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Toward an Understanding of the Fluorescence Intensity Changes Observed on Fluorescein 5'-Isothiocyanate-Na^+,K^+-ATPase[1]

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The fluorescence emission intensity between the Na^+ and the K^+ complex of Na^+,K^+-ATPase, labeled with fluorescein 5'-isothiocyanate (FITC), differs by 30 to 40%. Experimental studies are carried out to elucidate the physical reasons which account this intensity difference. The dissociation constant of protolysis of the covalently bound FITC and its fluorescence decay times are determined in media of different ionic compositions and are compared with the corresponding properties of a synthetic model compound. The fluorophore bound to the protein is characterized by two decay times in the nanosecond range; the model compound, by a single one. The static fluorescence intensity changes are discussed on the basis of these results.

KEY WORDS: Fluorescence spectra; fluorescence decay; dissociation constants; fluorescein 5'-isothiocyanate; FITC—fluorescein 5'-isothiocyanate-Na^+,K^+-ATPase.

INTRODUCTION

The integral membrane protein Na^+,K^+-ATPase, consisting of the catalytic α and β peptide subunit, acts as an ATP-driven cation pump in the cellular membranes of all higher organisms. To obtain information about molecular aspects of individual reaction steps, proteins are often covalently labeled with fluorescent dyes. A typical example is the labeling of Na^+, K^+-ATPase with fluorescein 5'-isothiocyanate (FITC) [1], where it can be shown that the fluorescence intensity increases upon Na^+ and decreases markedly upon K^+ binding [1–5], as depicted in Fig. 1. The fluorophore is assumed not to be directly involved in cation binding and acts as an indirect reporter group that is sensitive to changes in its environment, for example, due to protein conformational rearrangements. In biochemical studies, Xu (6) and Abott *et al.* [7] provided evidence that the FITC residue appears to be preferentially bound to Lys-501 and Lys-480 of the α peptide in the case of the renal canine and lamb enzyme. Thus, the covalently bound FITC is distributed among at least two different Lys residues (cf. Fig. 2). However, one equivalent of covalently bound FITC inhibits ATP-hydrolytic activity but neither phosphorylation from P_i nor nitrophenyl phosphatase activity.

EXPERIMENTAL

Membrane-bound Na^+,K^+-ATPase was prepared from pig kidney according to Ref. 8; the FITC-enzyme, according to Ref. 1. Details are given in Ref. 9. Fluorescence spectra are measured on a Spex fluorolog 212 instrument. The fluorescence decay data are obtained

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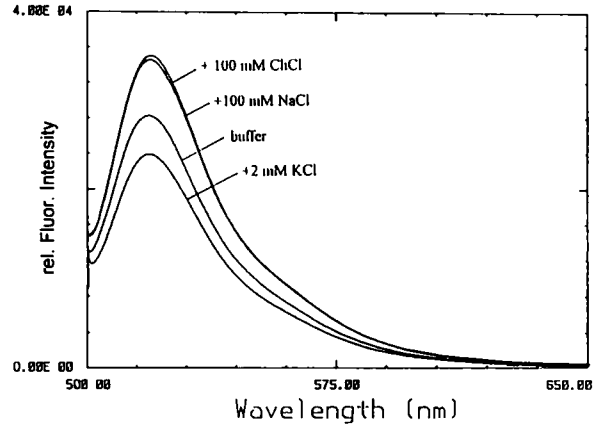

Fig. 1. Fluorescence emission spectra (excitation at 495 nm) of 0.2 μM FITC-Na^+,K^+-ATPase in 15 mM imidazole–HCl, pH 7.5 (buffer), as well as in the additional presence of NaCl, choline chloride (ChCl), and KCl at 25°C.

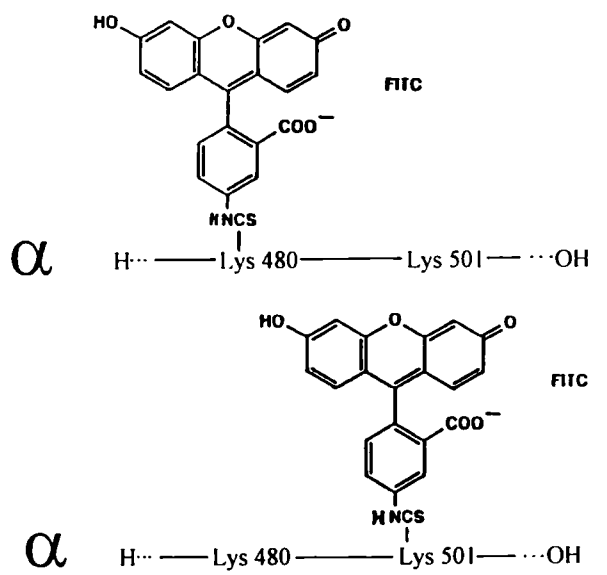

Fig. 2. Schematic illustration of the distribution of the covalently bound fluorescein 5'-isothiocyanate among two different Lys residues of the α unit of Na^+,K^+-ATPase.

employing a nanosecond (199 S, Edinburgh Instruments) and a picosecond laser single-photon counting (SPC) setup, described previously [10].

FACTORS INFLUENCING THE FLUORESCENCE PROPERTIES OF FITC

Stationary Fluorescence. Figure 1 illustrates the typical fluorescence emission intensity changes observed upon the addition of either NaCl or choline chloride or of KCl to the FITC-enzyme in 15 mM imidazole–HCl buffer at pH 7.5. To understand the fluorescence properties of FITC-Na^+,K^+-ATPase, a suitable model compound, N^{ε}-FITC,N^{α}-acetyl-L-lysine methylamide has been investigated. The fully deprotonated state is fluorescent; the mono- and higher protonated states are nonfluorescent. The negative logarithm of the corresponding dissociation constant (pK^D) is 6.3 in the 15 mM imidazole–HCl (25°C) and is assigned to the preferential deprotonation of the phenolic OH group. According to analogous studies on the FITC-enzyme in the same medium, a uniform pH titration curve with a pK^D value of 6.6 is found. This result suggests that the pK^D values of the FITC in both locations are likely to be similar, which, however, does not yet represent a conclusive statement. A location of FITC in a very hydrophobic local environment of the protein could have resulted in a drastic pK^D difference compared with that of the model compound. In addition, the FITC fluorescence emission intensity is expected to depend not only on the degree of deprotonation, but also on the ionic composition. In the case of the model compound the pK^D value decreases slightly at ionic strengths higher than 40 mM. This effect of the electrolyte concentration on the pK^D value is also observable for the FITC-enzyme but does not affect the measured properties markedly. Compared to the model compound, the emission intensity of the protein-bound FITC (1 equivalent per αβ) is reduced by a factor of about 1.4 in 15 mM imidazole–HCl, pH 8.0, which may be attributed to a number of quenching processes due to amino acid residues, ions, and solvent molecules within the fluorophore's environment. The position of the fluorescence emission maximum of fluorescein and its derivatives is not very sensitive to solvent polarity changes. The fully deprotonated state of the fluorophore in the model compound exhibits its emission maximum in aqueous solution at 516 nm and in methanol containing 3% water (v/v) at 518 nm, which corresponds to the value observed for the FITC-enzyme.

Dynamic Fluorescence. Since it is difficult to conclude the number of fluorescent states or species involved from static fluorescence studies, dynamic fluorescence studies were carried out. At pH 7.5 the model compound is characterized by a single fluorescence decay (decay time, 3.9 ns), which means that only one protolytic state exhibiting a high fluorescence intensity is populated (the remaining states are nonfluorescent and the rates of protolytic interconversions are considerably slower). According to Ref. 6, at least two fluorescence decay times due to the two different fluorophore locations are expected for FITC-Na^+,K^+-ATPase. The studies

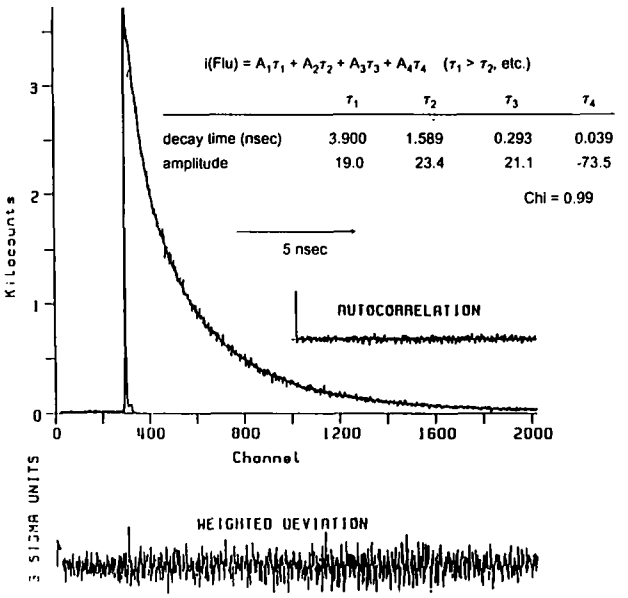

Fig. 3. Fluorescence decay of 1.1 μ*M* FITC-Na⁺,K⁺-ATPase in 10 m*M* imidazole–HCl, pH 7.5 (25°C). The parameters of the fit are given at the top. Experimental conditions: excitation at 296 nm, emission at 519 nm; 10.15 ps/channel.

in the nanosecond range reported in Ref. 7 provided evidence for only a single decay time. Our extensive decay studies in the picosecond and nanosecond time range provide a different result: The purified pig kidney FITC-enzyme exhibits four decay processes, characterized by two times in the picosecond (40 and 300 ps) and two in the nanosecond time range (Fig. 3). A detailed study of the fluorescein probe molecule itself can throw light on the photophysical processes leading to the 40- and 300-ps decay times. The observation that the shortest decay time has a negative amplitude (cf. Fig. 3) could originate from changes in the fluorophore's molecular conformation upon excitation. Further, primarily photophysical studies will be required to explain this experimental finding, which is not considered to be influenced by experimental artifacts because a comparatively low time resolution, about 10 ps/channel, had been chosen for the corresponding measurements. The fact, however, that clearly two slower decay times (around 1.6 and 3.9 ns) are found is indicative of the existence of two different fluorescent states, which is qualitatively consistent with biochemical evidence [6,7] concerning a localization of the fluorophore at, e.g., two different sites such as two amino acid side chains, as shown in Fig. 2. The amplitude of the 3.9-ns process is about 2.4 times higher than that of the 1.6-ns process and thus dominates the static fluorescence emission. If the majority of the FITC molecules were bound to Lys-501 in the case of the pig enzyme, the 3.9-ns decay could be directly assigned to this residue. The FITC molecules that are characterized by the 3.9-ns decay are likely to be located in a water-exposed part of the protein because the model compound exhibits the same decay time under these conditions. The shorter time constant, about 1.6 ns, appears to reflect the result of quenching by surrounding protein residues. Only the fluorophore molecules which give rise to the 1.6-ns process seem to be affected by the protein.

WHICH FACTORS ARE RESPONSIBLE FOR THE FLUORESCENCE INTENSITY DIFFERENCE BETWEEN THE Na⁺- AND THE K⁺-BOUND STATE?

Several physical factors could cause the fluorescence intensity difference (30 to 40%, depending on the preparation) between the high-intensity state of FITC-Na⁺,K⁺-ATPase, observed in the presence of Na⁺, choline, guanidinium, protonated diamines, bisguanidinium compounds (originally introduced in Ref. 11), or alkaline earth cations [5,9], and the low-fluorescence intensity state, found in the presence of K⁺, Rb⁺, Cs⁺, NH_4^+, or Tl^+ [1–5,9]: A shift of the pK^D value of the covalently bound FITC, for example, upon binding of K⁺, could lead to a fluorescence intensity change of the fluorophore linked to either one or to both Lys residues. Although no drastic pK^D changes are found experimentally under conditions of identical ionic strength (pK^D = 6.6 in the presence of 130 m*M* choline chloride or NaCl; pK^D = 6.2 with 30 m*M* KCl and 100 m*M* choline chloride), they are likely to contribute to the observed fluorescence change.

Based on a comparison of the static and dynamic fluorescence, one would expect that the intensity difference observed in the former case manifests itself at least in one of the nanosecond decay times. The experimental studies, however, indicate that the decay times are not markedly changed upon the addition of any buffer, salt, substrate, or inhibitor. Two currently indistinguishable possibilities could explain the result of the static measurements: Either the magnitude of the 1.6-ns decay time constant decreases upon the transition from the high- to the low-intensity fluorescence state or the amplitude, but not the time constant, of the 3.9-ns process, decreases. Both possibilities are consistent with the conclusion according to Ref. 7 that the fluorophore bound to only one of two possible Lys residues is sensitive to the change occurring in the protein environment and thus is capable of reporting cation binding processes. Further experi-

ments will be necessary to clarify these aspects to provide a rational basis for the understanding of the observed fluorescence intensity changes.

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