Journal of Fluorescence, Vol. 4, No. 4, 1994

DEDO: A Specific, Fluorescent Inhibitor for Spectroscopic Investigations of Na,K-ATPase

E. Lewitzki,[1] U. Frank,[1] E. Götz,[1] K. Brand,[2] F. W. Schneider,[2] and E. Grell[1]

Received October 18, 1993

The interaction between the fluorescent ouabain derivative DEDO and purified renal Na,K-ATPase (isolated from different animal species) is investigated. Equilibrium binding studies provide a pK value of about 7.5 and a stoichoimetric coefficient of 1. Nonmodified ouabain exhibits the same affinity to the rabbit enzyme; the enzyme originating from the other sources binds DEDO 10 times less strongly than ouabain. Kinetic studies indicate that this is the consequence of a 10 times higher dissociation rate constant of the complexes formed with DEDO. The fluorescence emission intensity of DEDO is enhanced, being dependent on the enzyme source. The single decay time of DEDO is 3 ns in the absence and 21 ns in the presence of the rabbit enzyme and 14 ns in the presence of the pig renal enzyme. This result suggests that the fluorophore of DEDO is bound to a very hydrophobic environment of the enzyme. Further characterization of the static fluorescence spectra provides evidence for energy transfer between Trp residues of the enzyme and DEDO. Distance estimations suggest that one or two Trp residues are likely to be located in the proximity of the fluorophore.

KEY WORDS: Na,K-ATPase; fluorescent inhibitor; kinetics; energy transfer.

INTRODUCTION

Na,K-ATPase is an enzyme found in nearly all animal cell membranes that uses energy from the hydrolysis of intracellular ATP to actively transport Na^+ out of and K^+ into the cell. The enzyme consists of an enzymatically active α and an inactive β (glycoprotein) subunit (MW of $\alpha\ \beta \sim 150{,}000$). Structure predictions based on known amino acid sequences suggest 8 or 10 transmembrane helices for the α and one for the β unit.

It is well-known that the Na,K pump is specifically inhibited by low concentrations of digitalis glycosides [1]. Many physiological indications correlate the inhibition of Na,K-ATPase with the observed inotropic effect of these drugs on heart muscle.

Since fluorescence spectroscopy is a useful tool for studying many structural and dynamic aspects, we have used (*N*-(dansyl)-*N'* (ouabain)-ethylenediamine (DEDO), a new fluorescent derivative [2] of digitalis glycoside ouabain of increased chemical stability compared, e.g., to anthroylouabain [3], to characterize the cardiac glycoside binding site of Na,K-ATPase isolated from tissues of different species. The dansyl residue is connected to the sugar moiety of the cardiac steroid (Fig. 1).

Kinetic experiments for determination of the dynamic parameters of complex formation and dissociation between DEDO and Na,K-ATPase were performed in the presence of different ligands and cofactors. Spectral characteristics of the fluorescence emission spectra, lifetime measurements of free and enzyme-bound DEDO, and energy transfer measurements provide information

[1] Max-Planck Institute for Biophysics, Kennedy-Allee 70, D-60596 Frankfurt, Germany.
[2] Institute for Physical Chemistry, University of Würzburg, Marcusstr. 9-11, D-97070 Würzburg, Germany.

1053-0509/94/1200-0287$07.00/0

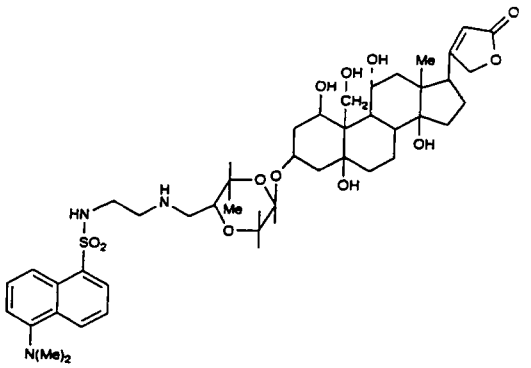

Fig. 1. Structure of DEDO.

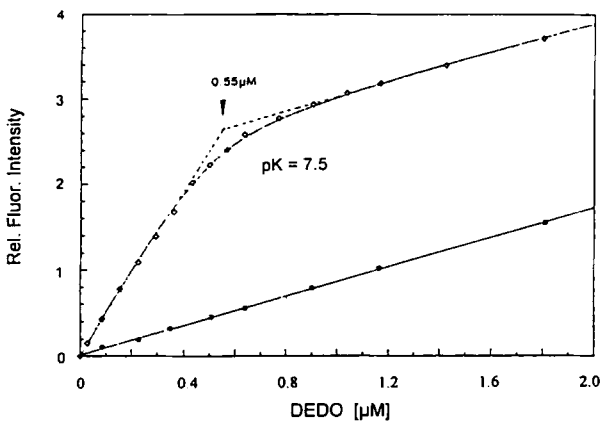

Fig. 2. Spectrofluorometric titration of Na,K-ATPase (0.52 μ*M*) from rabbit kidney with DEDO in 25 m*M* imidazole/HCl, pH 7.5, 3 m*M*, $MgCl_2$, 3 m*M* Tris–P_i, λ_{exc} = 325 nm, λ_{em} = 540 nm (complex II), at 25°C. The lower line indicates the dependence of the relative fluorescence intensity on the DEDO concentration in the absence of enzyme.

about the microenvironment of the dansyl reporter group.

MATERIALS AND METHODS

Membrane-bound Na, K-ATPase was prepared from dog, pig, sheep, and rabbit kidney according to Ref. 4 with an activity of 35–40 μmol P_i mg^{-1} min^{-1} under standard conditions. Buffers, salts, and synthetic reagents were obtained from Merck, Fluka, and Sigma in the form of Suprapur, Microselect, and pA quality. Monodansylethylenediamine was from Molecular Probes Eugene/USA. Spectrofluorometric titrations and spectra were carried out on a Spex Fluorolog 212 spectrofluorometer. Kinetic experiments were performed on a Jobin–Yvon-based fluorometer setup. Time-resolved fluorescence measurements (enzyme conc.:2.5μM) were done using single-photon counting technique on an Edinburgh Instruments 199S nanosecond fluorometer equipped with a hydrogen arc lamp.

States of formed complexes between Na,K-ATPase and inhibitor are denoted according to Allen *et al.* [5]: complex I, binding of cardiac glycoside in the presence of Na^+, ATP, and Mg^{2+}; and complex II, in the presence of Mg^{2+} and inorganic phosphate (P_i). Binding of cardiac glycoside in the presence of Mg^{2+} only is denoted complex III.

DEDO was synthesized by forming an adduct between 0.6 mmol of oxidized ouabain [6] and 0.8 mmol of monodansylethylenediamine in MeOH, followed by the $NaCNBH_3$ reduction under standard conditions. Purification of the product was performed by liquid chromatography on LH 20 (MeOH) and on silica gel (acetone). Fractions with a TLC R_f value of 0.36 $CHCl_3$/MeOH, 8/2 (v/v), were further purified by preparative TLC. A uniform pure product (oil) was obtained by NMR and FAB mass spectrometry. Details of structure determination will be given elsewhere.

RESULTS AND DISCUSSION

Equilibrium Binding and Kinetic Parameters for Complex Formation Between DEDO and Na,K-ATPase

Binding of DEDO to Na,K-ATPase leads to an increase in the fluorescence emission intensity as shown in Fig. 2. The solid line in Fig. 2, concerning data obtained in the presence of Na,K-ATPase, represents the best fit according to a 1:1 complex formation model, characterized by a p*K* value of 7.5. The dashed line in Fig. 2 represents the dependence expected for an indefinitely high p*K* value. The point of intersection reflects the binding stoichiometry between DEDO and Na,K-ATPase (0.55:0.52 μ*M* = 1.06), which is consistent with a stoichiometric coefficient of about 1 (assumed MW = 1.5×10^5).

Rapid mixing of DEDO and the solution containing the enzyme allowed us to resolve the binding process and to determine the formation rate constant (k_{on}) provided that k_{off} was known. Determination of the dissociation rate constant was carried out by adding quickly a 10^3-fold excess of unlabeled ouabain. Under such conditions the dissociation of fluorescent DEDO is rate-limiting. DEDO dissociation could always be characterized by a single-exponential function. If k_{on} and k_{off} are de-

Table I. Kinetic Parameters for DEDO Binding to Na,K-ATPase of Different Sources Leading to Complex II, in 25 m*M* Imidazole/HCl, pH 7.5, Containing 3 m*M* $MgCl_2$ and 3 m*M* Tris–P_i (37°C)

Renal enzyme	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	p*K*
Dog	3.0×10^4	9.0×10^{-4}	7.5
Pig	2.2×10^4	10.0×10^{-4}	7.4
Sheep	4.5×10^4	11.5×10^{-4}	7.6
Rabbit	2.8×10^4	3.5×10^{-4}	7.9

Table II. Wavelength of Emission Maximum and Maximum Emission Amplitude of DEDO Bound to Renal Na,K-ATPase, Isolated from Different Animal Species in 25 m*M* Imidazole/HCl, pH 7.5, λ_{exc} = 334 nm, 3 m*M* $MgCl_2$, 3 m*M* Tris–P_i (Complex II) at 25°C

Source of renal enzyme	Emission maximum (nm)	Rel. emission intensity (%)
None	554	100[a]
Dog	534	180
Pig	532	200
Sheep	536	175
Rabbit	496	850

[a]Fluorescence intensity of unbound DEDO in buffer is designated 100%.

Table III. Fluorescence Decay Times of DEDO, Free and Bound to Na,K-ATPase, in 25 m*M* Imidazole/HCl, pH 7.5, λ_{exc} = 337 nm, λ_{emi} = 521 nm, 25°C

Complex	Renal enzyme source	τ (ns)
No enzyme	Unbound DEDO	3.2
I	Rabbit	20.3
II	Rabbit	20.9
III	Rabbit	19.5
II	Pig	13.7

termined, *K* can be calculated according to $K = k_{off}/k_{on}$. The kinetic parameters are summarized in Table I.

As reported earlier [7] the determined formation rate constants of complexes I and II depend only slightly on the animal species from which the renal enzyme was isolated. No significant difference is found in the formation rate constants for complexes I and II determined with DEDO and the unmodified ouabain [2,8,9]. In the case of DEDO complex III the formation rate constant is 100 times lower than that observed for complex I or II.

Generally, the DEDO dissociation rate is about 10 times higher than that of the unlabeled ouabain, which corresponds to the value reported for anthroylouabain [3]. An exception is found for the rabbit kidney enzyme, where the rate constants of DEDO, as well as ouabain dissociation, are similar.

Spectroscopic Properties

Upon binding of DEDO to Na,K-ATPase a large blue shift (~60 nm) and an eightfold higher fluorescence intensity are observed in the case of rabbit Na,K-ATPase. This high spectral shift is indicative of a very hydrophobic binding microenvironment of the dansyl reporter group [10]. The proteins isolated from the kidneys of the other species exhibit only blue shifts between 10 and 20 nm, with an increase in maximum emission intensity ranging between 1.5- and 2-fold. The corresponding spectral characteristics are summarized in Table II. These spectroscopic results allow us to suggest that DEDO can act as a very suitable and sensitive probe molecule for achieving correlations between site-directed mutagenesis and structural aspects concerning the identification of interacting single amino acid side chains.

Time-Resolved Fluorescence Studies: Decay Time Measurements

Upon binding of DEDO to the enzyme a marked increase in fluorescence decay time is observed, which is largest for the rabbit enzyme. The τ value is similar to that of DEDO dissolved in nonpolar solvents (e.g. DIOXANE), which suggests that the dansyl moiety of the bound DEDO is located in a similar hydrophobic environment in the case of complexes I, II, and III.

From the τ values (Table III), a static fluorescence emission intensity increase by a factor of 7 is predicted upon binding of the fluorophore to the rabbit kidney enzyme. This is in good agreement with the experimental observation (cf. Table II). The same, simple correlation, however, does not hold for the pig enzyme, which implies that additional factors, such as static quenching, must be considered here. The change in the single decay time of DEDO from 3 to 21 ns upon binding to pure renal rabbit kidney enzyme and to 14 ns upon binding to pure renal pig kidney enzyme suggests that the fluorophore of DEDO is bound to an even more hydrophobic environment in case of the rabbit enzyme, where it remains essentially unquenched. When bound to the pig enzyme it

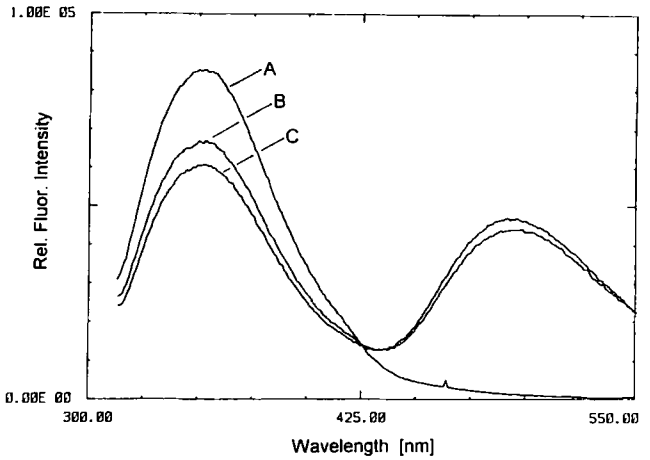

Fig. 3. Fluorescence emission spectra indicating energy transfer between tryptophan residues of rabbit kidney Na,K-ATPase (0.3 μM) and DEDO in 25 mM imidazole/HCl, pH 7.5, λ_{exc} = 295 nm, 25°C. Emission spectra of rabbit kidney Na,K-ATPase (A) without DEDO and in the presence of (B) 3 mM Tris–P_i, 3 mM $MgCl_2$, 0.6 μM DEDO (complex II), and (C) 100 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM Tris–ATP, 0.6 μM DEDO (complex I).

appears to be partially quenched. The fluorescence decay of free and bound DEDO is monoexponential.

Measurement of Energy Transfer

The interaction between tryptophan (Trp) residues and bound DEDO was studied by investigating the efficiency of energy transfer (11) of the Trp donor in the presence and absence of acceptor. Evidence for energy transfer results from the observed decrease in the steady-state fluorescence intensity of the Trp residues of the enzyme in the presence of DEDO by about 30% as shown in Fig. 3. To estimate distances between the donor (Trp) and the bound inhibitor, the efficiency (E) is calculated from the quantum yield of the donor in the absence (Q_O) and presence (Q_T) of the acceptor (DEDO) according to $E = 1 - Q_T/Q_O$.

The fluorescence emission of the tryptophan residues (Trp) of Na,K-ATPase is quenched by 20–30% upon binding of DEDO. Except for the renal sheep enzyme, the energy transfer efficiency of complex II always appears to be lower than that of complex I.

Calculations according to Förster's theory [11], assuming that DEDO interacts with only a single Trp residue, indicate a distance of about 26 Å in the case of the sheep kidney enzyme. Since the α and β sequence of this enzyme [12] contain 16 Trp residues (2 in putative transmembrane segments of the α chain, 4 localized in the β subunit), it is not possible to calculate such distances for multiple Trp sites in a realistic way. If all Trp residues were equally separated from the dansyl group, the transfer efficiency would result in a decrease in Trp emission intensity of only 9% for a distance of 50 Å. The observed transfer efficiencies of between 0.2 and 0.3 may suggest that one or two Trp residues are located close to the dansyl group of the bound DEDO.

ACKNOWLEDGMENTS

We thank Professor W. J. Richter for analytical support, Mrs. A. Schacht and Mr. G. Schimmack for expert technical assistance, and the Deutsche Forschungsgemeinschaft (SFB 169) for financial support.

REFERENCES

1. I. M. Glynn (1964) *Pharmacol. Rev.* **16**, 381–407.
2. E. Grell, E. Lewitzki, and A. Ifftner (1985) in I. Glynn and C. Ellory (Eds.), *The Sodium Pump*, The Company of Biologists Cambridge, pp. 289–294.
3. P. A. G. Fortes (1977) *Biochemistry* **16**, 531–540.
4. P. L. Jorgensen (1974) *Biochim. Biophys. Acta* **336**, 36–52.
5. J. C. Allen, R. A. Harris, and A. Schwartz (1971) *Biochem. Biophys. Res. Commun.* **42**, 366–370.
6. B. Forbush III, J. H. Kaplan, and J. F. Hoffman (1978) *Biochemistry* **17**, 3667–3676.
7. E. T. Wallick, B. J. R. Pitts, L. K. Lane, and A. Schwartz (1980) *Arch Biochem Biophys* **202**, 442–449.
8. A. Askari, S. S. Kakar, and Wh. Huang (1988) *J. Biol. Chem.* **263**, 235–242.
9. E. Erdmann and W. Schoner (1973) *Biochim. Biophys. Acta* **307**, 386–398.
10. R. F. Chen (1967) *Arch. Biochem. Biophys.* **120**, 609–620.
11. Th. Förster (1948) *Ann. Phys. (Leipzig)* **2**, 55–75.
12. G. E. Shull, A. Schwartz, and J. B. Lingrel (1985) *Nature* **316**, 691–695.