Petersen and Lene Jacobsen for excellent technical help. The study was supported by the Danish Medical Research Council and Novo's Foundation. K.D. received a stipendium from the Ministry of Education, Copenhagen, as part of a cultural agreement between Denmark and the U.S.S.R.

References

1 Jørgensen, P.L. (1980) Physiol. Rev. 60, 864–917
2 Jørgensen, P.L. and Skou, J.C. (1971) Biochim. Biophys. Acta 233, 366–380
3 Skriver, E., Maunsbach, A.B. and Jørgensen, P.L. (1983) Curr. Top. Membrane Transp. 19, 119–122
4 Forbush, B. (1982) J. Biol. Chem. 257, 12678–12684
5 Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529–539
6 Langone, J.J. (1980) Methods Enzymol. 70, 221–246
7 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
9 Jørgensen, P.L. and Petersen, J. (1982) Biochim. Biophys. Acta 705, 38–47
10 Jørgensen, P.L., Skriver, E., Hebert, H. and Maunsbach, A.B. (1982) Ann. NY Acad. Sci. 402, 207–225
11 Laemmli, U.K. (1970) Nature (London) 227, 680–685
12 Hubbart, A.L. and Cohn, Z.A. (1976) in Biochemical Analysis of Membranes (Maddy, A.H., ed.), Chapman & Hall, London
13 Sharkey, R. (1983) Biochim. Biophys. Acta 730, 327–341
14 Jørgensen, P.L., Karlish, S.J.D. and Gitler, C. (1982) J. Biol. Chem. 257, 7435–7442
15 Dzhandzhugazyan, K.N., Modyanov, N.N. and Ovchinnikov, Yu.A. (1981) Bioorg. Khim. 7, 847–857
16 Freytag, J.W. and Reynolds, J.A. (1981) Biochemistry 20, 7211–7214
17 Jørgensen, P.L. and Brunner, J. (1983) Biochim. Biophys. Acta 735, 291–296
18 Modyanov, N., Babakov, A., Arzamazova, N., Dzhandzhugazyan, K. and Kocherginskaya, S. (1982) in Chemistry of Peptides and Proteins (Voelter, W., Wünsch, E., Ovchinnikov, J. and Ivanov, V., eds.), Vol. I, pp. 85–92, Walter de Gruyter, Berlin
19 Herbette, L., Scarpa, A., Blasie, J.K., Wang, C.T., Hymel, L., Seelig, J. and Fleisher, S. (1983) Biochim. Biophys. Acta 730, 369–378
20 Allen, G., Trinnaman, B.J. and Green, N.M. (1980) Biochem. J. 187, 591–616
21 Hall, C. and Ruoho, A. (1980) Proc. Natl. Acad. Sci. USA 77, 4529–4533
22 O'Connell, M.A. (1982) Biochemistry 21, 5984–5991
23 Kagan, H.M. and Vallee, B.L. (1969) Biochemistry 8, 4223–4231
24 Girardet, M., Geering, K., Frantes, J.M., Geser, D., Rossier, B.C. and Krahenbuhl, J.P. (1981) Biochemistry 20, 6684–6691
25 Dzhandzhugazyan, K.N., Modyanov, N.N. and Ovchinnikov, Yu.A. (1981) Biorg. Khim. 7, 1790–1800