

Solubilization of Na,K-ATPase from rabbit kidney outer medulla using only C₁₂E₈

H.L. Santos,
R.P. Lamas and
P. Ciancaglini

Departamento de Química, Faculdade de Filosofia,
Ciências e Letras de Ribeirão Preto, Universidade de São Paulo,
Ribeirão Preto, SP, Brasil

Abstract

SDS, C₁₂E₈, CHAPS or CHAPSO or a combination of two of these detergents is generally used for the solubilization of Na,K-ATPase and other ATPases. Our method using only C₁₂E₈ has the advantage of considerable reduction of the time for enzyme purification, with rapid solubilization and purification in a single chromatographic step. Na,K-ATPase-rich membrane fragments of rabbit kidney outer medulla were obtained without adding SDS. Optimum conditions for solubilization were obtained at 4°C after rapid mixing of 1 mg of membrane Na,K-ATPase with 1 mg of C₁₂E₈/ml, yielding 98% recovery of the activity. The solubilized enzyme was purified by gel filtration on a Sepharose 6B column at 4°C. Non-denaturing PAGE revealed a single protein band with phosphomonohydrolase activity. The molecular mass of the purified enzyme estimated by gel filtration chromatography was 320 kDa. The optimum apparent pH obtained for the purified enzyme was 7.5 for both PNPP and ATP. The dependence of ATPase activity on ATP concentration showed high ($K_{0.5} = 4.0 \mu\text{M}$) and low ($K_{0.5} = 1.4 \text{ mM}$) affinity sites for ATP, with negative cooperativity. Ouabain (5 mM), oligomycin (1 $\mu\text{g/ml}$) and sodium vanadate (3 μM) inhibited the ATPase activity of C₁₂E₈-solubilized and purified Na,K-ATPase by 99, 81 and 98.5%, respectively. We have shown that Na,K-ATPase solubilized only with C₁₂E₈ can be purified and retains its activity. The activity is consistent with the form of ($\alpha\beta$)₂ association.

Key words

- Na,K-ATPase
- Rabbit kidney medulla
- Membrane solubilization
- C₁₂E₈
- ($\alpha\beta$)₂ Dimer

Correspondence

P. Ciancaglini
Departamento de Química
FFCLRP, USP
Av. Bandeirantes, 3900
14040-901 Ribeirão Preto, SP
Brasil
Fax: + 55-16-633-8151
E-mail: pietro@ffclrp.usp.br

Research supported by FAPESP and
CNPq. H.L. Santos is the recipient
of a student fellowship from
FAPESP, and P. Ciancaglini is a
CNPq research fellow.

Received April 20, 2001
Accepted January 2, 2002

Introduction

Detergents have been successfully used for the solubilization of membrane proteins. Several excellent reviews can be found on detergents, their physical properties and their use in the solubilization of membrane proteins (1-4). Due to three important factors, non-ionic detergents are frequently used for the solubilization of protein membranes: i)

their efficiency in breaking the lipid-lipid and lipid-protein interactions, ii) their inefficiency in weakening protein-protein interactions, and iii) their property of being less denaturing than the ionic detergents (3-6).

The foremost challenge in the solubilization of a membrane protein is obtaining a soluble protein in a stable form that retains its original function. A detergent that is very much used in the procedure of extraction

and/or Na,K-ATPase solubilization, although it partially denatures the enzyme molecule, is sodium dodecyl sulfate (SDS). Other detergents that have also been used to inactivate the enzyme in a concentration-dependent way are cholate, deoxycholate and 3-((3-cholamidopropyl)dimethylamino)-1-propane sulfonate (CHAPS) (7). For example, sodium cholate (1-2%, w/v) solubilizes 50% of renal Na,K-ATPase with retention of only half of the original specific activity (7). On the other hand, 0.2% (w/v) deoxycholate combined with 0.1% (w/v) saponin and high concentrations of NaCl (~360 mM) and cholesterol was efficient in Na,K-ATPase solubilization without losses of specific activity (7,8). However, CHAPS when used at a 1:2 (w/w) ratio of detergent:enzyme at a final concentration of 9 mM (0.55%, w/v) solubilized about 25% of the enzyme without loss of specific activity (7).

Another class of detergents used in Na,K-ATPase solubilization are octylglucosides and polyoxyethylenes (Triton, Lubrol, Brij, dodecyloctaethyleneglycol (C₁₂E₈) and Tween). Octylglucoside was not efficient for Na,K-ATPase solubilization because it needed a long incubation time with the protein, with the consequent inactivation of the enzyme. In contrast, polyoxyethylenes are good agents for Na,K-ATPase solubilization (7). Triton X-100, when used at a concentration of 0.25% (w/v), solubilizes 92% of Na,K-ATPase from kidney outer medulla, but partially inactivates the reconstituted enzyme (9).

A detergent that has been used with great success in Na,K-ATPase solubilization is C₁₂E₈ (4,5,7,10,11). Different results showed that Na,K-ATPase previously treated with SDS can be solubilized with C₁₂E₈ in an active form where most of the kinetic and conformational properties of the enzyme are preserved after solubilization (4,7,11).

Na,K-ATPase is an integral membrane protein found in all superior eukaryotic cells which are responsible for the active trans-

port of sodium and potassium ions through the plasma membrane coupled with ATP hydrolysis (8,12-14). This enzyme is a heterodimer of three subunits: α subunit, with a molecular mass of about 110 kDa; β subunit, highly glycosylated, with a molecular mass of about 50 kDa, and γ subunit, with a molecular mass of about 15 kDa (8,14,15). The α subunit is responsible for the enzymatic activity. It contains the binding and phosphorylation ATP domain, as well as essential amino acids for the binding of sodium, potassium and some inhibitors (8,16,17). The β subunit has a structural function and is important for membrane enzyme insertion (8,14,18,19). The γ peptide has hydrophobic characteristics associated with the Na,K-ATPase molecule; however, it is not required for the enzyme catalytic activity or for ion transport. Its association with the α and β subunits seems to involve a modulator function for Na,K-ATPase obtained from several tissues (14). In the plasma membranes, the α and β subunits build functional oligomers of the ($\alpha\beta$)₂ type or oligomers with superior structure ($\alpha\beta$)_n (20-24). Na,K-ATPase is specifically inhibited by cardiac glycosides such as ouabain, that bind to the extracellular domains. Its binding and inhibition depend on the combination of magnesium, sodium and potassium ions (8,25,26). Another potent inhibitor of the enzyme is orthovanadate (25,27).

The present study describes the solubilization using only C₁₂E₈, and a single step purification of Na,K-ATPase from the outer medulla of rabbit kidney. Our data suggest that this method is efficient, and the kinetic and structural characteristics of the solubilized enzyme are preserved.

Material and Methods

All solutions were prepared with Millipore MilliQ ultrapure apyrogenic water. SDS, Tris(hydroxymethyl)aminomethane (Tris), trichloroacetic acid (TCA), 2-N-morpholine

ethanesulfonic acid (MES), N-(2-hydroxyethyl) piperazine-N'-ethanesulfonic acid (HEPES), 2-amino-2-methyl-propan-1-ol (AMPOL), α -naphthyl phosphate, fast blue RR, oligomycin, ouabain, adenosine 5'-triphosphate Tris salt (ATP), sodium orthovanadate, p-nitrophenyl phosphate (di)Tris salt (PNPP), BSA, CHAPS, and 3-((3-cholamidopropyl)dimethylamino)-2-hydroxy-1-propane sulfonate (CHAPSO) were from Sigma (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), potassium chloride, sodium chloride and magnesium chloride were from Merck (São Paulo, SP, Brazil). C₁₂E₈ was from Calbiochem (San Diego, CA, USA). Analytical grade reagents were used without further purification.

Isolation of Na,K-ATPase from the outer medulla of rabbit kidney

Isolation of membrane enzyme. Membrane-bound Na,K-ATPase was obtained from the dark red outer medulla of the kidney of adult New Zealand white rabbits as described by Jorgensen (28), with some modifications, as follows. The kidney was removed immediately after sacrifice and stored in ice-cold 20 mM imidazole buffer, pH 6.8, containing 250 mM sucrose, 6 mM EDTA and 6 mM Tris. The kidney was cut into transversal sections and the light gray inner medulla was removed and discarded. The tissue from the dark red outer medulla was obtained by incisions along the inner side of the boundary with the cortex. The extracted tissue was homogenized in 20 mM imidazole buffer, pH 6.8, containing 250 mM sucrose, 6 mM EDTA and 6 mM Tris (7 ml buffer to 1 g of moist weight) in a high-speed shearing homogenizer for 30 s. The homogenate was centrifuged at 10,000 g for 35 min at 4°C and the supernatant was ultracentrifuged at 180,000 g for 1 h at 4°C. The pellet corresponding to membrane-bound Na,K-ATPase was resuspended in 20 mM imidazole buffer, pH 6.8, contain-

ing 250 mM sucrose, 6 mM EDTA and 6 mM Tris, and stored in 1-10 ml aliquots at -20°C.

Solubilization of Na,K-ATPase membrane fragments with C₁₂E₈. To determine optimum conditions to solubilize membrane-bound enzyme, an assay was carried out by varying the protein/C₁₂E₈ ratio and the time and temperature of incubation with the detergent. Optimum conditions were obtained at 4°C after rapid mixing of 1 mg of Na,K-ATPase membrane fragments with 1 mg of C₁₂E₈/ml. The non-solubilized residues were removed by centrifugation at 4°C for 1 h at 100,000 g in a Hitachi centrifuge. The supernatant was assayed for protein concentration and ATPase and PNPPase activities.

Purification of solubilized Na,K-ATPase. The C₁₂E₈-solubilized enzyme was concentrated through a YM-10 Amicon filter and purified by gel filtration at 4°C on a Sepharose 6B column (1.4 x 119 cm) equilibrated and eluted with 5 mM Tris-HCl buffer, pH 7.0, containing 1 mM EDTA, 150 mM KCl and 0.005 mg/ml C₁₂E₈ at a flow rate of 21 ml/h. The fractions showing ATPase activity were pooled, concentrated through a YM-10 Amicon filter, and dialyzed for 2 h at 4°C against 5 mM Tris-HCl buffer, pH 7.5, containing 15 mM KCl, 6 mM EDTA and 0.005 mg/ml C₁₂E₈, and stored in 1-ml aliquots at -20°C.

Enzyme assay

PNPPase activity was assayed discontinuously at 37°C with a Genesys 2 spectrophotometer by monitoring the liberation of p-nitrophenolate ion (ϵ 1 M, pH 13 = 17,600 M⁻¹ cm⁻¹) at 410 nm, in 50 mM HEPES buffer, pH 7.5, containing 10 mM PNPP, 15 mM KCl and 10 mM MgCl₂ in a final volume of 1.0 ml. The reaction was initiated by the addition of the enzyme and stopped with 1.0 ml of 1 M NaOH. Alternatively, ATPase activity was assayed discontinuously at 37°C by quantification of phosphate release as

described by Santos and Ciancaglini (5), adjusting the assay medium to a final volume of 1.0 ml. The reaction was initiated by the addition of the enzyme, stopped with 0.5 ml of cold 30% TCA at appropriate intervals and centrifuged at 4,000 *g* immediately prior to phosphate determination. Standard conditions were 50 mM HEPES buffer, pH 7.5, containing 3 mM ATP, 10 mM KCl, 5 mM MgCl₂ and 50 mM NaCl.

For both enzyme activities the determination was carried out in triplicate and the initial velocities were constant for at least 30 min, provided that less than 5% of substrate was hydrolyzed. Controls without enzyme were included in each experiment to quantify the non-enzymatic hydrolysis of the substrate. One enzyme unit (U) is defined as the amount of enzyme hydrolyzing 1.0 nmol of substrate per minute at 37°C.

Protein analysis

Protein concentrations were estimated as described by Santos and Ciancaglini (5) in the presence of 2% (w/w) SDS, with BSA used as standard.

Sucrose density gradient centrifugation of membrane-bound Na,K-ATPase

Membrane-bound Na,K-ATPase (1 mg) was layered onto a continuous sucrose gradient (10-50%, w/v) in imidazole buffer and centrifuged for 2 h at 180,000 *g* using a Hitachi PV50TA vertical rotor at 4°C. Fractions of 0.5 ml were collected and assayed for protein concentration (29), refractive index (% sucrose), and ATPase and PNPPase activities.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out on 5% gel according to Davis (30) using silver nitrate for protein staining (31). Phosphohydrolytic activity on

the gel was detected in 50 mM HEPES buffer, pH 7.5, containing 10 mM PNPP, 15 mM KCl, 10 mM MgCl₂, 0.12% α -naphthyl phosphate, and 0.12% fast blue RR at 37°C.

Molecular mass determination

The molecular mass of the solubilized and purified Na,K-ATPase was estimated by filtration on a Sepharose 6B column (0.8 x 133 cm) equilibrated and eluted with 5 mM Tris-HCl buffer, pH 7.0, containing 1 mM EDTA, 150 mM KCl and 0.005 mg/ml C₁₂E₈. BSA (66 kDa), alcohol dehydrogenase (150 kDa), β -amylase (200 kDa), apoferritin monomer (110 kDa), apoferritin dimer (221 kDa) and apoferritin tetramer (443 kDa) were used as molecular markers.

The molecular mass of the α and β units of kidney Na,K-ATPase was estimated by SDS-PAGE on 10% gels according to Laemmli (32), using silver nitrate for protein staining (31). Myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase B (97.4 kDa), BSA (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa) were used as molecular markers.

pH sensitivity of catalysis

The effect of pH on ATPase and PNPPase activities was measured in 50 mM buffer over the pH range of 5.0 to 9.5. MES buffer was used in the 5.0 to 6.0 range, Bis-Tris in the 6.0 to 6.5 range, imidazole in the 6.5 to 7.5 range, Tris in the 7.0 to 9.0 range, and AMPOL in the 8.5 to 9.5 range. The pH of the reaction mixture was measured both before and after the assay and did not vary by more than 0.05 units. ATPase and PNPPase activities were assayed as described above.

Estimation of kinetic parameters

Maximum velocity (*V*), initial velocity (*v*), apparent dissociation constant (*K*_{0.5}) and Hill coefficient (*n*), obtained from sub-

strate hydrolysis, were determined with a microcomputer program as described by Leone et al. (33). Data are reported as the mean of triplicate determinations in which $P < 0.05$ was considered to be statistically significant.

Results

Membrane fractions rich in Na,K-ATPase were obtained by homogenization of the dark red outer medulla of the kidney without the addition of SDS. Figure 1 shows the results of the centrifugation of the membrane enzyme in a 10 to 50% continuous sucrose gradient. A single protein peak, presenting PNPPase and ATPase activities, was obtained between 35.5 and 41% sucrose.

In order to obtain efficient solubilization of Na,K-ATPase using only C₁₂E₈, the effects of detergent concentration, protein concentration and incubation time were studied. Figure 2 illustrates the variation in protein concentration for a fixed C₁₂E₈ concentration. The amount of solubilized enzyme increased proportionally, reaching a maximum at a 1 mg protein/1 mg detergent ratio. At protein detergent ratios > 1.0 ATPase activity in the supernatant decreased, suggesting that the detergent was not sufficient to solubilize a larger amount of enzyme.

Variation of C₁₂E₈ concentration for a fixed protein concentration in the solubilization of membrane Na,K-ATPase (Figure 3) showed that the amount of solubilized enzyme was maximum (about 98%) when C₁₂E₈ was used at a ratio of 1 mg protein/1 mg detergent. Above this maximum value, the ATPase activity of the solubilized enzyme decreased, but the amount of solubilized protein for C₁₂E₈ concentrations above 1 mg/ml remained constant (Figure 3). This decrease in ATPase activity can be interpreted as inactivation by excess detergent. Under these conditions, the ATPase activity of solubilized enzyme was similar to that detected for the membrane fraction, with

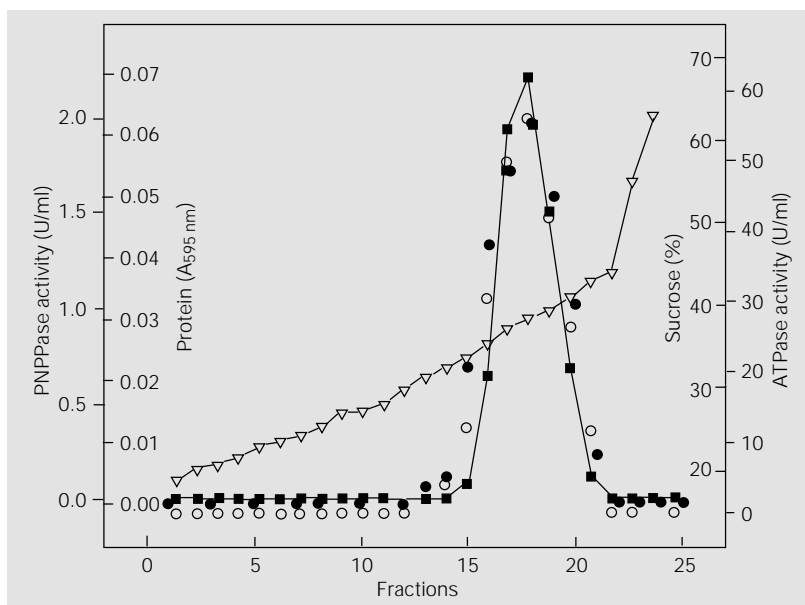


Figure 1. Sucrose density gradient centrifugation of membrane-bound Na,K-ATPase from rabbit kidney outer medulla. A sample of 1.0 mg protein in 20 mM imidazole buffer, pH 6.8, containing 250 mM sucrose, 6 mM Tris and 6 mM EDTA was layered on a continuous sucrose density gradient (10-50%, w/v) in 20 mM imidazole buffer, pH 6.8, and centrifuged at 180,000 g for 2 h using a Hitachi (Model 55P-72) centrifuge with a vertical rotor (PV50TA), at 4°C. Fractions of 0.5 ml were collected and assayed for protein concentration (A₅₉₅ nm; filled circles); refractive index (% sucrose; triangles); PNPPase activity (squares) and ATPase activity (open circles). ATPase, adenosine 5'-triphosphatase; PNPPase, p-nitrophenyl phosphatase.

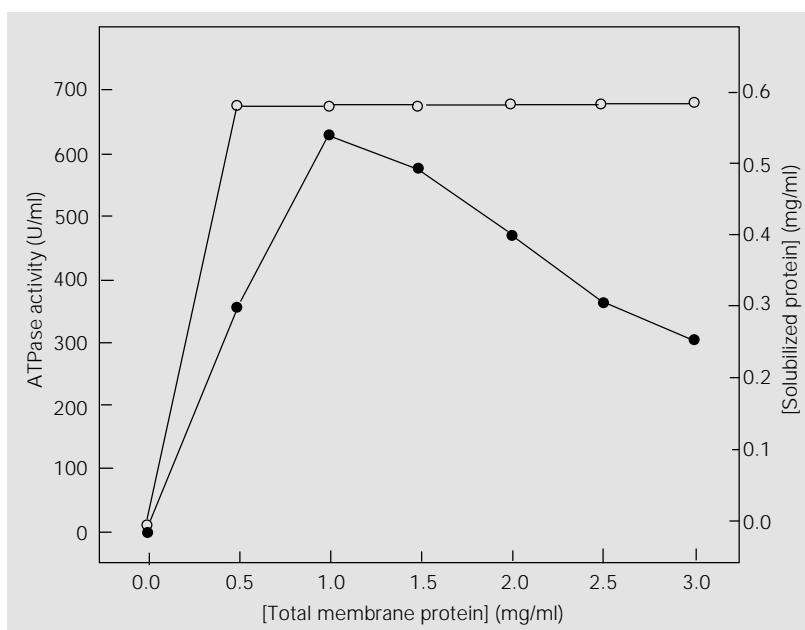


Figure 2. Solubilization of membrane-bound Na,K-ATPase with 1 mg/ml C₁₂E₈. Aliquots containing different concentrations of membrane-bound Na,K-ATPase in 20 mM imidazole buffer, pH 6.8, containing 250 mM sucrose, 6 mM Tris and 6 mM EDTA were mixed at 4°C with 1 mg/ml C₁₂E₈ and centrifuged for 1.5 h at 100,000 g using a Hitachi (Model 55P-72) centrifuge. The supernatant was assayed for ATPase activity (filled circles) and solubilized protein (open circles). ATPase, adenosine 5'-triphosphatase.

values of about 600-700 U/mg. Solubilization of Na,K-ATPase using $C_{12}E_8$ at the 1:1 ratio (w/w) was instantaneous and this pro-

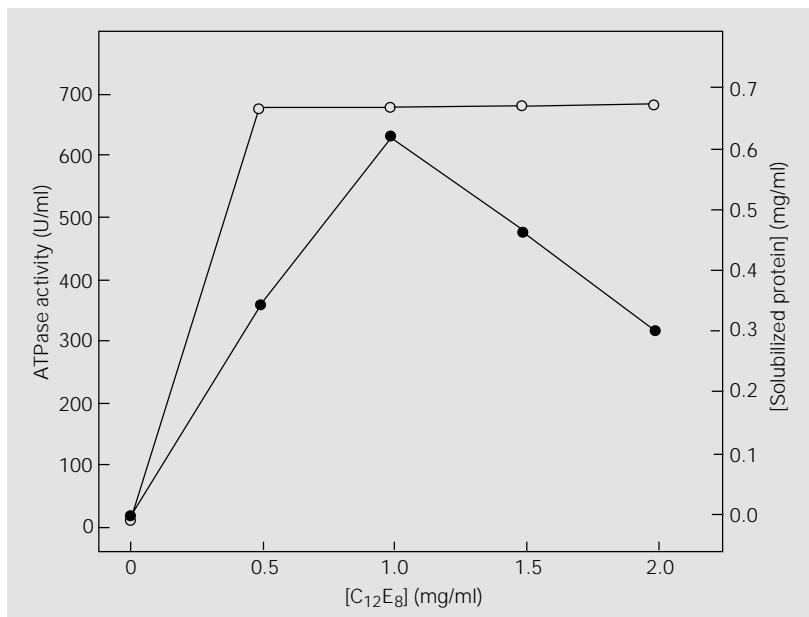


Figure 3. Effect of $C_{12}E_8$ concentration on the solubilization of membrane-bound Na,K-ATPase. Aliquots containing 1 mg of membrane-bound Na,K-ATPase in 20 mM imidazole buffer, pH 6.8, containing 250 mM sucrose, 6 mM Tris and 6 mM EDTA were mixed at 4°C with $C_{12}E_8$ at different concentrations and centrifuged for 1.5 h at 100,000 g using a Hitachi (Model 55P-72) centrifuge. The supernatant was assayed for ATPase activity (filled circles) and solubilized protein (open circles). ATPase, adenosine 5'-triphosphatase; $C_{12}E_8$, dodecyl-octaethyleneglycol.

cess was more efficient at 4°C (data not shown).

$C_{12}E_8$ -solubilized Na,K-ATPase (1 mg of protein/1 mg of detergent) was purified through a Sepharose 6B column (1.4 x 119 cm). The chromatogram obtained presented at least four protein peaks (Figure 4), and at least two peaks presented as much ATPase as PNPPase activity. Fractions 100-138 obtained in peak 11 were pooled, concentrated, analyzed and found to correspond to solubilized and purified Na,K-ATPase. Moreover, this pool presented a specific activity of 930 U/mg, with a recovery of about 21.4% of the total activity present in the outer medulla extract. The protein recovery corresponding to this peak represented about 27.5% of the total protein applied to the column, 17.4% of a membrane fragment and only 1.8% of the total protein present in the outer medulla extract. The purification factor for these purification steps of Na,K-ATPase was about 11.8 (see Table 1).

Non-denaturing PAGE showed that the solubilized extract presented a relatively large number of protein bands. In contrast, the purified sample presented only one discrete

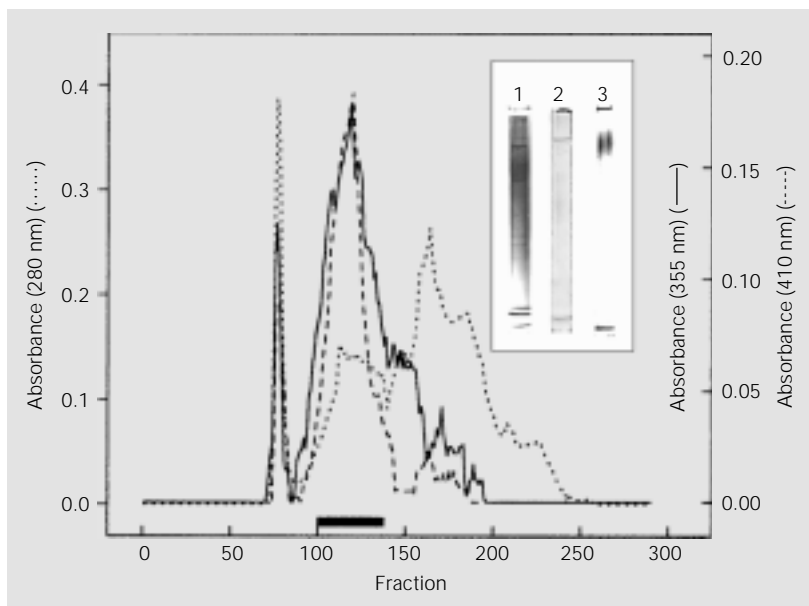


Figure 4. Chromatography of Na,K-ATPase solubilized with $C_{12}E_8$ on a Sepharose 6B column. A sample of Na,K-ATPase solubilized with $C_{12}E_8$ (1:1, w/w) containing 154 U/mg was applied to a Sepharose 6B column (1.4 x 119 cm) equilibrated and eluted with 5 mM Tris-HCl buffer, pH 7.0, containing 1.0 mM EDTA, 150 mM KCl and 0.005 mg/ml $C_{12}E_8$, at a flow rate of 21 ml/h. Fractions of 3.5 ml were collected and aliquots were assayed for protein concentration (dotted line) (A_{280} nm); PNPPase activity (broken line) and ATPase activity (solid line). The fractions indicated (peak II) were pooled and correspond to solubilized and purified Na,K-ATPase. Inset: Non-denaturing polyacrylamide gel electrophoresis of Na,K-ATPase in different purification steps: gel 1 - $C_{12}E_8$ -solubilized extract stained with silver nitrate; gel 2 - purified enzyme (peak II) stained with silver nitrate, and gel 3 - phosphohydrolytic activity of purified enzyme developed with 0.12% (w/v) 1-naphthyl phosphate and 0.12% (w/v) fast blue RR, in the normal kinetic assay medium.

band with relatively diffuse phosphomono-hydrolase activity (Figure 4, inset). These results indicate that the solubilized sample was relatively homogeneous and purified after chromatography through a Sepharose 6B column.

The molecular mass of C₁₂E₈-solubilized and purified Na,K-ATPase determined by chromatography was about 320 kDa (Figure 5). Note that the molecular mass was not corrected for the amounts of phospholipids and detergent that remained bound to the purified enzyme.

In order to determine the integrity of C₁₂E₈-solubilized and purified Na,K-ATPase, some kinetic studies were performed. Figure 6 shows the pH effect of ATP and PNPP hydrolysis by purified Na,K-ATPase. The same optimum pH of about 7.5 was found for both substrates. Table 2 shows the effects of some inhibitors on the ATPase and PNPPase activities of purified Na,K-ATPase. It should be stressed that 5 mM ouabain or 3 μM vanadate totally inhibited both assayed activities. Oligomycin (10 μg/ml) and sodium azide (0.1 mM) inhibited activity by about 70 and 15%, respectively. Note that 10 μM ouabain inhibited Na,K-ATPase activity by 50% (results not shown).

The results reported for pH optimum (Figure 6) and inhibition studies (Table 2) attest to the purity of the enzyme using these solubilization and purification steps.

The dependence on ATP concentration of the rate of hydrolysis by purified Na,K-ATPase at pH 7.5 is shown in Figure 7. Relatively complex results suggested that at least two classes of hydrolyzing sites were involved in these experimental conditions. The high-affinity sites appearing in the range of 1 μM to 0.1 mM ATP correspond to about 15% of total activity ($K_{0.5} = 4 \mu\text{M}$ and $V = 120.9 \text{ U/mg}$) while the low-affinity sites observed above 0.1 mM ATP represent 85% of total activity ($K_{0.5} = 1.4 \text{ mM}$ and $V = 703.9 \text{ U/mg}$). Cooperative effects were found for both hydrolysis sites (0.8 and 0.9, respec-

tively) and inhibition of ATPase activity was observed for ATP concentrations above 3 mM (results not shown).

The dependence on PNPP concentration

Table 1. Extraction, C₁₂E₈ solubilization and purification steps of an Na,K-ATPase from rabbit kidney outer medulla.

Step	Volume (ml)	Protein (mg/ml)	U/ml	U/mg	Yield (%)	Purification
Outer medulla extract	63	11.30	890.4	78.8	100	1
Membrane fragment	53	1.40	702.9	689.2	66.4	7.5
Supernatant of solubilized membrane	64	0.893	409.1	458.1	46.7	5.8
Pellet of solubilized membrane	74.2	0.501	154.1	307.4	20.3	3.9
Concentrate of solubilized extract	35	1.34	382.9	285.8	23.9	3.6
*Peak II	85	0.152	141.4	930.3	21.4	11.8

All assays were carried out in 50 mM HEPES buffer, pH 7.5, containing 3 mM ATP, 10 mM KCl, 5 mM MgCl₂ and 50 mM NaCl, at 37°C, as described in Methods.

*Peak of a Sepharose 6B column.

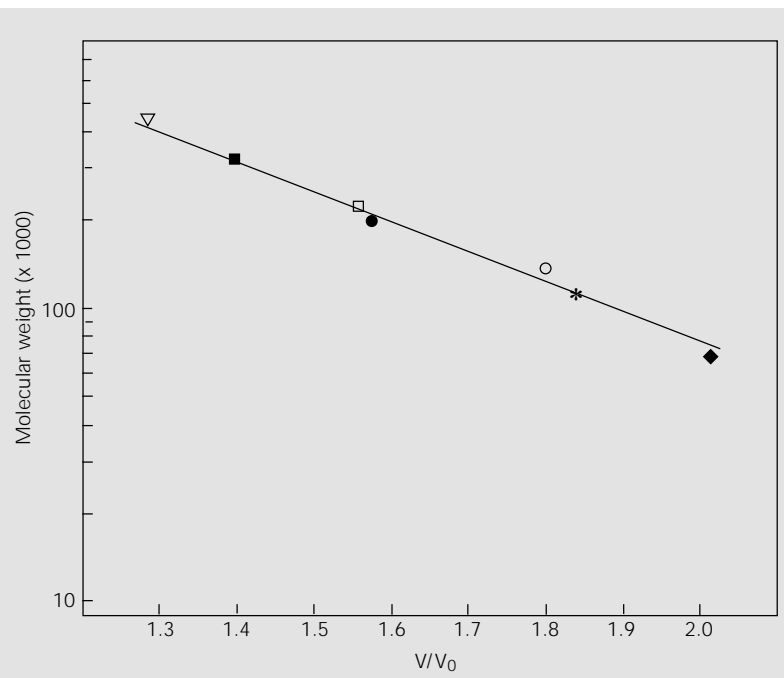


Figure 5. Molecular mass determination of C₁₂E₈-solubilized Na,K-ATPase. Molecular mass was determined using a Sepharose 6B column (0.8 x 133 cm) equilibrated and eluted with 5 mM Tris-HCl buffer, pH 7.0, containing 1 mM EDTA, 150 mM KCl and 0.005 mg/ml C₁₂E₈ as described in Material and Methods. BSA (lozenge); alcohol dehydrogenase (open circle); β-amylase (filled circle); apoferritin tetramer (triangle); apoferritin dimer (open square); apoferritin monomer (asterisk), and solubilized and purified Na,K-ATPase (filled square).

of the rate of hydrolysis by purified Na, K-ATPase at pH 7.5 in the presence of 10 mM MgCl₂ and 15 mM KCl showed a single saturation curve with cooperative effects ($n = 1.3$), $K_{0.5} = 0.74$ mM and

$V = 42.1$ U/mg (results not shown).

Discussion

Obtaining Na,K-ATPase from a highly specialized tissue with enhanced capacity for Na⁺ and K⁺ transport such as the outer medulla of the kidney has the advantage of obtaining enzyme activity in relatively large amounts (7,8,28).

Generally, Na,K-ATPase-rich membranes were obtained by mechanical tissue disruption followed by differential centrifugation steps. However, other methods, such as cloning techniques using the enzyme expressed in different systems, have also been described and used for enzyme production and characterization (14,34).

Kidney outer medulla membrane extraction and homogenization followed by a sucrose gradient resulted in a single protein peak (Figure 1). The fact of not finding any other soluble protein component along the whole gradient suggests that the membrane obtained in the preparation of Na,K-ATPase was homogeneous. However, it should be emphasized that membranes contain many proteins and solubilization and purification methods are necessary for isolation of the pure Na,K-ATPase enzyme (8,19,25,35). The presence of other enzymes in the membrane fraction was easily proven by specific inhibitors such as ouabain. In fact, 5 mM ouabain inhibited only 11.5% of the ATPase activity of the membrane fraction (data not shown).

Therefore, Na,K-ATPase could be separated from the other proteins present in the membrane that can directly or indirectly interfere with the kinetic properties of the enzyme. The development of a rational approach to the solubilization of membrane proteins always requires a review of the literature to determine the detergent type and the conditions that are generally used for the solubilization of the protein of interest (1,3-8,10).

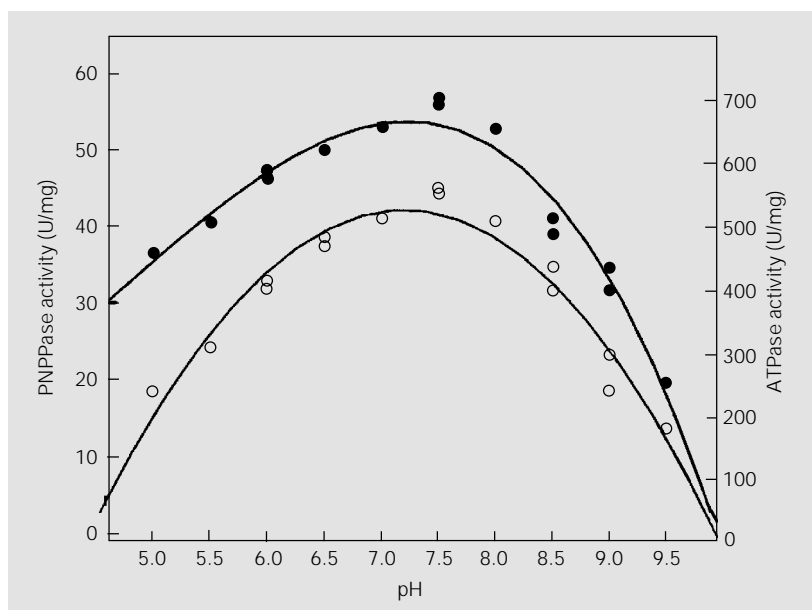


Figure 6. pH sensitivity of the hydrolysis of p-nitrophenyl phosphate (PNPP) and adenosine 5'-triphosphate (ATP) by Na,K-ATPase solubilized with C₁₂E₈ (1:1, w/w) and purified on a Sepharose 6B column. PNPPase (filled circles) and ATPase activities (open circles) are shown. Assays were buffered with 50 mM buffer containing 5 mM MgCl₂, 10 mM KCl and 50 mM NaCl and the substrate, i.e., 10 mM PNPP or 3 mM ATP (for details, see Material and Methods). The reaction was started by the addition of 3.5 µg protein.

Table 2. Effect of several inhibitors on adenosine 5'-triphosphatase (ATPase) and p-nitrophenyl phosphatase (PNPPase) activities of purified Na,K-ATPase from rabbit kidney outer medulla.

Inhibitors	Concentration	Inhibition (%)	
		ATPase	PNPPase
Ouabain	5 mM	99.1	99.9
Oligomycin	1 µg/ml	81.1	63.7
Sodium azide	100 µM	14.5	16.1
Vanadate	3 µM	98.5	93.2

All assays were carried at 37°C as described in Methods. ATPase activity was assayed in 50 mM HEPES buffer, pH 7.5, containing 3 mM ATP, 10 mM KCl, 5 mM MgCl₂ and 50 mM NaCl, using 10 µg of enzyme. Specific activity of 100% corresponds to 603.2 U/mg. PNPPase activity was assayed in 50 mM HEPES buffer, pH 7.5, containing 10 mM PNPP, 15 mM KCl and 10 mM MgCl₂, using 10 µg of enzyme. Specific activity of 100% corresponds to 38.5 U/mg.

The treatment of Na,K-ATPase membrane fractions with different concentrations of SDS resulted in a time-dependent inactivation of the enzyme activity (5). This inactivation is also determined by the SDS/protein ratio. Moreover, apparently many SDS molecules are involved in the inactivation of a single Na,K-ATPase molecule. The use of SDS is only recommended for the selective removal of some contaminant proteins of the membrane without significantly affecting the Na,K-ATPase molecule, which remains in the membrane fraction after this treatment (28).

The incubation of the Na,K-ATPase-rich membrane fraction with SDS is also recommended in the presence of ATP because this substrate protects the enzyme structure against inactivation. Another important factor is that the purification procedure using SDS should be combined with density gradient centrifugation steps. This slow purification procedure resulted in a significant loss and irreversible inactivation of ATPase activity, even when controlling the detergent/protein relationship, temperature, pH, incubation time and buffer ionic forces (7,28).

Our method using only C₁₂E₈ is based on the methodology described by Santos and Ciancaglini (5) and others who used it combined with other detergents (7,10). The advantage of this method is the considerable reduction in the time needed to obtain the purified enzyme, which is solubilized instantaneously and purified in a single chromatographic step and has a smaller denaturing effect on the solubilized enzyme.

The amount of solubilized enzyme activity was maximum, about 98%, when C₁₂E₈ was used at a ratio of 1 mg enzyme/1 mg detergent (Figures 2 and 3). Under these conditions, the specific ATPase activity of the solubilized Na,K-ATPase was in the range of 600-700 U/mg, which is similar to the activity of the membrane enzyme, as reported by Cornelius (7) for the enzyme obtained from rabbit kidney. However, activi-

ties 20-30-fold higher have been reported both for membrane-bound and solubilized enzyme (8,10,28). In our study the initial activity for the membrane-bound enzyme was lower than reported by others but a purification factor of about 6-10-fold, which is typical for enzyme isolated from membrane of specialized tissue, suggests a selective extraction of Na,K-ATPase.

It should be noted that quite different values of specific ATPase activity can be obtained in different conditions for tissue source, solubilization and purification, as well as methods for the determination of enzymatic activity (4,5,7,10,36).

The enzyme obtained from the kidney external medulla showed a purification factor of about 11 (Figure 4 and Table 1). In a single chromatographic step, rabbit kidney ($\alpha\beta$)₂ protomer of Na,K-ATPase was purified and separated from non-solubilized pro-

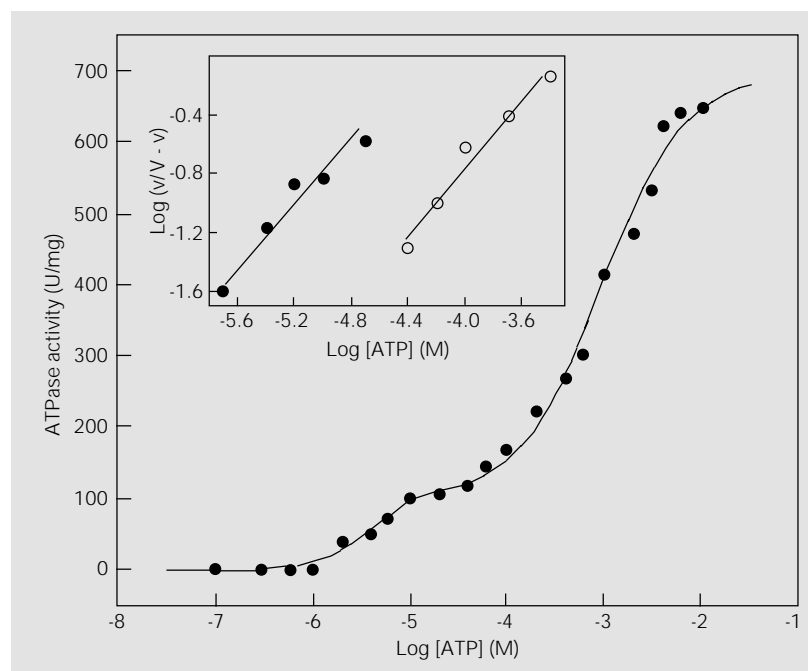


Figure 7. Effect of adenosine 5'-triphosphate (ATP) concentration on ATPase activity of Na,K-ATPase solubilized with C₁₂E₈ (1:1, w/w) and purified on a Sepharose 6B column. Assays were buffered with 50 mM HEPES buffer, pH 7.5, containing ATP, 5 mM MgCl₂, 10 mM KCl and 50 mM NaCl. The reaction was started by the addition of 3.5 µg protein. Inset: Hill plot for the respective results: high-affinity sites (filled circles) and low-affinity sites (open circles).

teins that were excluded in the column “void”, and other proteins that were also solubilized by the detergent, and from different other forms of association between the α and β subunits and even the individual subunits (10,28). The purification factor, the specific activity and the stability of the enzyme solubilized and purified by gel filtration obtained by several authors with Na,K-ATPases from different sources show different values. This fact can be attributed to several causes, including i) variation in the methodology for the extraction and preparation of the membrane fraction; ii) differences in the determination of activity such as different substrate concentrations, buffer, and metal ion used at different concentrations, and iii) differences in the duration and in the temperature of the solubilization and purification steps (7,10,28).

In the present study the molecular mass found for rabbit kidney Na,K-ATPase solubilized with $C_{12}E_8$ and purified on a Sepharose 6B column was about 320 kDa (Figure 5), suggesting an association of the $(\alpha\beta)_2$ type, considering the molecular mass of the α and β subunits to be about 100 and 57 kDa, respectively. Note that, under our experimental conditions, amounts of phospholipids and detergent that remained bound to the purified enzyme were not taken into account. Moreover, when the purification step used 10-fold more detergent, a significant decrease in the activity of the fractions corresponding to the oligomer $(\alpha\beta)_2$ was observed (results not shown). Another important factor which may be responsible for the loss of catalytic activity of the enzyme can be attributed to the oligomer delipidation that might occur in the presence of high detergent concentrations (7,10).

Although most of the structural studies indicate a 1:1 stoichiometry of the α and β subunits, the exact quaternary structure of Na,K-ATPase, as well as its functional association with the membrane, are not understood. There are controversies about whether

the enzyme exists as a protomer ($\alpha\beta$) (21-23,37) or as a diprotomer $(\alpha\beta)_2$ involving a balance between protomer-diprotomer, or even in the form of oligomers $(\alpha\beta)_n$ (24,38).

Rabbit kidney Na,K-ATPase, when solubilized with $C_{12}E_8$ and purified by fractionated centrifugation, showed that the solubilized form of the enzyme consists predominantly (80-85%) of associations of the $(\alpha\beta)$ type, with molecular mass between 140 and 170 kDa (7,20-22,28). However, when other isolation methods are used, such as gel filtration, a relatively stable association of subunit $(\alpha\beta)_2$ types and/or superior oligomers with molecular mass of 257-380 kDa was observed. It is important to note that these associations are necessary for the enzyme to have a catalytic activity and are related to the incubation time with the detergent and to detergent concentration (10,20-22,24).

The apparent optimum pH for ATP and PNPP hydrolysis by Na,K-ATPase (Figure 6) was 7.5 for the same substrate, and was similar to that reported by other investigators with enzymes obtained from several tissues (28). The effect of some Na,K-ATPase inhibitors showed that the solubilized enzyme is completely inhibited by 5 mM ouabain or 3 μ M vanadate (see Table 2), as described by several authors (8,13,19,25). However, oligomycin does not completely inhibit the activity of Na,K-ATPase (80%), and it is known that the effect of this inhibitor depends on the enzyme orientation and the presence of sodium ions (8,13,25).

Finally, the inhibition of only about 15% of Na,K-ATPase by sodium azide confirmed the absence of FoF1-ATPase (25). It is important to emphasize that the effect of these inhibitors depends on the presence of divalent metal ions (sodium, potassium and/or magnesium) and the substrate used in the reaction medium, as well as on the tissue origin of Na,K-ATPase (8,25).

Kinetic results for $C_{12}E_8$ -solubilized and purified Na,K-ATPase (Figure 7) revealed two classes of ATP-hydrolyzing sites under

these experimental conditions, one in the micromolar range (high-affinity site) and the other in the millimolar range (low-affinity site), presenting negative cooperativity.

Results using microsomal fractions of several vertebrate tissues showed that the stimulation of Na,K-ATPase activity by ATP occurs through two-phase curves, one with $K_{0.5}$ from 0.1 to 1 μ M and the other with $K_{0.5}$ from 0.01 to 0.4 mM, which suggests the existence of two sites of substrate hydrolysis (21,39). Dual effects were also found on ATP hydrolysis by C₁₂E₈-solubilized and purified Na,K-ATPase with $K_{0.5}$ values of about 0.1 and 4 μ M for the high-affinity site and of about 0.1 and 0.5 mM for the low-affinity site, both with negative cooperativity, suggesting that the enzyme environment or oligomer formation is very important for substrate hydrolysis (21-23,40). It should be emphasized that the contribution of the high-affinity site to the ATPase specific activity of the enzyme generally corresponds to values about 1 to 10% of the total activity, which hinders its kinetic characterization

(21-23,39). It is very difficult to determine whether these arose from the presence of more than one ATP site per ($\alpha\beta$) protomer or from a single site whose function and affinity change during the catalytic cycle (21-24,40), and the functional relationship between the two hydrolysis sites and their behavior during the reaction cycle remains to be explored.

The kinetic data shown for rabbit kidney Na,K-ATPase solubilized with C₁₂E₈ and purified suggest that the enzyme molecule is preserved and presents some important characteristics reported by some investigators, i.e., stability, ($\alpha\beta$)₂ protomer association, biphasic behavior during ATP hydrolysis, and ouabain and vanadate inhibition, which makes the method suitable for obtaining the enzyme for studies in reconstitution systems.

Acknowledgments

We thank Dr. Richard John Ward and Mrs. Priscila C. Ciancaglini for revising the manuscript.

References

- Helenius A, McCaslin DR, Fries E & Tanford C (1979). Properties of detergent. *Methods in Enzymology*, 56: 734-749.
- Tanford C (1980). *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*. 2nd edn. Wiley-Interscience, New York, NY, USA.
- Koepsell H (1986). Methodological aspects of purification and reconstitution of transport proteins from mammalian plasma membranes. *Reviews of Physiology, Biochemistry and Pharmacology*, 104: 65-137.
- Silvius JR (1992). Solubilization and functional reconstitution of biomembrane components. *Annual Review of Biophysics and Biomolecular Structure*, 21: 323-348.
- Santos HL & Ciancaglini P (2000). A practical approach to the choice of a suitable detergent and optimal conditions to solubilize a membrane protein. *Biochemical Education*, 28: 178-182.
- Le Maire M, Champeil P & Moller JV (2000). Interaction of membrane proteins and lipids with solubilizing detergents. *Biochimica et Biophysica Acta*, 1508: 86-111.
- Cornelius F (1991). Functional reconstitution of the sodium pump. Kinetics of exchange reactions performed by reconstituted Na,K-ATPase. *Biochimica et Biophysica Acta*, 1071: 19-66.
- Skou JC & Esmann M (1992). The (Na⁺, K⁺)-ATPase. *Journal of Bioenergetics and Biomembranes*, 24: 249-261.
- Jackson RL, Verkleij AJ, Van Zoelen EJJ, Lane LK, Schwartz A & Van Deenen LLM (1980). Asymmetric incorporation of Na,K-ATPase into phospholipid vesicles. *Archives of Biochemistry and Biophysics*, 200: 269-278.
- Brotherus JR, Jacobsen L & Jorgensen PL (1983). Soluble and enzymatically stable Na,K-ATPase from mammalian kidney consisting predominantly of protomer $\alpha\beta$ -units. Preparation, assay and reconstitution of active Na,K transport. *Biochimica et Biophysica Acta*, 731: 290-303.
- Esmann M & Skou JC (1985). Occlusion of Na⁺ by the Na,K-ATPase in the presence of oligomycin. *Biochemical and Biophysical Research Communications*, 127: 857-863.
- Jorgensen PL (1992). Functional domains of Na,K-ATPase: conformational transitions in the α -subunit and ion occlusion. *Acta Physiologica Scandinavica*, 146: 89-94.
- Skou JC (1998). The identification of the sodium-potassium pump (Nobel Lecture). *Angewandte Chemie International Edition*, 37: 2320-2328.
- Pressley TA (1996). Structure and function of the Na,K pump: ten years of molecular biology. *Mineral and Electrolyte Metabolism*, 22: 264-271.
- Jorgensen PL, Nielsen JM, Rasmussen JH & Pedersen PA (1998). Structure-

- function relationships based on ATP binding and cation occlusion at equilibrium in Na,K-ATPase. *Acta Physiologica Scandinavica*, 163: 79-87.
16. Jorgensen PL, Rasmussen JH, Nielsen JM & Pedersen PA (1997). Transport-linked conformational changes in Na,K-ATPase. Structure-function relationships of ligand binding and E1-E2 conformational transitions. *Annals of the New York Academy of Sciences*, 834: 161-174.
 17. Lingrel JB, Croyle LM, Woo AL & Arguello JM (1998). Ligand binding sites of Na,K-ATPase. *Acta Physiologica Scandinavica*, 163: 69-77.
 18. O'Brien WJ, Lingrel JB & Wallick ET (1994). Ouabain binding kinetics of the rat alpha two and alpha three isoforms of the sodium-potassium adenosine triphosphate. *Archives of Biochemistry and Biophysics*, 310: 32-39.
 19. Emery AM, Billingsley PF, Ready PD & Djamgoz MBA (1998). Insect Na,K-ATPase. *Journal of Insect Physiology*, 44: 197-209.
 20. Brotherus JR, Moller JV & Jorgensen PL (1981). Soluble and active renal Na,K-ATPase with maximum protein molecular mass $170,000 \pm 9,000$ daltons: formation of larger units by secondary aggregation. *Biochemical and Biophysical Research Communications*, 100: 146-154.
 21. Ward DG & Cavieres JD (1993). Solubilized $\alpha\beta$ Na,K-ATPase remains protomeric during turnover yet shows apparent negative cooperativity toward ATP. *Proceedings of the National Academy of Sciences, USA*, 96: 5332-5336.
 22. Ward DG & Cavieres JD (1996). Binding of 2'-(3')-O-(2,4,6-trinitrophenyl)ADP to soluble alpha beta protomers of Na,K-ATPase modified with fluorescein isothiocyanate. Evidence for two distinct sites. *Journal of Biological Chemistry*, 271: 12317-12321.
 23. Ward DG & Cavieres JD (1998). Photoin-activation of fluorescein isothiocyanate-modified Na,K-ATPase by 2'-(3')-O-(2,4,6-trinitrophenyl)8-azidoadenosine 5'-diphosphate. *Journal of Biological Chemistry*, 273: 14277-14284.
 24. Linnertz H, Urbanova P, Obsil T, Herman P, Amler E & Schoner W (1998). Molecular distance measurements reveal an (alpha beta)(2) dimeric structure of Na,K-ATPase - high affinity ATP binding site and K⁺-activated phosphatase reside on different alpha-subunits. *Journal of Biological Chemistry*, 273: 28813-28821.
 25. MacGregor SE & Walker JM (1993). Inhibitors of the Na,K-ATPase. *Comparative Biochemistry and Physiology*, 105C: 1-9.
 26. Kasturi R, Yuan J, McLean LR, Margolies MN & Ball WJ (1998). Identification of a model cardiac glycosid receptor: comparisons with Na,K-ATPase. *Biochemistry*, 37: 6658-6666.
 27. Fedosova NU, Cornelius F & Klodos I (1998). E2P phosphoforms of Na,K-ATPase. I. Comparison of phosphointermediates formed from ATP and Pi by their reactivity toward hydroxylamine and vanadate. *Biochemistry*, 37: 13634-13642.
 28. Jorgensen PL (1988). Purification of Na,K-ATPase. Enzyme sources, preparative problems and preparation from mammalian kidney. *Methods in Enzymology*, 156: 29-42.
 29. Read SM & Northcote DH (1981). Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Analytical Biochemistry*, 116: 53-64.
 30. Davis BJ (1964). Disc electrophoresis II - Method and application to human serum proteins. *Annals of the New York Academy of Sciences*, 121: 404-427.
 31. Blum H, Beir H & Gross HJ (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, 8: 93-99.
 32. Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
 33. Leone FA, Degreve L & Baranauskas JA (1992). Sigraf-A versatile computer-program for fitting enzyme kinetic data. *Biochemical Education*, 20: 94-96.
 34. Pedersen PA, Rasmussen JH & Jorgensen PL (1996). Consequences of mutations to the phosphorylation site of the alpha-subunit of Na,K-ATPase for ATP binding and E1-E2 conformational equilibrium. *Biochemistry*, 35: 16085-16093.
 35. Yingst DR, Yang SY & Schiebinger R (1998). Purification of active Na,K-ATPase using a new ouabain-affinity column. *American Journal of Physiology*, 275C: 1167-1177.
 36. Rigaud JL, Pitard B & Levy D (1995). Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. *Biochimica et Biophysica Acta*, 1231: 223-246.
 37. Jorgensen PL & Andersen JP (1986). Structural basis for E1-E2 conformational transitions in Na,K-pump proteins. *Journal of Membrane Biology*, 103: 95-120.
 38. Tsuda T, Kaya S, Yokoyama T & Taniguchi K (1997). Are pyridoxal and fluorescein probes in lysine residues of α -chain in Na⁺,K⁺-ATPase sensing ATP binding? *Annals of the New York Academy of Sciences*, 834: 186-192.
 39. Robinson JD (1976). Substrate sites for the Na,K dependent ATPase. *Biochimica et Biophysica Acta*, 429: 1006-1019.
 40. Thoenges D, Linnertz H & Schoner W (1997). A two-site model of interacting ATP sites. *Annals of the New York Academy of Sciences*, 834: 322-326.