Journal of Fluorescence, Vol. 3, No. 4, 1993

The Frequency-Domain Method Reveals the Dimeric Structure of Na,K-ATPase

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Received October 18, 1993

Lucifer yellow and lissamine rhodamine sulfonyl hydrazine were used as the donor and the receptor, respectively, for Förster energy transfer measurements to determine the location of the β subunit in the native Na,K-ATPase from pig kidney. It was found that (1) the β subunits are located in one functional complex, i.e., the dimer $(\alpha\beta)_2$ appears to be the functional complex of Na,K-ATPase, and (2) the β subunits in the functional enzyme complex in the membrane are not located next to each other but are rather well separated. The distance between fluorophores covalently attached to the β subunits was found to be 5.3 nm.

KEY WORDS: Na,K-ATPase; frequency domain; Lucifer yellow; lissamine rhodamine sulfonylhydrazine; dimeric structure.

INTRODUCTION

Na,K-ATPase is a plasma membrane protein of animal cells, the primary function of which is to maintain the Na^+ and K^+ cation gradients across the membranes. The enzyme is composed of at least two subunits, the catalytic α subunit (M_r = 112,000) and the smaller glycoprotein β subunit (M_r = 45,000, including its oligosaccharides). However, whether the minimal functional unit of the enzyme in the membrane is a monomer or a dimer still remains a matter of debate.

In the present study we made extensive use of fluorescent labels of carbohydrates [Lucifer yellow (LY), lissamine rhodamine sulfonyl hydrazone (LRSH)] as a donor–acceptor pair for structural and dynamic measurements of β–β interactions on the basis of Förster energy transfer (FET) and tried to elucidate the organization of the functional Na,K-ATPase complex in the membrane, with special emphasis on the β subunit.

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METHODS

Enzyme and Subunit Preparation

Na,K-ATPase was purified from pig kidney as previously described [1]. 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 m*M* EDTA, 3 m*M* ATP, and 3% sodium dodecyl sulfate (SDS) [2]. For disruption of disulfide bridges, 0.3 *M* β-mercaptoethanol was added to the mixture. The enzyme was labeled with LY and LRSH as described by Lee and Fortes [3].

Dynamic Fluorescence Measurements

Frequency-domain measurements were done to determine the fluorescence decay lifetimes for the FET efficiency determination using an ISS K2 Multifrequency Cross-Correlation Phase and Modulation Fluorometer. The experimental data were analyzed by a nonlinear least-squares routine for multiexponential fitting (and/or lifetime distribution), correction for back-

1053-0509/93/1200-0245$07.00/0

ground [4], and additional software available at the Institute of Biochemistry, University of Ancona. The orientation factor κ^2 was taken as 2/3 for all calculations because the anisotropies of bound fluorophores were sufficiently low [5].

The distance between the donor and the acceptor pairs was calculated by determining the R_0 value and the efficiency of the energy transfer as $d = R_0[(1/E)+]^{1/6}$.

RESULTS AND DISCUSSION

The distance between bound LY and bound LRSH was measured by FET. The necessary condition for the calculation of the distance between the labels was the knowledge of the critical distance R_0 where the efficiency of FET was 50%. This distance was calculated to be 4.3 nm.

Donor (LY) fluorescence was used for distance calculations. To avoid trivial contributions to donor fluorescence intensity decrease (e.g., scattering or static quenching), we determined all the efficiencies of FET from the lifetimes of the excited states, using the phase ISS K2 phase fluorometer. After labeling the enzyme with 3 m*M* LY, the stoichiometric ratio of LY bound to the β subunit (using the extinction coefficient for LY of 12,000 M^{-1} cm^{-1} at 428 nm) was found to be 1.2.

The fluorescence lifetime of the excited state of LY–Na,K-ATPase in 0.1 *M* Tris–HCl at pH 7.4 was measured with glycogen as the reference sample and the background scattering was subtracted. The fluorescence data showed a single population of LY connected with the β subunit after labeling. The lifetime of the excited state of LY–Na,K-ATPase could be fitted to a monoexponential decay curve with a lifetime of the excited state of 5.03 ns and a χ^2_R of 4.2 (Table I). Only an insignificant improvement of the fit was achieved by applying the lifetime distribution approach. The center of distribution was at 5.02 ns with the narrowest width the program allowed, *viz.*, $w = 0.05$ ns. As mentioned above, LY is a hydrophilic label and thus the essentially homogeneous population of the fluorophore lifetimes supports the view that the LY molecules are attached mainly to water-exposed sites.

When the enzyme was doubly labeled under the same conditions as above, both with 3 m*M* LY and with 6 m*M* LRSH, the stoichiometry of binding of LY was preserved ($S_1 = 1.2$ mol/mol β subunit), while for LRSH S_2 was 2.4. The lifetime distribution was found to be the best fit of the frequency response of such doubly labeled samples. The distribution was shifted to shorter lifetimes when the enzyme was labeled with both LY and LRSH (Table I). The mean lifetime reduction indicated the presence of FET. The greater width of the peak can be explained as a consequence of more than a single fixed distance between the two labels on the oligosaccharides of the β subunit.

Table I. Lifetime Analysis of LY Covalently Bound to Na,K-ATPase and Apparent Efficiencies of Energy Transfer to LRSH[a]

Quantity	Variant I	II	III	IV	V
τ (ns)	5.03	5.02	2.32	3.49	4.36
w (ns)	0.05	0.05	0.25	0.63	0.12
χ^2_R	4.2	2.7	5.4	4.3	7.8
E_{app}	NA	NA	0.54	0.30	0.13

[a]The frequency response of LY fluorescence was measured for samples in 0.1 *M* Tris buffer (I) as well as for samples after solubilization with 3% SDS (II) and also for doubly labeled samples in buffer (III) or after solubilization with 3% SDS without (IV) or with (V) β-mercaptoethanol. NA, not applicable.

This shorter lifetime of LY in the doubly labeled sample was obviously a consequence of energy transfer between LY and LRSH both on the same subunit (intramolecular interaction) and between LY and LRSH bound to different subunits (intermolecular interaction).

Finally, we calculated the rate of energy transfer between both fluorophores (corrected for nonspecific labeling) as 0.26 nm. The average intermolecular distance between the fluorophores was then determined as 5.3 nm.

Consequently, it may be concluded that the Na,K-ATPase we examined was an $(\alpha\beta)_2$ dimer.

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