Journal of Fluorescence, Vol. 8, No. 2, 1998

Characterizing Protein Conformational Transitions of Na,K-ATPase with Antibodies by Fluorescence Spectroscopy

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Received October 27, 1997; accepted January 9, 1998

Stationary and time-resolved fluorescence of FITC–Na,K-ATPase is investigated as a function of pH in the presence of different ligands, cations, and the monoclonal anti-FITC antibody 4-4-20. The binding of K^+ and of the antibody leads to the same decreased fluorescence intensity level. Antibody binding is observed only under conditions where the enzyme exists in the conformational state F_1, and not in the form of the Na^+ or K^+ complex or when it is phosphorylated with inorganic phosphate in the presence of Mg^{2+}. For the interpretation of the results it is shown that the fluorophore is not essentially affected by an acidity change of the bound dye, so that p*K* variations responsible for the observed intensity changes can be excluded in favor of a static quenching process

KEY WORDS: FITC; antibodies; fluorescence decay; Na, K-ATPase; p*K*.

INTRODUCTION

The transport enzyme Na,K-ATPase (Na^+ pump) is integrated in the plasma membranes of most eukariotic cells. It transduces the free energy obtained from the hydrolysis of Mg-ATP to build up and maintain the required Na^+ and K^+ cation gradients across the cellular membranes [1]. The enzyme consists of an enzymatically active α and an inactive β (glycoprotein) unit [MW (αβ) ≈ 150,000]. Structural predictions based on known amino acid sequences suggest the formation of 10 putative transmembrane segments for the α unit and 1 for the β unit.

The coupling between alkali ion transport and enzymatic activity is, among other facts, thought to be linked to conformational transitions of the protein. One particular attempt to provide information about the conformational properties of the enzyme consists in covalently attaching fluorophores, such as fluorescein 5'-isothiocyanate (FITC), to the protein. The FITC group is linked to the nucleotide domain of the enzyme. The fluorescence emission intensity of the bound dye changes upon binding of certain ligands and of cations in a reversible manner [2,3].

The fluorescence emission intensity of renal FITC–Na,K-ATPase in a standard buffer solution, such as 10 m*M* imidazole/HCl, pH 7.5, increases by about 25% as a consequence of the nonselective binding of different mono- and divalent cations (formation of the F1 state [4]). On the other hand, selective binding of K^+ and its congeners can be followed by an intensity decrease of approximately 20%, leading to the formation of a state denoted E2 [2–4].

In contrast to the extensive use of FITC as a fluorescence label, and to attempts to understand the basis of its fluorescence intensity changes [5,6], there is still a lack of information concerning the physical, resp. molecular origins of these experimentally observed effects.

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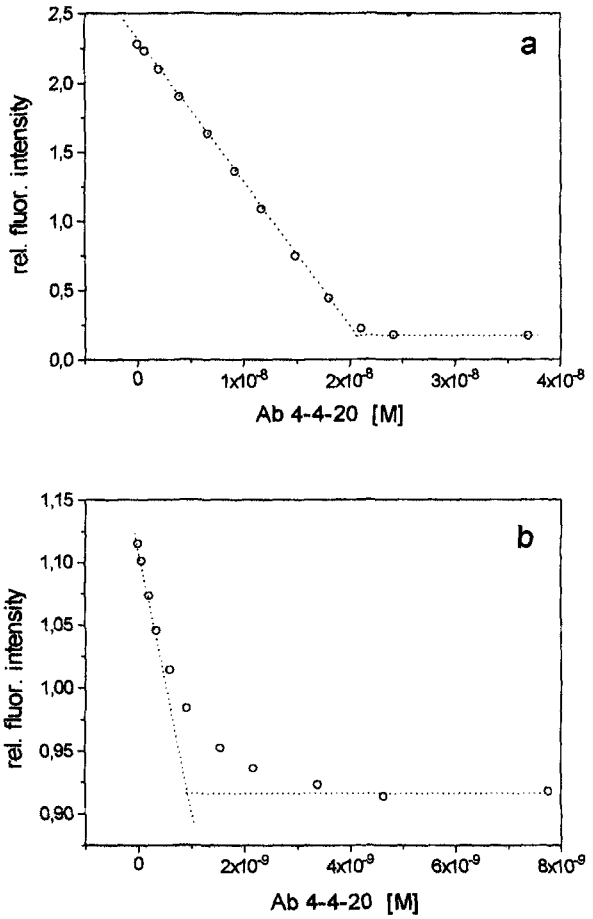

Fig. 1. Spectrofluorometric titration of 3.5×10^{-8} *M* fluorescein with mouse monoclonal antibody (4-4-20). Quenching efficiency = 93% (a) and of 4×10^{-9} *M* FITC–Na,K-ATPase (b). Buffer (a and b), 10 m*M* imidazole/HCl, pH 7.5; $T = 25°C$.

We therefore try in this study to characterize the binding of antifluorescein antibodies to FITC-labeled Na,K-ATPase. Stationary and time-resolved fluorescence measurements were applied to gain information about the accessibility and therefore the possible location of the labeling sites. Spectrofluorometric pH titrations of FITC–Na,K-ATPase of different conformational states were investigated to estimate whether the observed fluorescence intensity changes can be attributed to an acidity change (p*K* change) of the bound fluorophore.

MATERIALS AND METHODS

Membrane-bound Na,K-ATPase is isolated from pig kidney according to Ref. 7. The purified enzyme exhibits a maximum activity of 35 μmol P_i mg^{-1} min^{-1} under standard conditions and is obtained in the form of membrane disks which are accessible from both sides. The mean diameter is about 200 nm.

For spectrofluorometric binding studies, the enzyme is labeled covalently according to a modified procedure of Karlish [2] and contains about 1 equiv of FITC. It is stored as a concentrated stock solution in the presence of 0.2 m*M* dithiothreitol on ice for not more than 4 days. For stationary fluorescence measurements with a Spex fluorolog 222 instrument, an excitation wavelength of 495 nm is used and emission is observed at 518 nm unless specified otherwise. Determination of pK_a values for the characterization of protolytic equilibria of enzyme-bound FITC is performed with a combined glass pH electrode (Moeller/Zürich). To avoid KCl release from the electrode during pH measurements, the internal 3 *M* KCl solution in the reference compartment was removed and exchanged against 500 m*M* tetramethylammonium chloride.

Time-resolved fluorescence studies are carried out with an Edinburgh 199s nanosecond fluorometer (λ_{exc} = 337 nm; λ_{emi} = 518 nm).

Three antibodies (Ab) are used for quenching experiments with FITC-Na,K-ATPase: (a) polyclonal antifluorescein Ab, rabbit IgG fraction (Molecular Probes); (b) monoclonal antifluorescein Ab, mouse 4-4-20 (Molecular Probes); and (c) antifluorescein single-chain Ab (Pan Vera, Madison, WI), a polypeptide of approximately 27,000 Da, consisting of a 112-amino acid V_L chain joined to a 121-amino acid V_H chain by an 18-amino acid linker and a small N-terminal extension.

All three antifluorescein antibodies induce 95% quenching of the fluorescence of free fluorescein, as shown in Fig. 1a, for example, for mouse monoclonal 4-4-20 Ab.

RESULTS AND DISCUSSION

Fluorophore Accessibility to Antibodies

Time-resolved fluorescence decay measurements of FITC–Na,K-ATPase are characterized by two nanosecond processes (around 3.9 and 1.6 ns) [6]. This is indicative of the existence of two fluorescent states due to the distribution of the FITC label among two lysine side chains [8], for example. The 3.9-ns decay dominates the stationary fluorescence and is tentatively attributed to FITC at Lys-501. The fluorophore is present in an aqueous environment, whereas the 1.6-ns process is consistent with a protein environment for the fluorophore [6]. Because the current studies have not allowed us to ex-

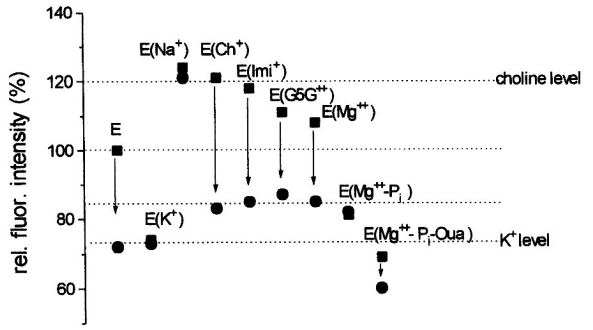

Fig. 2. Relative fluorescence intensity levels of FITC–Na,K-ATPase (100% corresponds to the observed intensity in 10 m*M* imidazole/HCl, pH 7.5) in the presence of different bound cations (■) as well as after addition of a saturating amount of Ab 4-4-20 (●). Ch, choline chloride; G5G, 1.5-diguanidiniumpentane dichloride; Imi, imidazole; Oua, ouabain.

plain unambiguously the physical nature of the fluorescence intensity decrease observed upon K^+ binding, additional studies are required to characterize the properties of the bound FITC.

If the interpretations concerning the local environments of the bound fluorophores are correct, the fluorophore giving rise to the 3.9-ns process should be accessible to anti-FITC antibodies, and in these circumstances it should be quenchable upon specific antibody binding.

All three investigated antibodies are able to quench extensively the fluorescence of free fluorescein, but only the monoclonal 4-4-20 Ab can quench markedly the fluorescence of FITC–Na,K-ATPase, as shown in Fig. 1b. A maximum quenching of about 25% is observed for a molar ratio of Ab/FITC of about 0.3. The dense packing of proteins in the membrane disk is assumed to hinder sterically the interaction of accessible fluorophores with antibody molecules.

The antifluorescein single-chain antibody, which was investigated because possible aggregation effects due to binding to two antigen binding sites can be avoided, surprisingly showed no detectable quenching of FITC–Na,K-ATPase prepared from pig kidney and shark rectal gland enzyme. The reason may be due to unspecific binding of the Ab to other protein epitopes, thus preventing the interaction with the enzyme-bound fluorophore. In the case of the polyclonal Ab, evidence for additional unspecific binding is found. Detailed quenching experiments thus were carried out preferentially with the monoclonal 4-4-20 Ab.

The degree of quenching and thus also the accessibility of the fluorophore depend in a sensitive way on the buffer concentration as well as on the nature of the bound cations, as indicated in Fig. 2, and thus on the conformational properties of the enzyme. The fluorophore is essentially not accessible to the 4-4-20 Ab under conditions where the FITC enzyme exists in the form of its Na^+ or K^+ complex and when it is phosphorylated with P_i in the presence of Mg^{2+}. In contrast to this observation, complexes with nonselectively bound cations, characteristic of the E1 state (choline level; cf. Fig. 2), clearly exhibit fluorescence quenching to about the level of that of the K^+ complex (K^+ level), as observed for the free FITC enzyme.

Surprisingly, the addition of 100 m*M* NaCl to the quenched choline complex of the enzyme reverses the quench and leads back to the high intensity level of the Na^+ complex. The high-affinity binding of Na^+ appears to control the accessibility of the fluorophore much more extensively than the weaker, nonselective binding of this cation.

With regard to dynamic fluorescence, the amplitude of the 3.9-ns process is reduced, resulting in a decrease in fractional intensity from an initial 75% to about 50% upon antibody binding.

Protolysis of the Fluorophore

Protolysis of fluorescein and, accordingly, also of FITC is characterized by three pK values (3:1 stoichiometry; $pK_1 = 2.08$, $pK_2 = 4.31$, and $pK_3 = 6.43$ for fluorescein [9]). Only the dianionic state, which predominates in alkaline solutions, exhibits strong fluorescence. A fluorescence emission intensity decrease of FITC–Na,K-ATPase, as observed upon K^+ binding, among other effects, could possibly also be attributed to a decrease in the pK_3 value of the FITC as an indirect consequence of K^+ or congener binding. In order to investigate this aspect, spectrofluorometric pH titrations are carried out on FITC–Na,K-ATPase in media of different ionic compositions and are compared with the results obtained with the model compound N^{ε}-FITC,N^{α}-acetyl-L-lysine methylamide (FITC-lys). Its pK_3' is found to be 6.35 in 10 m*M* imidazole/HCl. As in the case of fluorescein or free FITC, the pK_3' of FITC-lys decreases by about 0.25 pK unit with 0.1 *M* increasing ionic strength. Thus, also the emission intensity at a constant pH (below pH 8.0) will increase with increasing ionic strength.

Figure 3 shows a spectrofluorometric pH titration of FITC–Na,K-ATPase. Although the second FITC acid dissociation step characterized by pK_2 contributes to some extent to the observed dependence below pH 5.5, the evaluation is based on a 1:1 stoichiometry, leading to an apparent value for pK_3, denoted pK_3' (cf. Table I).

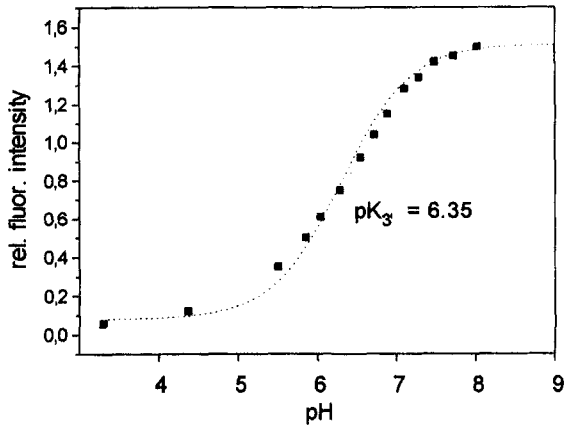

Fig. 3. Spectrofluorometric pH titration of 7×10^{-8} *M* FITC–Na,K-ATPase in 10 m*M* imidazole/HCl containing 3 m*M* KCl at 25°C. Evaluation based on 1:1 stoichiometry.

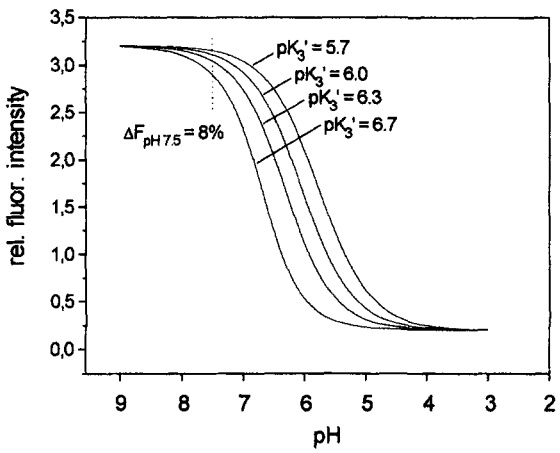

Fig. 4. Hypothetical fluorescence pH titrations for pK_3' values ranging from 5.7 to 6.7. The vertical dotted line corresponds to the expected intensity change ΔF observable at pH 7.5.

Table I. Apparent Protolytic Constants Determined from Spectrofluorometric pH Titrations for Different Ionic States and Complexes of FITC—Na,K-ATPase at 25°C

Medium	pK_3'
25 m*M* histidine/HCl	5.70
2.5 m*M* imidazole/HCl	6.10
10 m*M* imidazole/HCl	6.35
10 m*M* imidazole/HCl containing Ab 4-4-20	6.40
3 m*M* KCl	6.35
3 m*M* KCl/Ab 4-4-20	6.15
100 m*M* NaCl	6.10
100 m*M* ChCl	6.00
100 m*M* ChCl/Ab 4-4-20	5.85
3 m*M* G5G-Cl_2	6.30
4 m*M* $BaCl_2$	6.30

Concerning FITC–Na,K-ATPase, the pK_3' value is very low in 25 m*M* histidine/HCl and increases upon transfer to imidazole/HCl buffers. Otherwise, the pK_3' values decrease with increasing ionic strength, as reported for fluorescein [9]. In addition, the remaining fluorescence observed upon binding of the antibody is characterized by a lower pK_3' value than in the absence of the antibody. As a general result, the pK_3' value, essentially assigned to the protonation of the phenolic OH group of the FITC residue, depends on the composition of the ionic medium and is changed upon antibody binding.

CONCLUSIONS

The experimentally observed nanosecond fluorescence decay studies on FITC–Na,K-ATPase indicate that at least two fluorophore states exist. According to the corresponding amplitudes, about one-half of the fluorophores are located in an aqueous (3.9 ns) and the second half in a protein-like (1.6 ns) environment [6]. The 25% total fluorescence intensity decrease observed in 10 m*M* imidazole/HCl, pH 7.5, upon K^+ binding is attributed to a static quenching process since there is no measureable change of the corresponding decay times. This quench could be due to a complex formation of the fluorophore with an amino acid side chain that has changed its position subsequent to cation binding, and this interaction should lead to a change of at least one of the amplitudes [6].

Such an interpretation can be valid only if, for example, the fluorescence intensity change observed upon K^+ binding is not simply the result of a pK_3' increase of the bound FITC. The binding of this cation could, for example, lead to a subsequent structural rearrangement that could induce the corresponding pK_3' shift. The spectrofluorometric pH titrations of FITC–Na,K-ATPase in different media (Table I) indicate that the resulting pK_3' values are all very similar, except for those determined at very low ionic strengths or in the presence of the monoclonal antibody 4-4-20. Even if we assume a hypothetical pK_3' increase of 1 unit, for example, from 5.7 to 6.7 (cf. Fig. 4), which is much larger than what has been observed experimentally (Table I), a maximum fluorescence intensity decrease at pH 7.5 of around 8% would be expected. Thus, this consideration leads to the conclusion that the fluorescence intensity changes observed upon cation binding can be attributed only to a minor extent to an acidity change of the bound fluorophore. Therefore, this possibility is excluded in favor of the static quenching process mentioned before.

With regard to dynamic fluorescence, the aqueous exposed part of the fluorophore, which is represented by the 3.9-ns decay time, is expected to be accessible to anti-FITC antibodies, independent of the adopted conformational state. Surprisingly the fluorophore is essentially not influenced by the monoclonal 4-4-20 Ab under conditions where the FITC enzyme exists in the form of its Na^+ or K^+ complex. Only conformations formed with divalent or monovalent cations, leading to the F1 state [4], are partially quenched by the antibody. Nearly independent of the initial intensity of the fluorescence of the FITC–Na,K-ATPase in the presence or absence of cations or ligands, the addition of the monoclonal antibody 4-4-20 leads to almost the same intensity level for all of the investigated states of the enzyme (cf. Fig. 2). This intensity level is virtual identical to that of the K^+ complex in the presence or absence of the antibody. This correspondence may suggest that the same fraction of fluorophores is not only accessible to the antibody but also quenched upon K^+ binding.

Preliminary time-resolved fluorescence measurements indicate a decrease in the amplitude of the 3.9-ns in favor of an amplitude increase in the 1.6-ns decay process. This implies that the 3.9-ns process is significantly affected by K^+ as well as antibody binding. Since there is an experimentally observed wavelength-dependent change of the measured lifetimes, detailed time-resolved decay measurements will be requiered to support this interpretation in quantitative terms.

However, it should be taken into consideration that other explanations are possible. For example, protein conformational-dependent molecular events could lead to the formation of the lactone form of the bound fluorophore, which would also lead to a fluorescence intensity decrease. This possibility would have to be investigated by different techniques.

ACKNOWLEDGMENTS

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

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