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Oligosaccharide Organization on the β-subunits of Pig Kidney Na^+/K^+-ATPase

E. AMLER[1,2], R. STAFFOLANI[2], J. BARANSKA[3], T. OBŠIL[1], P. URBANOVÁ[1], E. BERTOLI[2], L. MAZZANTI[2]

[1]*Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic,* [2]*Institute of Biochemistry, University of Ancona, Ancona, Italy and* [3]*Nencki Institute of Experimental Biology, Warsaw, Poland*

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Summary

The distance between the β-subunits of Na^+/K^+-ATPase isolated from pig dark red kidney medulla was determined by Förster energy transfer. First, oligosaccharides of the β-subunit were shown to be labelled with three fluorophores: Lucifer yellow (LY), Lissamine rhodamine B sulfonyl hydrazine (LRSH) and Cascade blue (CB). Further, LY and LRSH were used as the donor and the acceptor, respectively, for Förster energy transfer studies to determine the localization of the β-subunit in the native enzyme which is known to be formed as a tetramer $(\alpha\beta)_2$. It was found that the β-subunits in the functional enzyme complex in the membrane are not localized next to each other but are spatially separated. The distance between fluorophores covalently attached to the β-subunits was found to be 5.1 nm. This conclusion was confirmed by measurements with another donor-acceptor pair CB-LY. The results also support the idea of a direct interaction of the β-subunit with the extracellular part of the α-subunit. These interactions were modified in the presence of millimolar concentrations of magnesium ions. This indicates a crucial role of magnesium in extracellular interactions between the alpha and beta subunits.

Key words
Na^+/K^+-ATPase – Quartenary structure – Förster energy transfer – β-subunit

Abbrevations
LY – Lucifer yellow, LRSH – Lissamine rhodamine sulfonyl hydrazine, CB – Cascade blue, MERC – β-mercaptoethanol, SDS – sodium dodecyl sulfate, FET – Förster energy transfer, PAGE – polyacrylamide gel electrophoresis

Introduction

Na^+/K^+-ATPase is a plasma membrane protein whose primary physiological function is to maintain the Na^+ and K^+ cation gradients across animal cell membranes. Despite years of intense study, the molecular mechanism of this pump and its regulation are still not fully understood. The enzyme is known to be composed of at least two subunits, the catalytic α-subunit (Mr = 112 000) and the smaller glycoprotein β-subunit (Mr = 45 000, including its oligosaccharides). There may also be an associated low-molecular-weight polypeptide gamma (Collins and Leszyk 1987). Knowledge of the amino acid sequence of cloned cDNAs has provided us with information on the folding of the main subunits of Na^+/K^+-ATPase in the membrane (for reviews see Chow and Fortes 1995, Kotyk and Amler 1995). The α-subunit carries all presently known functional properties of the enzyme, such as the ability to hydrolyze ATP, to bind Na^+ and K^+ and cardiac glycosides, specific inhibitors of Na^+/K^+-ATPase (see e.g. Chow and Fortes 1995, Anner 1985). The β-subunit, on the other hand, does not appear to have a well-defined role, although its presence has been identified even in crystalline Na^+/K^+-ATPase (e.g. Zampighi *et al.* 1986). In

addition, the β-subunit is a glycoprotein, and the role of the sugar moiety remains obscure, as is the case with many other glycoproteins. Interestingly, in *Xenopus* oocytes, efficient expression of functional pumps was only observed after injection of both alpha and beta mRNAs (Noguchi *et al.* 1987). This result clearly demonstrates that only an enzyme complex composed of α- and β-subunits becomes functionally active. Either subunit alone has no catalytic or transport activity. The reason why the coexistence of the α- and β-subunit is essential for forming an active enzyme is not known, although it is becoming increasingly clear that subunits assembly is likely to play a crucial role in the regulation of the export of multimeric proteins from the endoplasmic reticulum and their functional maturation.

Even less is known about the organization of subunits in the functional native enzyme complex. Various biophysical approaches have been used to investigate the functional assembly of the enzyme (Ottolenghi and Ellory 1983, Cavieres 1988, Norby and Jensen 1989, Pachence *et al.* 1987, Brotherus *et al.* 1983, Skriver *et al.* 1981, Beall *et al.* 1989). However, there was no clear conclusion even about the minimum functional unit of Na^+/K^+-ATPase: whether such a unit was $(\alpha\beta)$ or $(\alpha\beta)_2$. Our recent fluorescence study (Amler *et al.* 1992) supported the idea of the $(\alpha\beta)_2$ oligomer. A Förster energy transfer study also showed the distance between high affinity ATP-binding sites of the two α-subunits in the complex to be 5.6 nm. However, where the β-subunit is localized in the native functional complex remains unresolved.

In the present study we made extensive use of fluorescent labels of carbohydrates (LY, LRSH, CB) as donor-acceptor pairs to carry out structural and dynamic measurements of β-β interactions using Förster energy transfer. These fluorophores were chosen because they have significant spectral overlap giving a large overlap integral for an efficient energy transfer.

Methods

Enzyme assays and subunit preparation

Na^+/K^+-ATPase was purified from pig kidney as previously described (Jorgensen and Andersen 1988). Membrane protein concentrations were determined by Lowry's method (1951) using bovine serum albumin as standard. The Na^+/K^+-ATPase activity was assessed as described previously (Linnertz *et al.* 1995, 1997). The α- and β-subunits were separated by heating (10 min at 100 °C) of Na^+/K^+-ATPase (about 1 mg/ml) in 0.1 M Tris-HCl, (pH 7.4), 1 mM EDTA, 3 mM ATP and 3 % SDS. For disruption of disulfide bridges 0.3 M mercaptoethanol was added to the mixture. Protein purity was confirmed by PAGE (Laemmli 1970) and the labelling with fluorophores in the gels was examined using a UV transilluminator. The proteins were visualized in the gel by Coomassie brilliant blue staining.

Absorption and steady-state fluorescence measurements

Absorption measurements were done on a Uvikon 810 spectrophotometer. For stoichiometry purposes, an adequate amount of SDS was added to the samples to clarify the solution. The steady-state fluorescence data were taken either on a Perkin-Elmer LS-5 or a Perkin-Elmer 7300 fluorometer (Amler *et al.* 1994).

Dynamic fluorescence measurements

Frequency-domain measurements were done to obtain the fluorescence decay lifetimes for the Förster energy transfer efficiency determination using an ISS K2 multifrequency cross-correlation phase and modulation fluorometer. Phase and modulation measurements were performed in the range of 5 to 200 MHz with a xenon lamp and monochromator as a source of excitation light. Fluorescence emissions from the samples were observed through appropriate Schott filters. The temperature of the samples was maintained at 25 °C. An aqueous suspension of glycogen was used as the reference sample. Corrections for background fluorescence and/or light scattering were taken by doing frequency-domain measurements with nonlabelled enzyme as control and then correcting the data in the manner described by Lakowicz *et al.* (1987). The experimental data were analyzed using a nonlinear least-squares routine for multiexponential fitting (and/or lifetime distribution) and additional software available at the Institute of Biochemistry, University of Ancona. The parameter χ_R^2 was used for judging the quality of the fit of the phase (ϕ) and modulation (M) data.

Förster energy transfer measurements

The distances (R) between donor and acceptor pairs were calculated from the FET measurements. The apparent efficiency of transfer E was calculated by the decrease in the donor lifetime of the excited state (Abbott *et al.* 1991). The orientation factor was taken as $\kappa^2 = 2/3$ for all our calculations because the anisotropies of bound fluorophores were sufficiently low (Dale and Eisinger 1976). The distance between the donor and acceptor pairs was calculated upon determination of the R_O value and the efficiency of the energy transfer as:

$$R^6 = R_O^6(1/E-1) \quad (1)$$

Results

Fluorescent labelling of the β-subunit

Na^+/K^+-ATPase isolated from pig dark red outer kidney medulla was labelled with three fluorophores: Lucifer yellow (LY), Lissamine

rhodamine sulfonyl hydrazine (LRSH) and Cascade blue (CB). The labelling procedure was essentially the same that was described previously for labelling of the β-subunit with LY (Amler *et al.* 1992, Lee and Fortes 1985). The results are shown in Figure 1. Polyacrylamide gel electrophoresis of the enzyme was employed to confirm the specificity of the labelling by LY on the β-subunit and to demonstrate that also the labelling of the other fluorophores used (LRSH and CB) virtually all took place on the β-subunit. No labelling was observed on the α-subunit. The activity of the enzyme was slightly affected by the labelling procedure a less than 10 % reduction in ATPase activity) but the actual reaction with the fluorophores had no additional effect on enzyme activity (Table 1).

Determination of the fluorescent properties of free and enzyme-bound fluorophores

a) *Free fluorophores*

Absorption spectra and some fluorescence properties of LY (a free probe) have already been published (Lee and Fortes 1985). Absorption of LRSH (free fluorophore) was pH-sensitive and the absorbance was much lower at pH 9.0 (0.1 M Tris-HCl, 1 mM EDTA, pH 9.0) than in 0.1 M Tris-HCl, 1 mM EDTA at pH 7.4. No shift of the peaks was observed (Fig. 2). These data indicate the effect of pH on the ground state. A shift of the peak, however, was clearly visible in the fluorescence spectra. The maximum of the fluorescence spectrum was blue-shifted (to lower wavelengths) at pH 9.0 (0.1 M Tris-HCl, 1 mM EDTA, pH 9.0) compared to the fluorescence spectrum in 0.1 M Tris-HCl, 1 mM EDTA at pH 7. The spectral shift (a specific effect due to acid-base chemistry) was substantial (about 9 nm) (Fig. 3). In addition, the emission spectrum of LRSH, was also sensitive to the general solvent effects. We observed a red shift of the fluorescence emission spectra with higher polarity of the solvents (Table 2). Absorption and fluorescence spectra of CB in 0.1 M Tris-HCl, 1 mM EDTA at pH 7.4 are shown in Fig. 2b. The fluorescence spectrum is uncorrected and normalized to unity. The fluorescence emission spectrum of CB is seen to overlap with the absorption spectrum of LY (absorption maximum at $\lambda = 428$ nm). While CB did not prove to be as pH-sensitive as LRSH, its fluorescence emission spectrum was sensitive to the polarity of the surrounding environment. Increasing solvent polarity also resulted in a shift of the emission spectrum to longer wavelengths (a red shift). The positions of the higher energy maximum are presented in Table 2; one can observe a 20 nm red shift between the fluorescence emission maximum in dimethylformamide and water.

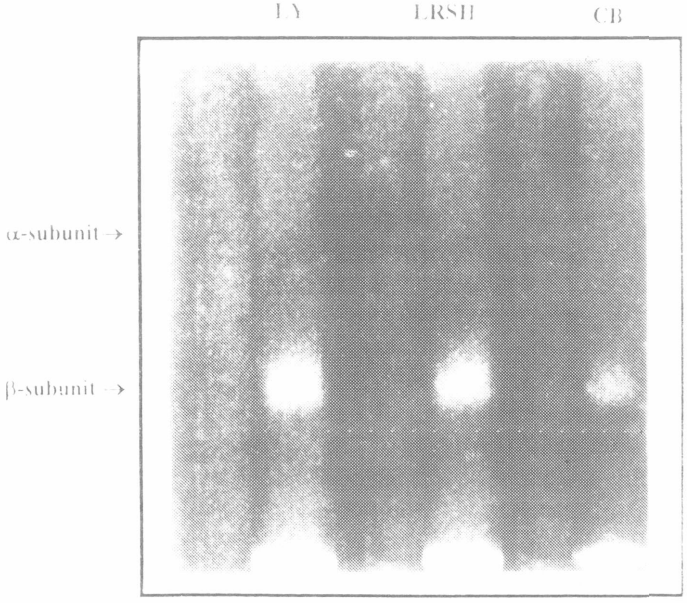

Fig. 1. *Polyacrylamide gel electrophoresis of labelled Na^+/K^+-ATPase*

Table 1. The activity of Na^+/K^+-ATPase after labelling with fluorophores

Fluorophore	None	LY	LRSH	CB
Activity (%)	91±7	90±6	93±8	91±7

Means ± S.D. were calculated from two independent experiments with three vials for each assay. The untreated (intact) enzyme activity was taken as 100 %.

b) *Bound fluorophores*

Upon covalent binding to the enzyme's carbohydrate, the absorption and fluorescence emission spectra of LY were essentially unchanged (data not shown). This suggested that the bound probe remained largely exposed to the polar, aqueous

environment of the buffer. A completely different situation, however, was observed for LRSH. Absorption and fluorescence spectra of the LRSH-labelled Na^+/K^+-ATPase were similar to the spectra of the free label (data not shown). Surprisingly, however, the peak of the fluorescence spectra of LRSH-labelled Na^+/K^+-ATPase was blue-shifted when measured in 0.1 M Tris-HCl, 1 mM EDTA at pH 7.4 (about 3 nm compared to the spectrum of the free fluorophore in the same buffer). This resembled the effect of higher pH on the free labell. No such effect was observed for LY. It can conclude that the fluorescence properties of LRSH were highly influenced by its enzyme microenvironment, one possible explanation being that the fluorophore was localized at a locally higher pH or, alternatively, at lower polarity. In terms of rotational freedom of the bound fluorophores, the anisotropies of bound labels were found to be significantly higher ($r=0.16$ for LY-Na^+/K^+-ATPase and $r=0.14$ for LRSH-Na^+/K^+-ATPase; Table 3) than the anisotropy of free labels which was $r=0.01$ for both free LY and LRSH. This clearly indicated an immobilization of the labels on the oligosaccharides of the β-subunit. Anisotropy of LY-labelled Na^+/K^+-ATPase was even higher than the anisotropy of the LRSH-labelled enzyme.

Table 2. The shift of the maximum of fluorescence emission spectra of free fluorophores in different solvents.

Solvent	Water	Methanol	Acetone	Dimethylformamide
LRSH	598	582	576	576
CB	419	407	402	399

Excitation wavelengths were 556 nm for LRSH and 376 nm for CB. The maxima of uncorrected spectra are presented in nm. The solvent polarity decreases from left to right.

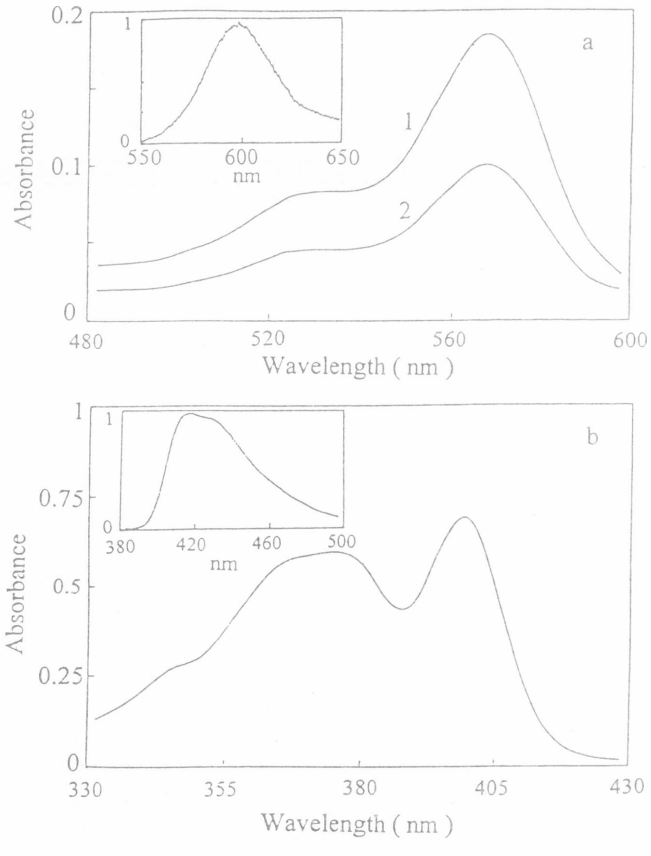

Fig. 2. *Absorption and fluorescence spectra (inserts) of LRSH (a) and CB (b) as free fluorophores. LRSH was dissolved in dimethylformamide (DMF) and subsequently in 0.1 M Tris-HCl, pH 7.4 (line 1) or 0.1 M Tris-HCl, pH 9.0 (line 2). The final concentration of DMF was kept below 0.5 %. CB was dissolved in water and the spectrum was taken in 0.1 M Tris-HCl, pH 7.4. The final concentrations of LRSH for absorbance measurements were 2.5 mM; for fluorescence determinations the concentrations were 0.2 mM. The fluorescence spectrum (inset) is uncorrected. Excitation wavelengths were $\lambda=556$ nm. Temperature of measurement was 22 °C.*

To discuss these anisotropy values, one needs to know the initial anisotropies (theoretical maximum – this anisotropy depends only on the mutual orientation of absorption and emission dipoles of the respective fluorophore) and the average lifetimes of the excited states of LY-Na^+/K^+-ATPase and LRSH-Na^+/K^+-ATPase. The initial anisotropies were determined in glycerol solutions with increasing viscosity and were $r_0=0.33$ for LY and $r_0=0.39$ for LRSH. Nevertheless, this did not allow us to make any conclusions about the rotational restrictions of bound fluorophores because the lifetimes of the excited states of the fluorophores were still unknown. The average lifetimes of bound fluorophores in a liquid buffer (0.1 M Tris-HCl, 1 mM EDTA, pH 7.4) were determined using a phase fluorometer with modulation

frequencies over the range of 5–200 MHz. The polarizer and analyzers were placed at the magic angle and a glycogen solution was used as reference. The average lifetime of the excited state was several times higher for LY-Na^+/K^+-ATPase ($\tau = 5.0$ ns) than for LRSH-Na^+/K^+-ATPase ($\tau = 1.5$ ns). Consequently, the lower lifetime with simultaneously lower anisotropy of LRSH-Na^+/K^+-ATPase indicated a higher rotational freedom of bound LRSH compared to bound LY. Taking into account that we found LY as a water-exposed fluorophore and LRSH as a label localized close to the protein (and, thus, influenced by its microenvironment), this is somewhat unexpected.

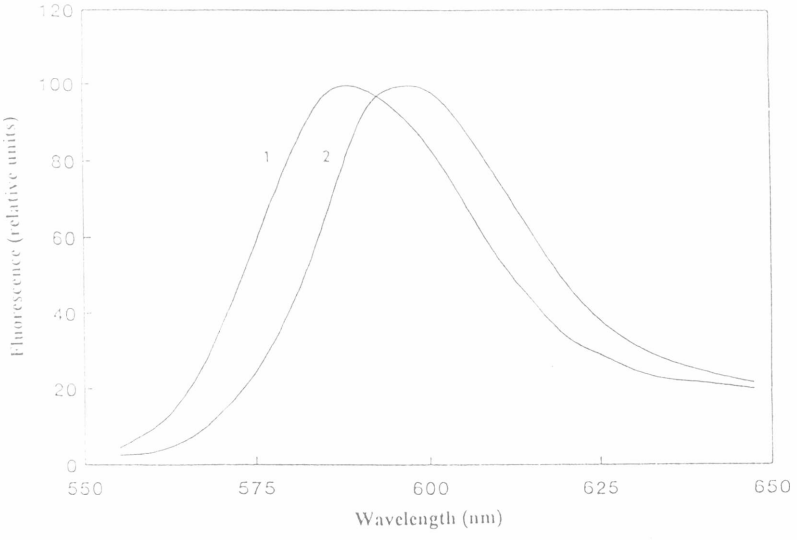

Fig. 3. *Fluorescence spectra of LRSH covalently linked with sheep kidney Na^+/K^+-ATPase Uncorrected fluorescence (final fluorophore concentration in the cuvette was 0.2 μM) spectrum was taken in 0.1 M Tris-HCl, pH 7.4 at 22 °C (line 1). The excitation wavelength was 556 nm. For comparison, the fluorescence spectrum of free LRSH in the same buffer and under the same conditions is presented in line 2.*

Table 3. Effect of magnesium and SDS on the anisotropy of LY-labelled and LRSH-labelled Na^+/K^+-ATPase.

	0 mM Mg^{2+}	3 mM Mg^{2+}	10 mM Mg^{2+}	1 % SDS
LY-Na^+/K^+-ATPase	0.159 ± 0.005	0.163 ± 0.004	0.167 ± 0.004	0.091 ± 0.005
LRSH-Na^+/K^+-ATPase	0.138 ± 0.004	0.135 ± 0.005	0.136 ± 0.004	0.185 ± 0.006

Fluorescence anisotropy was measured at excitation wavelengths 428 nm for LY and 556 nm for LRSH and at emission wavelengths 535 nm for LY and 589 nm for LRSH in 0.1 M Tris-HCl at pH 7.4 and room temperature. Magnesium was added subsequently directly into the cuvette. Solubilization effects were observed after treatment with 1 % SDS.

The effect of magnesium and solubilization

Because magnesium is an essential activator of the enzyme, it was used to further determine the effects of divalent cations upon the fluorescence properties of the labelled Na^+/K^+-ATPase. In the millimolar concentration range, magnesium slightly increased the anisotropy (from r = 0.159 in the absence of magnesium to r = 0.167 in the presence of 10 mM $MgCl_2$) and also slightly decreased the fluorescence intensity (about 6 %) of the LY-labelled enzyme. Magnesium, however, did not influence the anisotropy of LRSH-Na^+/K^+-ATPase (Table 3) and it did not also affect the anisotropy of the free fluorophores. Consequently, the observed effect of magnesium on fluorescently labelled Na^+/K^+-ATPase has to be ascribed to an interaction of magnesium with the oligosacharides of the β-subunit. Due to the significantly higher anisotropy of both labels after binding to the enzyme, one would naturally expect an anisotropy decrease after solubilization. Therefore, we performed an experiment with the solubilized enzyme. The labelled protein was solubilized with 3 % SDS as described in the Methods. Solubilization of the enzyme was accompanied by a loss of enzyme activity. The solubilization process affected the fluorescence intensities of neither LY-Na^+/K^+-ATPase nor LRSH-Na^+/K^+-ATPase and the

anisotropy of solubilized LY-Na^+/K^+-ATPase dramatically decreased as was expected (from r=0.159 for the labelled unsolubilized enzyme to r=0.091 for the enzyme after solubilization, Table 3). Surprisingly, however, we observed no decrease but an increase of anisotropy of solubilized LRSH-Na^+/K^+-ATPase (from r=0.138 to r=0.185, Table 3). No such effect was observed for the free labels.

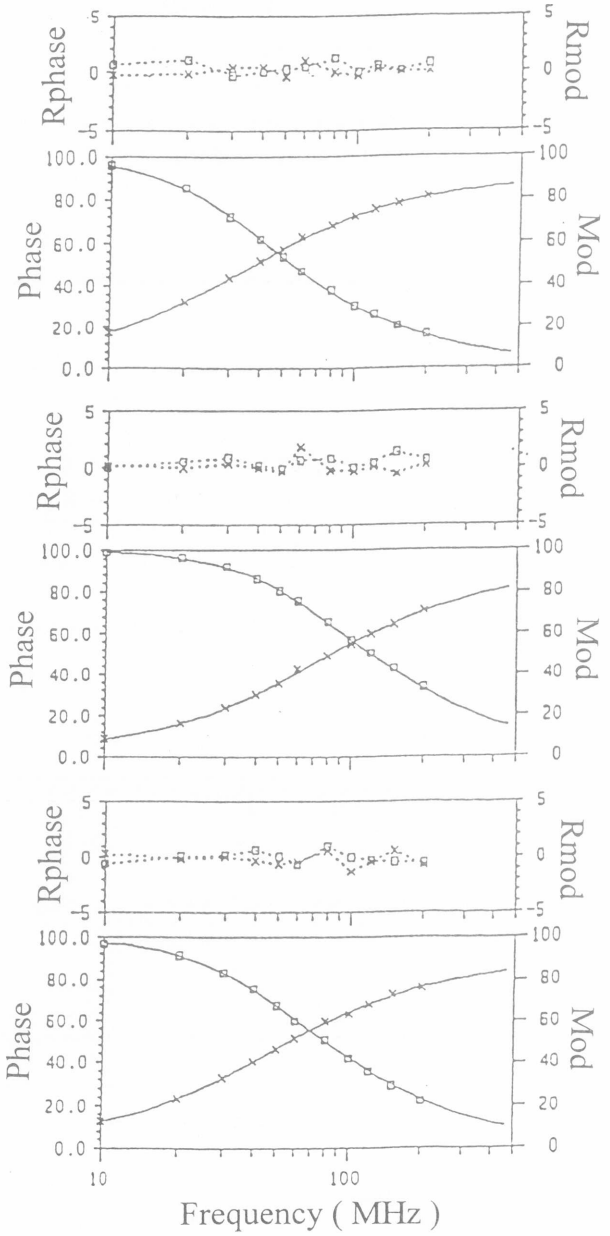

Fig. 4. *Frequency-response of LY bound to Na^+/K^+-ATPase. Frequency-responses of LY-Na^+/K^+-ATPase from sheep kidney (100 μg/ml) were observed over the frequency range 10–200 MHz. Na^+/K^+-ATPase was labelled with LY (top), both with LY and LRSH (middle) and the double-labelled sample (both with LY and LRSH) was boiled with 3 % SDS for 10 min (bottom). The background was subtracted according to (Lakowicz et al. 1987). Excitation wavelength was 428 nm, emission wavelength was 530 nm with the broadest slits of the fluorometer.*

Determination of the distance between LY and LRSH labelled β-subunit carbohydrates

In order to better understand the relative location and potential interaction between the β-subunits in the functional complex of Na^+/K^+-ATPase, the distance between the LY and LRSH was measured by FET. The fluorophores were found as a suitable donor-acceptor pair for long-distance measurements because the emission spectrum of LY (emission maximum at $\lambda=540$ nm) overlaps with the absorption spectrum of LRSH (see above). Thus, LY can serve as an energy donor and LRSH as an energy acceptor for FET experiments. A necessary condition for the calculation of the distance between the labels was the knowledge of the critical distance where the efficiency of FET was 50 %. This distance was determined to be $R_0=4.3$ nm. Because the anisotropies of both LY-Na^+/K^+-ATPase and LRSH-Na^+/K^+-ATPase were lower than r=0.2 in all samples, the orientational factor in our calculation was taken as 0.67. The donor fluorescence (LY) was used for distance calculations. To avoid trivial contributions to donor fluorescence intensity decrease (e.g. a static quenching), we determined all efficiencies of FET from the lifetimes of the excited states. Phase fluorometry and phase fluorometer ISS GREG 200 was used for all these experiments (see Methods). The lifetime of the donor (LY) excited state was measured over the frequency range of 5–200 MHz. The enzyme was labelled with 3 mM LY as described in the Methods. First to be determined was the stoichiometric excess of LY bound to the β-subunit. Using an extinction coefficient of LY ($\epsilon=12\ 000\ cm^{-1}.M^{-1}$ at $\lambda=428$ nm), we determined the stoichiometric excess as 1.2 mole/mole β-subunit.

Then we measured the fluorescence lifetime of the excited state of LY-Na^+/K^+-ATPase in 0.1 M Tris-HCl at pH 7.4. Glycogen was used as a reference sample and the background scattering was subtracted as described in the Methods. Our fluorescence data clearly show a single population of LY connected with the β-subunit after labelling (Fig. 4, Table 4). The lifetime of the excited state of LY-labelled Na^+/K^+-ATPase could be fitted to a monoexponential decay with the lifetime of the excited state of $\tau=5.03$ ns and $\chi_R^2=3.8$. Only a little improvement of the fit was achieved by using the lifetime distribution. The center of distribution was $\tau=5.02$ ns with the narrowest width allowed by the program: w=0.05 ns. As mentioned above, LY is a hydrophilic label and, thus the essentially homogeneous population of the fluorophore lifetime supports the view that the LY molecules are preferably attached to water-exposed sites.

The enzyme was double labelled both with 3 mM LY and 6 mM LRSH. We again determined the stoichiometry of binding. Both labellings proved to be independent and the stoichiometry of labelling with LY was preserved ($S_1=1.2$ mole/mole β-subunit), while

$S_2 = 2.4$ molecules of LRSH were bound as an average to one β-subunit. The fluorescence lifetime of such a double labelled sample was measured. A lifetime distribution was found to be the best fit of the measured data. The lifetime distribution became broader and the centre of the distribution was shifted to shorter lifetime when the enzyme was labelled both with LY and LRSH (Table 4). The mean lifetime reduction indicated the presence of FET. The width of the peak can be explained as a consequence of not only a single fixed distance between the two labels on the oligosaccharides of the β-subunit; in other words, there are more than one site on the oligosacharide(s) with highest affinity for LY and/or LRSH.

Table 4. Lifetime analysis of LY covalently bound to Na^+/K^+-ATPase and apparent efficiencies of energy transfer to LRSH.

	LY	LY+SDS	LY, LRSH	LY, LRSH+SDS	MERC
Lifetime τ(ns)	5.02	5.02	2.32	3.49	4.36
w(ns)	0.05	0.05	0.25	0.63	0.12
χ_R^2	2.1	1.7	2.4	2.3	3.8
App. efficiency	N/A	N/A	0.54	0.30	0.13

Frequency response of LY fluorescence was measured for samples in buffer as well as for samples after solubilization with SDS (+SDS) and also for the double labelled sample which was solubilized with SDS in the presence of 0.3 M β-mercaptoethanol (MERC). The lifetime distribution was used as the best fit for the obtained data. The mean lifetimes (τ) were determined for LY-Na^+/K^+-ATPase (LY) in 0.1 M and LY, LRSH-Na^+/K^+-ATPase (LY, LRSH), both in 0.1 M Tris-HCl buffer, and after solubilization treatment with 3 % SDS (LY+SDS and LY, LRSH+SDS, respectively). The apparent efficiencies were calculated from the lifetimes of the excited states of LY as described in Methods. N/A – not applicable.

Table 5. Lifetimes of CB-Na^+/K^+-ATPase and efficiencies of Förster energy transfer between CB and LY covalently bound to the β-subunit of the Na^+/K^+ATPase.

	CB	CB+SDS	CB,LY	CB,LY +SDS	CB,LY +SDS+MERC
Lifetime τ(ns)	2.61	2.63	2.19	2.45	2.46
χ_R^2	6.5	4.8	4.4	3.0	4.0
App. efficiency	N/A	N/A	0.17	0.07	0.06

Frequency response of CB fluorescence was measured for samples in buffer as well as for samples after solubilization with SDS in the presence of 0.3 M β-mercaptoethanol (MERC). The lifetime distribution was used as the best fit for the obtained data. The mean lifetimes (τ) were determined both in 0.1 M Tris-HCl buffer, after solubilization treatment with 3 % SDS and with 0.3 M MERC, respectively. The apparent efficiencies were calculated from the lifetimes of the excited states of CB as described in Methods. Lifetime distribution was found to be the best fit as judged from parameter χ_R^2 of the frequency response data of CB attached to Na^+/K^+-ATPase. N/A – not applicable.

Decrease of the mean lifetime of LY in a double labelled sample indicated an energy transfer between LY and LRSH both on the same subunit and between LY attached to one and LRSH bound to the other subunit. Due to the random labelling, we can write for the apparent rate constant k_{app} which is evidently a sum of the rate constants between the donor and acceptor on the same (k') and on different (k") subunits:

$$k_{app} = E_{app}/(1-E_{app}) = k' + k" \qquad (2)$$

where k' is the rate constant for energy transfer on the same subunit and k" is the rate of energy transfer

between LY and LRSH, each attached to a different subunit of the enzyme complex. We estimated this apparent rate constant from the mean lifetimes of LY-Na^+/K^+-ATPase and LY, LRSH-Na^+/K^+-ATPase (the apparent efficiency of FET was $E_{app}=0.54$) according to Eqn. (2) to be $k_{app}=0.234\ ns^{-1}$.

We were able to separate the two contributions using a solubilization treatment with SDS. Such treatment had no influence on the donor lifetime and its fluorescence intensity remained preserved (see above). Separation of the Na^+/K^+-ATPase complex, however, resulted in a new apparent FET, E'_{app}, which was commensurate with the energy transfer between labels attached to the same subunit and from which the rate constant k' can be calculated:

$$k' = E'_{app}/(1-E'_{app}) \quad (3)$$

Now, one could calculate the rate constant for energy transfer between subunits, $k'= 0.085\ ns^{-1}$ from the value of $E'_{app}=0.30$ (Table 4). However, there was no one-to-one ratio between the donor and acceptor on the β-subunit. Let τ be the ratio between stoichiometries S_2 (LRSH per β-subunit) and S_1 (LY per β-subunit). The corrected rate constant k_{corr} for Förster energy transfer between LY and LRSH attached to different β-subunits of the Na^+/K^+-ATPase complex is:

$$k_{corr} = k''/t. \quad (4)$$

One can obtain after substitution of Eqns. (2) and (3) into Eqn. (4) :

$$k_{corr}=S_1/S_2*[E_{app}/(1-E_{app})-E'_{app}/(1-E'_{app})] \quad (5)$$

After solubilization of the enzyme (treatment with SDS at 100 °C), we observed only a shift of the distribution center to longer lifetimes (Fig. 4, bottom, Table 4). The mean lifetime of the excited state for the solubilized complex LY, LRSH-Na^+/K^+-ATPase was $t=3.49$ ns. We calculated the apparent efficiency of FET for such solubilized complex according to Eqn. (3) to be $E'_{app}=0.30$. This apparent constant was only slightly lower than the apparent efficiency constant of Förster energy transfer for the unsolubilized complex E_{app}. This indicated that Förster energy transfer was partially due to energy transfer between two different β-subunits of the same functional enzyme comlex ($\alpha\beta$). Preferentially, however, the energy transfer occurred between donors and acceptors attached to the same β-subunit. The corrected rate constant was determined according to Eqn. (5) as $k_{corr}=0.075\ ns^{-1}$. The corrected efficiency of Förster energy transfer E_{corr} was then calculated from k_{corr} as $E_{corr}=0.27$. Finally, introducing E_{corr}, the critical distance R_o and the lifetime of the excited state of LY-Na^+/K^+-ATPase into Eqn. (1), one can obtain the average distance between fluorophores attached to different subunits as $R=5.1$ nm.

To confirm the calculated distance between the oligosacharides of the β-subunits in native Na^+/K^+-ATPase, we used CB and LY as a donor-acceptor pair. The emission spectrum CB overlaps with the absorption spectrum of LY and, thus, CB and LY can serve as an appropriate donor-acceptor pair for FET. First, we calculated the critical distance as described in the Methods. Because the anisotropies of both bound fluorophores were lower than $r=0.2$, the orientation factor was taken as $K^2=2/3$. We obtained the critical distance $R_o= 4.2$ nm.

Na^+/K^+-ATPase was first labelled with CB alone. The labelling procedure of the enzyme with the fluorophore was the same as was described above for both LY and LRSH, the concentration of the fluorophore in the labelling buffer was 3 mM. The labelling was successful (Fig. 1), the CB being essentially covalently bound only to the β-subunit. Subsequently, the enzyme was double labelled both with CB and LY. Both fluorophores (3 mM) were present simultaneously in the labelling buffer. We determined the stoichiometry of bound fluorophores to be 1.3 mole CB/mole β-subunit for monolabelling with CB alone, and 1.8 mole CB/mole β-subunit and mole LY/mole β-subunit for double-labelling with both CB and LY. The distance between the labelled oligosaccharides was determined from a FET experiment using the donor lifetime of the excited state for the calculation. The lifetimes of the excited state of CB were measured on a phase fluorometer as described above with the modulation frequency range of 10–200 MHz. The background was subtracted as described in the Methods. The best model to fit the measured data was shown to be based on the lifetime distribution. The mean lifetimes τ, and parameters χ_R^2 are summarized in Table 5. The mean lifetime of the CB-labelled Na^+/K^+-ATPase was $\tau=2.6$ ns. This lifetime, naturally, decreased when the enzyme was double labelled with CB and LY ($\tau=2.19$ ns). This lifetime decrease clearly indicated the presence of FET from CB to LY. Solubilization of the enzyme resulted in a decrease of the lifetime of the excited state ($\tau=2.45$ ns) due to separation of subunits and presence of only a transfer between fluorophores attached to the same subunit. Treatment with MERC to break the disulfide bonds and tertiary structure resulted only in a minuscule change of the lifetime of the excited state of CB ($\tau=2.46$ ns). Knowledge of the lifetime of the excited state of CB in the absence of any acceptor, and the lifetimes of the double-labelled sample in the buffer and after solubilization in the absence and in the presence of MERC permit us to calculate the apparent efficiencies of Förster energy transfer (Table 5) according to Eqn. (2). Subsequently, we have determined the average distance between the donor and the acceptor on the same β-subunit as $R_s= 5.3$ nm.

Discussion

The β-subunit, the function of which has been somewhat puzzling until recently, now attracts the attention of biochemists, molecular geneticists and biophysicists. The hypothesis that the β-subunit regulates, through the assembly of $(\alpha\beta)_2$ heterodimers, the number of sodium pumps transported to the plasma membrane (for review see McDonough *et al.* 1990) is now widely accepted. Amino acid sequence analysis deduced from cDNA revealed a high similarity (over 90 %) (for review see Geering 1990) among mammalian species (e.g. Brown *et al.* 1987, Kawakami *et al.* 1986, Shull *et al.* 1986). Hydropathy analysis of the amino acid sequence also revealed that in contrast to the α-subunit, the β-subunit has a short cytoplasmic tail, one transmembrane segment and a large extracellular domain containing at least three glycosylation sites – at Asn 157, next at Asn 192 and at Asn 264 (numbering for our enzyme from lamb kidney – see Shull *et al.* 1986). The protein appears to be primarily α-helical from the amino terminal until just before the first glycosylation site. There are three disulfide bonds between cysteines 125 and 148, 158 and 174 and 212 and 275. Sufficient data, however, are not available for deducing the tertiary structure of the β-subunit. Also the quaternary structure (mutual organization of the two subunits in the functional complex in the membrane) is unknown.

Using FET study with LY-LRSH and CB-LY as donor-acceptor pairs, respectively, we found that the average mutual separation of labels was quite large, more than 5 nm. This distance is comparable with the diameter of the α-subunit (Amler *et al.* 1992). Conseqently, the β-subunits are neither neighbours (situated, for example, just between the two large α-subunits) nor are they separated by the α-subunits.

It follows from the hydropathy analysis that there are three α-helical regions between amino acid residue 60 at the outside membrane face and the first glycosylation site (residue 157), namely 104–120, 129–138 and 141–148 which exhibit a significantly amphipathic character and may have both hydrophobic and electrostatic interactions with each other, other protein or even with the lipid bilayer. The presence of the disulfide bonds between cysteines 125 and 148, and 158 and 174 indicates that the domain must maintain a compact tertiary structure. This cluster can electrostatically interact with an extracellular part of the α-subunit which is also known to bear a negative charge. Such interaction need not be direct but can be ion-mediated or ion-modulated. Magnesium is, without any doubt, a candidate for the mediation of such interactions. The high concentration of aspartate and glutamate residues, negatively charged at physiological pH, at the extracellular part of the α-subunit favours such a hypothesis. In addition, such interactions would be a plausibile explanation of the magnesium effects on anisotropy of LY-labelled oligosaccharides of the β-subunit presented in this work as well as of its effect on the motional properties of oligosaccharides of the β-subunit (Amler *et al.* 1996).

A cluster of the β-subunit attached to the extracellular part of the α-subunit would also make sense for the enzyme function from a dynamic point of view. As follows from the secondary structure, the extracellular part of the α-subunit is negligible with respect to its cytoplasmic part. Nevertheless, the α-subunit, undergoes a rather extensive movement during the transport cycle as can be concluded from the large distance of the ATP-binding site and the phosphorylation site (Amler *et al.* 1992). Movement of a large mass of the cytoplasmic part of the α-subunit would be facilitated by anchoring of the small extracellular part by the β-subunit. The β-subunit would thus serve as a dynamic counterbalance during the transport cycle.

All three fluorophores labelled sufficiently the oligosaccharides of the β-subunit. However, while LY and CB labelled probably a water-exposed oligosaccharide, LRSH was attached to a site which was close to the protein and/or membrane surface. This follows from the change of LRSH fluorescence spectra after binding to the enzyme. Surprisingly, LRSH showed a higher degree of rotational freedom than the water exposed LY. Still more surprising was the observation that the anisotropy of LRSH increased after solubilization (while anisotropy of LY decreased). This indicated that LRSH was bound to a site in which the fluorophore had relatively high rotational freedom due to the tertiary structure. The rotational volume was possibly created by hydrophobic and/or electrostatic interactions of the β-subunit with its neighbourhood. Such interactions were disrupted after solubilization and the motion of the label was restricted by the collapsed mass of the β-subunit. Consequently, the rotational freedom of the fluorophore decreased resulting in higher anisotropy of fluorescence.

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Reprint requests

E. Amler, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic