Journal of Fluorescence, Vol. 6, No. 3, 1996

Conformational Changes of Na,K-ATPase Probed with Eosin Y

E. Lewitzki,[1] E. Schick,[1] R. Hutterer,[2] F. W. Schneider,[2] and E. Grell[1]

Received September 24, 1995; accepted July 29, 1996

Time-resolved fluorescence and binding studies have been carried out on Na,K-ATPase in the presence of the fluorescent dye eosin Y to obtain thermodynamic and kinetic parameters for the interaction of the enzyme with different cations. Eosin Y binding is indicated by a 3 ns fluorescence decay process and is observed only in the presence of mono- and divalent cations. This type of cation binding is interpreted as a nonselective electrostatic interaction, with negatively charged groups of the enzyme providing a high-affinity eosin Y binding site. Eosin Y binding is observed only under conditions where the enzyme exists in the conformational state F_1. The kinetic parameters of eosin Y binding have been determined employing stopped-flow fluorometry.

KEY WORDS: Cation binding; fluorescence decay; kinetics; binding constants; Na,K-ATPase; eosin Y.

INTRODUCTION

Na,K-ATPase, an integral membrane protein found in nearly all cell membranes of most higher animals, converts energy from the hydrolysis of intracellular ATP to the active transport of Na^+ out of and K^+ into cells. This membrane transport of Na^+ and K^+ is thought to be coupled to a transition between two main conformational states of the enzyme, denoted E_1, indicative of a high-affinity Na^+, and E_2, indicative of the K^+ complex.

Fluorescent dyes such as fluorescein isothiocyanate (FITC), covalently coupled to lysine residues, and eosin Y upon reversible binding provide information on the state and the population of conformational states in the case of Na,K- ,[1] H,K-,[2] and Ca-ATPase.[3] Competition experiments suggest that eosin Y is bound to the ATP binding region.[4] This dye thus acts as a relevant model compound. In the case of Na,K- and H,K-ATPase, bound eosin Y exhibits a higher quantum yield in the presence of certain cations than the free dye.

Binding of the negatively charged eosin Y to Na,K-ATPase has been shown to exhibit a high affinity preferentially in the presence of Na^+.[4] This observation has been interpreted to be indicative of the formation of state E_1.[4,5] In the presence of K^+ or its congeners, where Na,K-ATPase is assumed to exist in state E_2, as well as in the absence of high concentrations of cations, no evidence for high-affinity binding has been observed.[5]

We apply time-resolved fluorescence decay measurements to characterize the interaction between Na,K-ATPase and eosin Y in the presence of many mono- and divalent cations. To characterize the dynamic aspects of eosin Y binding to Na,K-ATPase, the fluorescent properties of the ligand are used successfully to carry out stopped-flow studies.

MATERIALS AND METHODS

Membrane-bound and purified Na,K-ATPase was prepared from pig kidneys according to Ref. 6 with a

[1] Max-Planck-Institute of Biophysics, D-60596 Frankfurt, Kennedy-Allee 70, Germany.

[2] Institute of Physical Chemistry, University of Würzburg, D-97070 Würzburg, Marcusstr. 9-11, Germany.

1053-0509/96/0900-0165$09.50/0

Table I. Fluorescence Decay Times of Eosin Y (1 μ*M*) in Solvents of Different Polarities and in the Presence of Na,K-ATPase (T = 25°C, λ_{exc} = 337 nm, λ_{emi} = 539 nm)

Medium	Decay time (ns)
Methanol	3.3
Ethanol	3.1
5 m*M* imidazole/HCl pH 7.5	1.0
5 m*M* imidazole/HCl pH 7.5, containing 2 μ*M* Na,K-ATPase	1.0
5 m*M* imidazole/HCl pH 7.5, containing 2 μ*M* Na,K-ATPase + 100 m*M* NaCl	1.0 and 2.85

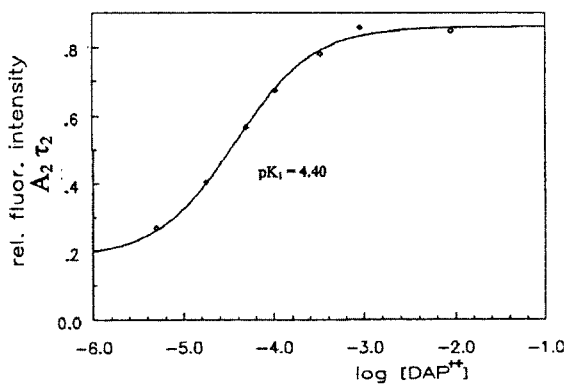

Fig. 1. Time resolved fluorescence titration of Na,K-ATPase (2 μ*M*) in the presence of eosin Y (0.72 μ*M*) with 1,3-diammonium propane in 5 m*M* imidazole/HCl, pH 7.5 (T = 25°C. λ_{exc} = 337 nm, λ_{emi} = 539 nm). The relative amplitude $A_2\tau_2$, characteristic of the slower decay time (2.85 ns), is plotted versus cation concentration.

specific activity around 35 μmol P_i mg^{-1} min^{-1}. Spectrofluorometric titrations were carried out on a Spex Fluorolog 212 spectrofluorometer. Stopped-flow experiments were performed on an Applied Photophysics Model 13-106 equipped with fluorescence detection. Time-resolved fluorescence measurements were carried out using single-photon counting technique on an Edinburgh Instrument 199S nanosecond fluorometer equipped with a hydrogen arc lamp. K_1 and K_2 are specified as dissociation constants.

RESULTS AND DISCUSSION

Time-Resolved Fluorescence Studies: Decay Time Measurements

Upon binding of eosin Y to the enzyme the stationary fluorescence level of the dye is increased by about 10 %, and only a small band shift is observed.[4] Previous equilibrium binding studies[5] suggest that only the Na^+ complex of Na,K-ATPase exhibits strong binding of eosin Y. It has thus been concluded that eosin Y binding is indicative of state E_1. Using time-resolved fluorescence spectroscopy it is possible to differentiate between free and enzyme-bound fluorophore.

According to our results eosin Y exhibits a single decay time in aqueous solution (1.0 ns) as well as in some organic solvents (Table I); the time constant depends on the solvent polarity.

Also, in the presence of the enzyme at low ionic strength, a single exponential decay (τ_1) is observed, which is attributed to unbound eosin Y because the time constant corresponds to that of the dye in aqueous buffer [cf. Eq. (1)]. However, in the presence of millimolar concentrations of different mono- and divalent cations where binding of eosin Y is observed, a second, slower decay process (τ_2), with a time constant around 3.0 ns (amplitude A_2), can be detected. The amplitude A_2, expressed as $A_2\tau_2$, increases with increasing cation concentration, while the corresponding time constant remains stable. Under these conditions the amplitude of the faster process decreases. Thus the slower decay is assigned to the enzyme—bound eosin Y. The postulated reaction schemes are (E, Na,K-ATPase; Y, eosin Y) as follows: in buffer,

$$E + Y \rightleftharpoons \text{no binding observed} \qquad (1)$$

in buffer containing a cation M^{n+},

$$M^{n+} + E + Y \underset{k_{21}}{\overset{K_1 \; k_{12}}{\rightleftharpoons}} M^{n+} - E + Y \underset{k_{32}}{\overset{K_2 \; k_{23}}{\rightleftharpoons}} M^{n+} - E \cdot Y \qquad (2)$$

In Eq. (2) it is assumed that binding occurs only between eosin Y and the cation complex M^{n+}–E of the enzyme. Provided that saturation conditions for the second equilibrium, characterized by K_2, are maintained, the apparent equilibrium constant K_1 can be obtained experimentally by determining the dependence of the amplitude $A_2\tau_2$ of the slower decay process on the cation concentration. A corresponding plot for, e.g., 1,3-diammonium propane as a divalent organic cation is shown in Fig. 1; evaluation is performed in terms of a 1:1 binding model. If ATP is added to a Na,K-ATPase solution containing bound eosin Y, the amplitude $A_2\tau_2$ of the slower decay process decreases, which is indicative of competition for the same enzyme site.

Our results clearly show that eosin Y binding is observed in the presence of all investigated mono- and divalent cations (Table II), and not just in the presence of Na^+ and Mg^{2+}. All pK_1 values are around 2.2 for the

Table II. Apparent Affinities of Na,K-ATPase for Different Cation Chlorides as Sensored with Eosin Y (Experimental Conditions as in Fig. 1)

Cation	pK_1
Choline	2.00
Sodium	2.65
Imidazolium	1.70
Potassium	1.70
Magnesium	3.90
1, 3-Diammonium propane	4.40

different monovalent cations and around 4.2 for the divalent cations. Similar values are obtained by spectrofluorometric equilibrium titrations employing stationary fluorescence.

The observed pK values are indicative of the existence of a nonselective cation binding site which, upon cation occupation, provides high-affinity eosin Y binding. Evidence for such a site has also been presented recently on the basis of cation binding studies of FITC-Na,K-ATPase.[7,8] The pK_1 value obtained for Na^+ is slightly higher than that for other monovalent cations. This may reflect a certain effect of the additional occupation of the selective Na^+ site, occurring at lower concentrations than that of the non-selective one.[7]

Dynamics of Eosin Y Binding

To avoid time-consuming binding studies as carried out previously[9] for the determination of pK_2, kinetic studies were carried out to determine the formation rate constants k_{23} and k_{32}. Knowing these parameters, K_2 can be calculated. Recent kinetic studies with FITC-Na,K-ATPase indicated that the formation of M^{n+}–E is very slow, and occurring in the time range of seconds. The rate-limiting step of cation binding and dissociation has been attributed to a conformational change leading to a state that has been denoted F_1.[10] State F_1 differs from the well-known Na,K-ATPase conformations E_1 and E_2.[7–10] Evidence for the existence of such a slow, non-selective cation binding step results also from kinetic cation binding studies in the presence of the eosin Y.[11,12]

Because eosin Y binding to M^{n+}–E is very fast compared to the adjustment of the slow, preceding, cation binding equilibrium [Eq. (2)], the latter was assumed as unperturbed during our kinetic study. In these circumstances, k_{23} and k_{32} can be obtained in a direct manner by performing stopped-flow experiments (shown in Fig. 2) in the presence of comparatively high concentrations

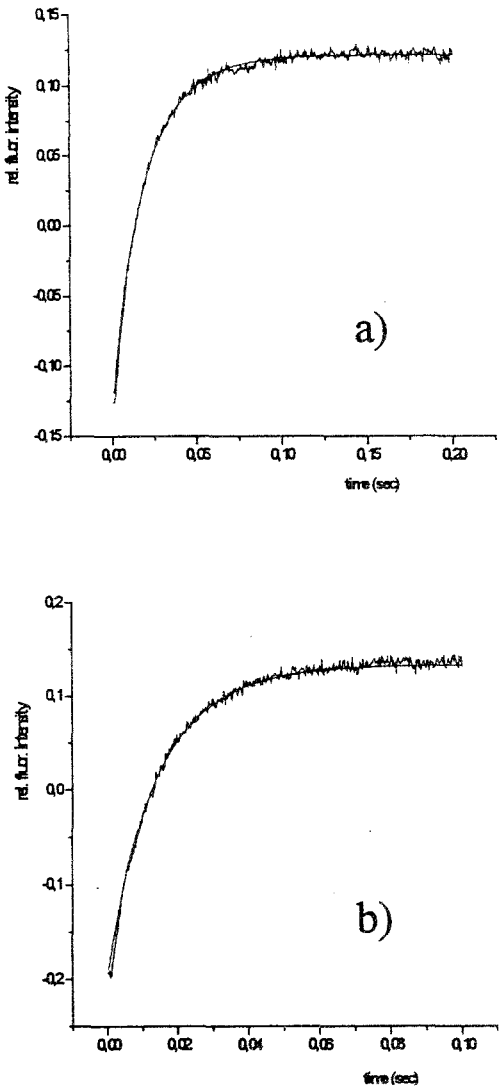

Fig. 2. Kinetics of eosin Y binding: fluorescence stopped-flow experiments at 25°C. Rapid mixing between eosin Y in the presence of (a) 24 mM NaCl or (b) 1 mM $BaCl_2$ with Na,K-ATPase in the presence of the same cation concentrations in 5 mM imidazole/HCl, pH 7.5 (concentration after mixing: 0.5 μM eosin Y, 0.33 μM Na,K-ATPase). The solid line is the result of a fit with (a) $k_{23} = 6.3 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{32} = 16\ s^{-1}$, and (b) $k_{23} = 8.7 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{32} = 22\ s^{-1}$.

of, e.g., NaCl or $BaCl_2$. Here k_{23} is determined as a second-order formation and k_{32} as a first-order dissociation rate constant.[13]

The pseudo-first-order condition generally applied for the evaluation of stopped-flow data does not hold under our conditions. The obtained results are in agreement with the postulated reaction model. The k_{23} values are close to what is expected for a diffusion-controlled binding reaction.

CONCLUSIONS

Time-resolved fluorescence allows, in a convenient and direct manner differentiation between free and bound eosin Y and therefore characterization of the enzyme bound state. Because negatively charged eosin Y competes with ATP and ADP for the same binding region within the enzyme, this fluorophore is considered as a relevant model compound with regard to adenine nucleotides. High-affinity eosin Y binding is observed only under conditions where a nonselective, regulatory cation binding site is occupied. Since this type of cation binding induces a conformational transition,[10] the observed eosin Y binding effects are indicative of a certain conformational change, leading to the formation of state F_1. From the diffent pK values for mono- and divalent cations, it is concluded, in accordance with the electrostatic ion binding concept of Ref. 9 and 14, that the cation site of the protein regulating eosin Y binding is likely to exhibit two negative charges. Because the enzyme is located in a negatively charged small membrane particle surface charge effects are also thought to influence eosin Y binding.

ACKNOWLEDGMENTS

The authors thank A. Schacht for excellent technical assistance, Dr. M. Esmann and Dr. H. Ruf for stimulating discussions, and SFB 169 for financial support.

REFERENCES

1. S. J. D. Karlish (1979) in J. C. Skou and J. G. Nørby (Eds.), *Na,K-ATPase Structure and Kinetics*, Academic Press, New York, pp. 115–128.
2. M. L. Helmich-de Jong, J. P. M. van Duynhoven, F. M. A. H. Schuurmans Stekhoven, and J. J. H. H. M. De Pont (1986) *Biochim. Biophys. Acta* **858**, 254–262.
3. C. Gatto, C. C. Hale, W. Xu, and M. A. Milanick (1995) *Biochemistry* **34**, 965–972.
4. J. C. Skou and M. Esmann (1981) *Biochim. Biophys. Acta* **647**, 232–240.
5. J. C. Skou and M. Esmann (1983) in *Current Topics in Membranes and Transport, Vol. 19*, Academic Press, New York, pp. 451–455.
6. P. L. Jørgensen (1974) *Biochim. Biophys. Acta* **336**, 36–52.
7. H. Ruf, E. Lewitzki, and E. Grell (1994) in E. Bamberg and W. Schoner (Eds.), *The Sodium Pump*, Steinkopff, Darmstadt, pp. 569–572.
8. E. Grell, E. Lewitzki, H. Ruf, and M. Doludda (1994) in E. Bamberg and W. Schoner (Eds.), *The Sodium Pump*, Steinkopff, Darmstadt, pp. 617–620.
9. E. Grell, R. Warmuth, E. Lewitzki, H. Ruf (1992) *Acta Physiol. Scand.* **146**, 213–221. ERRATUM: **147**, 343–344 (1993).
10. M. Doludda, E. Lewitzki, H. Ruf, and E. Grell (1994) in E. Bamberg and W. Schoner (Eds.), *The Sodium Pump*, Steinkopff, Darmstadt, pp. 629–632.
11. E. Lewitzki *et al.* unpublished.
12. M. Esmann (1994) *Biochemistry* **33**, 8558–8565.
13. E. Grell, E. Lewitzki, and A. Ifftner (1985) in I. M. Glynn and E. Ellory (Eds.), *The Sodium Pump*, The Company of Biologists Limited, Cambridge, pp. 289–294.
14. R. M. Fuoss (1958) *J. Am. Chem. Soc.* **80**, 5059–5061.