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Binding of pyrene isothiocyanate to the E_1ATP site makes the H_4-H_5 cytoplasmic loop of Na^+/K^+-ATPase rigid

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1-Pyreneisothiocyanate was shown to be an inhibitor of Na^+/K^+-ATPase. Reverse-phase HPLC and activity studies indicated binding of 1-pyreneisothiocyanate at the H_4-H_5 loop of the α subunit and competition with the fluorescein 5′-isothiocyanate for the E_1ATP site. While fluorescein 5′-isothiocyanate, the fluorescent ATP pseudo-analog, was shown to be immobilized at the E_1ATP site, there was no possibility to draw any conclusion about the flexibility of the E_1ATP site due to its short lifetime. Employing 1-pyreneisothiocyanate as a long-lived fluorophore and a label for the E_1ATP site, we found that the ATP-binding site of Na^+/K^+-ATPase and, in fact, the whole large intracellularly exposed H_4-H_5 loop of the catalytic α subunit is rigid and rotationally immobilized. This has important consequences for the molecular mechanism of the transport function.

Keywords: Na^+/K^+-exchanging ATPase; ATP-binding site; cytoplasmic loop; frequency-domain fluorometry.

Na^+/K^+-ATPase is a plasma membrane protein whose primary physiological function is to maintain the Na^+ and K^+ cation gradients across animal cell membranes. Therefore, it plays a central role in maintaining the resting membrane potential, in facilitating the transport of various ions and cell metabolites [1]. Na^+/K^+-ATPase is composed of at least two subunits, the catalytic α subunit and the smaller glycoprotein β subunit. All of the known functional aspects of the enzyme are attributed to the α subunit whose C- and N-termini are in the cytosol [2]. It contains the extracellular binding site for ouabain [3] and ATP [4, 5]. A key role in the enzyme function is undoubtedly played by the large cytoplasmic loop between the fourth and fifth transmembrane segments (the H_4-H_5 loop) where the high-affinity ATP-binding site is localized. It was reported to be involved in structural transitions coupled to the ion translocation process in the ($\alpha\beta$) unit of the Na^+/K^+-ATPase [5] even if the transmembrane ion translocation process is apparently associated with the transmembrane segments.

Fluorescent derivatives of isothiocyanate have been shown to bind to the ATP-binding site at Lys480 and/or at Lys501 (the specific fluorescein-5′-isothiocyanate(FITC)-binding site) and to block efficiently the access of ATP to its binding site [6−8]. The fluorescence of FITC-labeled Na^+/K^+-ATPase is highly anisotropic and, moreover, it increases in the presence of anti-FITC antibody, which quenches nonspecifically bound FITC molecules [9, 10]. This suggests a kind of immobilized ATP-binding site on the H_4-H_5 loop without any independent segmental motion. The lifetime of the excited state of FITC is less than 4 ns, too short to yield any information about the mobility of the whole large H_4-H_5 cytoplasmic loop. In the present study, we used 1-pyreneisothiocyanate, which is known to have a much longer lifetime of the excited state. It has a broader time-window that makes it possible to look at the mobility of the H_4-H_5 cytoplasmic loop of the catalytic subunit of Na^+/K^+-ATPase on the time scale of tens of nanoseconds. This study indicates that labeling of the H_4-H_5 loop by PITC makes it rigid.

MATERIALS AND METHODS

Materials. All chemicals were of the highest available purity and were obtained from Bio-Rad, Boehringer/Mannheim, Molecular Probes, and E. Merck. The Lab-Trol protein standard is a product of Merz & Dade; [γ-^{32}P]ATP was from Amersham Buchler.

Enzyme assays and preparation. Na^+/K^+-ATPase in the range 15−20 U/mg protein was isolated from pig kidney by a modification of Jørgensen's procedure [11] and measured by a coupled spectrophotometric assay [12, 13]. 1 U enzyme is defined as the hydrolysis of 1 μmol ATP/min at 37 °C. Protein was determined by the method of Lowry et al. [14] using Lab-Trol as a protein standard. Lab-Trol is a mixture of proteins and

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Abbreviations. FITC, fluorescein 5′-isothiocyanate; PITC, 1-pyreneisothiocyanate; H_4-H_5 loop, the cytoplasmic loop between transmembrane segments 4 and 5 of the α-subunit of Na^+/K^+-ATPase; E_1ATP site, ATP-binding site of Na^+/K^+-ATPase with high affinity; E_2ATP site, ATP-binding site of Na^+/K^+-ATPase with low affinity; f_i, fractional intensity of *i*th component; g_i, complex functions of the rates of rotation around the molecular axes of the asymmetric body; r, fluorescence anisotropy; w_i, half-width at the half maximum of *i*th component; α_i, respective pre-exponential factor of *i*th component; ϱ_i, rotational correlation time of the *i*th component; τ_i, lifetime of *i*th component; χ^2_r, goodness of the fit.

Enzyme. Na^+/K^+-exchanging ATPase (EC 3.6.1.37).

enzymes used for the calibration of assays in clinical chemical analysis. All buffers used were made up to their appropriate pH value at room temperature. Electrophoresis and Coomassie brilliant blue staining were carried out on analytical SDS/polyacrylamide gels containing 15% polyacrylamide according to Laemmli [15].

Inactivation of Na^+/K^+-ATPase by PITC. Inactivation by 1-pyreneisothiocyanate (PITC) was examined as follows: 1 U Na^+/K^+-ATPase was incubated in a total volume of 1 ml with various concentrations of PITC in 20 mM Tris/HCl, pH 7.25, and 15 mM NaCl at 37 °C. The residual Na^+/K^+-ATPase activity was measured spectrophotometrically in 50-µl aliquots [12, 13].

Inactivation of Na^+/K^+-ATPase by FITC. Na^+/K^+-ATPase in a final concentration of 1 U/ml (65 µg/ml) was incubated overnight at 37 °C in a solution containing 20 mM Tris/HCl, pH 7.25, and 10 µM FITC. The control enzyme was treated in the same way but without FITC. The inactivated enzyme (residual activity ≈1%) was centrifuged in Eppendorf tubes at 100 000 *g* in a Ti50 rotor of a Beckman L2-65B centrifuge using adapters of our own design. The pellet was washed in 20 mM Tris/HCl, pH 7.25, and resuspended in a solution of 20 mM Tris/HCl, pH 7.25, 15 mM NaCl and different PITC concentrations (0−50 µM). After 1 h incubation at 37 °C, the enzyme was centrifuged under the same conditions, washed twice in 20 mM Tris/HCl, and the K^+-activated *p*-nitrophenylphosphatase activity was subsequently determined.

Determination of K^+-activated *p*-nitrophenylphosphatase activity. K^+-activated *p*-nitrophenylphosphatase was measured on a multititer plate by incubating Na^+/K^+-ATPase in a total volume of 150 µl containing 61 mM Tris/HCl, pH 7.25, 6.4 mM $MgCl_2$, 12 mM KCl, and 5 mM *p*-nitrophenylphosphate [13]. After 10 min at 37 °C, the reaction was stopped by adding 200 µl 3 M NaOH to the media. The *p*-nitrophenolate formed was estimated at 405 nm in an ELISA reader.

Frontdoor (Na^+ dependent) phosphorylation of Na^+/K^+-ATPase. Na^+/K^+-ATPase at a final concentration of 1 U/ml (65 µg/ml) was incubated overnight at 37 °C in a solution containing 20 mM Tris/HCl, pH 7.25, and 50 mM $Co(NH_3)_4PO_4$. The inactivated enzyme was centrifuged at 100 000 *g*, washed in 20 mM Tris/HCl, pH 7.25, and incubated with 20 mM Tris/HCl, 15 mM NaCl, and different concentrations of PITC (0−50 µM). After 1 h at 37 °C, the enzyme was centrifuged, washed twice with 20 mM Tris/HCl, pH 7.25, and frontdoor (Na^+ dependent) phosphorylation was determined. The enzyme in 100 mM NaCl, 1 mM $MgCl_2$, and 10 mM imidazole/HCl, pH 7.25 was placed on ice. The reaction was started by the addition of 100 µl 0.1 mM [γ-^{32}P]ATP (200 cpm/pmol), so that the final concentration was 1 ml. After 1 min the reaction was stopped by addition of 250 µl 25% trichloroacetic acid, 10 mM Na_2HPO_4, and 1 mM non-radioactive ATP. The mixture was centrifuged for 30 min at 100 000 *g* in a Ti50 rotor of a Beckman Spinco L50 ultracentrifuge. The pellet was washed three times with 500 µl 5% trichloroacetic acid containing 2 mM Na_2HPO_4 and 0.2 mM non-radioactive ATP. 1 U Na^+/K^+-ATPase, which was quenched with 250 µl 25% trichloroacetic acid before phosphorylation, was used as the background value.

Tryptic digestion and HPLC. Tryptic digestion was performed according to Capasso et al. [16] as follows: Na^+/K^+-ATPase (3 mg/ml) was suspended in 2 ml 20 mM Tris/HCl, pH 7.25. Trypsin digestion (10 mg trypsin /100 mg protein) was carried out at 37°C for 24 h. Soybean trypsin inhibitor was added at 5:1 (by mass) with respect to trypsin. The suspension was diluted to 5 ml with a solution of 20 mM Tris/HCl, pH 7.25, and warmed at 37 °C for 30 min. Peptides were analyzed by reverse-phase HPLC using a Waters automated gradient controller (Millipore), Waters model 510 pumps and steel 250 mm×4 nm Nucleosil 5 C_{18} column (Macherey-Nagel). The sample (40 ml) was injected into the column and eluted with a gradient consisting of (A) Milli-Q water with 0.1% trifluoroacetic acid (by vol.) and (B) acetonitrile with 0.1% trifluoroacetic acid (by vol.) (flow rate 1.0 ml/min, 30 °C). The gradient started at 98% A and reached a 62:38 A/B ratio after 30 min. After a further 5 min, the gradient reached 100% B. Elution was monitored by a fluorescence detector RF-530 (Shimadzu, Kyoto, Japan) with excitation at 345 nm and emission at 410 nm for pyrene and 520 nm for fluorescein, respectively.

Sequencing of the PITC-labeled peptide. The purified PITC-labeled peptide was evaporated and dissolved in *n*-propanol/water (60:40, by mass), concentrated in a SpeedVac, and loaded onto a Beckman protein support glass fiber disc. The sample was subsequently inserted into the cartridge of a LF3600 protein sequencer (Beckman), and subjected to five cycles of automated Edman degradation.

Steady-state and dynamic fluorescence measurements. Steady-state experiments were performed on a Perkin-Elmer LS50 spectrofluorometer. The excitation wavelength was 345 nm for the pyrene derivative and 490 nm for the fluorescein derivative; the emission wavelength was 420 nm for pyrene and 520 nm for fluorescein, respectively. A frequency-domain approach was employed to determine the fluorescence decay lifetimes and anisotropies using an ISS K2 multifrequency cross-correlation phase and modulation fluorometer. This fluorometer has a tunable light modulation and phase and modulation measurements were performed in the range 1−200 MHz with a xenon lamp and a monochromator as a source of excitation light. The excitation wavelength for fluorescein was 490 nm. Fluorescence emissions from the samples were observed through appropriate Schott filters. The integration time was 20 s, with a minimum of 40 cycles in the sweep mode; the minimum number of trials was 16. The temperature of the samples was maintained at 25 °C. An aqueous suspension of glycogen or a solution of 1,4-bis(5-phenyl-2-oxazolyl)benzene in methanol was used as reference sample. The experimental data were analyzed using a non-linear least-squares routine for multiexponential fitting (and/or lifetime distribution). The χ_r^2 parameter was used to judge the quality of the fit. In the analysis, the uncertainties in the phase and modulation were taken as 0.2 and 0.003, respectively. The best fit of fluorescence intensity decays appeared to be the Lorentzian distribution.

RESULTS AND DISCUSSION

Inactivation of Na^+/K^+-ATPase. To verify that 1-pyreneisothiocyanate is a specific label of the E_1ATPase and therefore useful to monitor the rotational and vibrational freedom of the H_4-H_5 loop of the α subunit of Na^+/K^+-ATPase, we studied the inhibitory effect of PITC on Na^+/K^+-ATPase. First, the overall reaction of Na^+/K^+-ATPase was inactivated at pH 7.25 with a K_i of 100 µM (Fig. 1). The inhibition constant K_i was estimated by plotting the apparent velocity constants against the concentration of PITC [17, 18]. In addition, the frontdoor (Na^+ dependent) phosphorylation in a $Co(NH_3)_4PO_4$ pretreated enzyme, which is described as a partial activity of the E_1ATP binding site [17−19], showed a strong concentration-dependent effect of PITC (Fig. 2). However, when K^+-activated *p*-nitrophenylphosphatase, a partial activity of the E_2ATP site [13] was measured in untreated and FITC-blocked Na^+/K^+-ATPase preparations; no influence of PITC was detectable (Table 1) and, in both cases, *p*-nitrophenylphosphatase was unaffected by PITC in a concentration range of 0−200 µM.

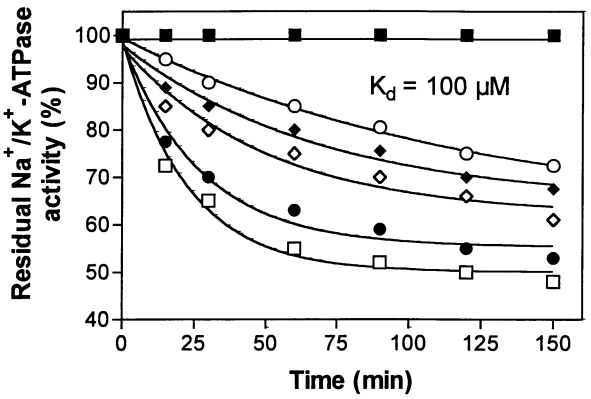

Fig. 1. Inactivation of Na^+/K^+-ATPase by various concentrations of PITC. Residual activity was measured by transferring 50 µl incubation medium containing 15 mM NaCl, 20 mM Tris/HCl, pH 7.25, and 1 U Na^+/K^+-ATPase to the spectrometric assay (for experimental details see Materials and Methods section); (■) control with 0 µM PITC; (○) 10 µM PITC; (◆) 20 µM PITC; (◇) 30 µM PITC; (●) 40 µM PITC; (□) 75 µM PITC.

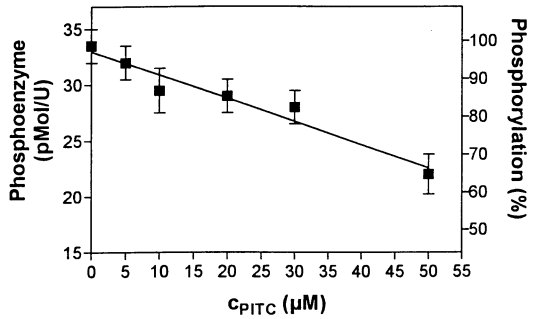

Fig. 2. Effect of various concentrations of PITC on the frontdoor phosphorylation from [γ-^{32}P]ATP in $Co(NH_3)_4PO_4$ pretreated enzyme. 1 U Na^+/K^+-ATPase was incubated overnight at 37°C in a solution containing 20 mM Tris/HCl, pH 7.25, and 50 mM $Co(NH_3)_4PO_4$. After washing, the enzyme was incubated with 20 mM Tris/HCl, 15 mM NaCl, and various concentrations of PITC (0−50 µM) for 1 h and frontdoor phosphorylation was measured. The enzyme was phosphorylated in 100 mM NaCl, 1 mM $MgCl_2$ and 10 mM imidazole/HCl (pH 7.25) by the addition of 100 µl 0.1 mM [γ-^{32}P]ATP (200 cpm/pmol). The reaction was quenched after 1 min by addition of 250 µl 25% trichloroacetic acid, 10 mM Na_2HPO_4, and 1 mM non-radioactive ATP. Mean values and standard deviations of three experiments are shown. The 100% value of phosphorylation is 33.3 pmol/unit according to [19].

The fact that PITC inactivated the Na^+/K^+-ATPase activity with a K_i of 100 µM and affected the E_1ATP site without influencing the partial activities of the E_2ATP site, clearly indicated that PITC is a specific label of Na^+/K^+-ATPase and binds at or close to the E_1ATP binding site, much like FITC [13].

Labeling of Na^+/K^+-ATPase by PITC at the H_4-H_5 loop. To get additional support for the conclusion that PITC modified like FITC the ATP binding site of Na^+/K^+-ATPase, a comparison of the tryptic labeled pattern was performed. Na^+/K^+-ATPase was divided into two parts, one labeled with FITC and the other with PITC. Both labeled enzyme preparations were extensively digested by trypsin. Na^+/K^+-ATPase (3 mg/ml) was suspended in 2 ml 20 mM Tris/HCl, pH 7.25, and trypsin digestion (10 mg trypsin /100 mg protein) was carried out at 37°C for 24 h. Soybean trypsin inhibitor was then added at a 5:1 (by mass) ratio with respect to trypsin. The peptides obtained were separated by reverse-phase HPLC and the fluorescence intensity of the fractions was monitored. The analysis revealed a large fluorescence peak at the same retention time (Fig. 3) both for the enzyme labeled with PITC and FITC in accordance with our conclusion from the inhibitory studies that PITC binds at or close to the FITC-binding site. The steady-state fluorescence of undigested FITC-Na^+/K^+-ATPase was also significantly higher than the fluorescence of doubly labeled PITC, FITC-Na^+/K^+-ATPase (Table 2), which also supported the inhibitory study.

Table 1. Effect of PITC on K^+-activated *p*-nitrophenylphosphatase in FITC pretreated and untreated Na^+/K^+-ATPase. 1 U Na^+/K^+-ATPase was incubated in a solution of 20 mM Tris/HCl, pH 7.25, and 10 µM FITC (control without FITC) overnight. The residual Na^+/K^+-ATPase activity was lower than 1%. After centrifugation and washing (for experimental details see Materials and Methods section), the enzyme was incubated for 1 h at 37°C in a solution containing 20 mM Tris/HCl, pH 7.25, 15 mM NaCl, and different concentrations of PITC. K^+-activated *p*-nitrophenylphosphatase was determined as described in the Materials and Methods section. Mean values and standard deviations of three experiments are shown.

c_{PITC}	*p*-Nitrophenylphosphatase activity			
	control		FITC-pretreated enzyme	
µM	U/mg protein	%	U/mg protein	%
0	0.75 ± 0.05	100	0.57 ± 0.03	100
25	0.76 ± 0.06	101	0.57 ± 0.06	100
50	0.70 ± 0.05	93	0.58 ± 0.05	102
75	0.72 ± 0.03	96	0.58 ± 0.03	102
100	0.72 ± 0.04	96	0.54 ± 0.07	95
200	0.76 ± 0.05	101	0.55 ± 0.05	97

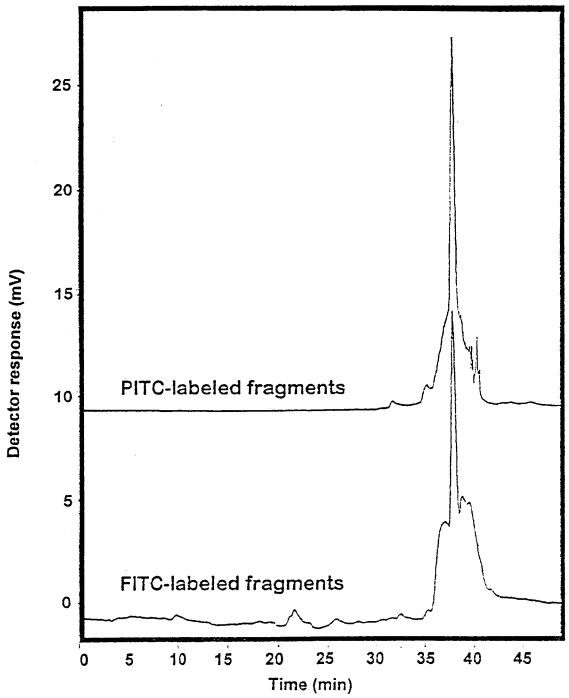

Fig. 3. HPLC analysis of cleaved Na^+/K^+-ATPase labeled with PITC and FITC. PITC-labeled Na^+/K^+-ATPase and FITC-labeled Na^+/K^+-ATPase were cleaved by trypsin (10 mg trypsin /100 mg protein) at 37°C for 24 h. The peptides were analyzed by reverse-phase HPLC. Elution was monitored by fluorescence of pyrene (a) and fluorescein (b). For details, see Materials and Methods section.

Table 2. Steady-state fluorescence of FITC conjugated with the pig kidney Na^+/K^+-ATPase. The fluorescence excitation and emission wavelengths were 490 nm and 520 nm, respectively. The protein concentration was 10 mg/ml; nonspecifically bound FITC was quenched by anti-FITC antibody (the concentration was 10 mg/ml).

Enzyme	Relative steady-state fluorescence	
	without antibody	with antibody
PITC, FITC-Na^+/K^+-ATPase	28.2 + 1.4	6.9 + 2.1
FITC-Na^+/K^+-ATPase	39.3 + 2.1	25.8 + 1.6

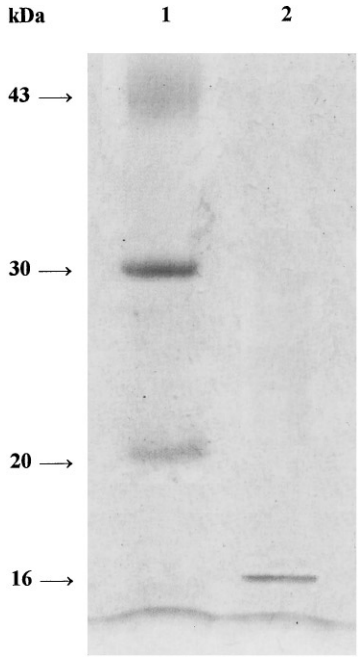

Fig. 4. SDS gel electrophoresis of the PITC-labeled fragment. SDS/PAGE of the PITC-labeled fragment (lane 2) obtained from the HPLC analysis. Lane 1 shows the mass markers. The sample was run and stained by Coomassie brilliant blue as described in the Materials and Methods section.

N-terminal sequence of PITC-labeled tryptic fragments of the α subunit of Na^+/K^+-ATPase. For direct evidence of PITC binding to the α subunit, the PITC-labeled peptide obtained from reverse-phase HPLC was run on an SDS/polyacrylamide gel containing 15% polyacrylamide and subjected to Edman degradation on a model LF3600 protein sequencer (Beckman). The SDS/polyacrylamide gel analysis showed that the molecular mass of the PITC-labeled fragment was about 16 kDa (Fig. 4). The Edman degradation of the PITC-labeled segment (from the N-terminus) showed the following amino acid sequence: P I L K R. This motif is identical with the primary structure of the α subunit of pig kidney Na^+/K^+-ATPase starting at position 439: P I L K R [20]. Moreover, the 16-kDa-labeled tryptic fragment indicated that the peptide was composed of about 80 amino acid residues. Consequently, the isolated PITC-labeled peptide should also contain Lys480 and Lys501. This section is a part of the H_4-H_5 loop of the α subunit, which is also where the ATP site is located [21] and, thus, this experiment provided direct evidence of the labeling of the H_4-H_5 loop of the α subunit by PITC.

Determination of rotational mobility of the H_4-H_5 cytoplasmic loop. As expected for a covalent label with hydrophobic properties, the steady-state fluorescence of PITC-treated Na^+/K^+-ATPase increased about three times as compared to PITC in buffer (data not shown). Dynamic measurements performed on the tunable multifrequency cross-correlation phase and modulation fluorometer ISS K2 clearly confirmed the steady-state data (Fig. 5). The lifetime of the excited state of PITC revealed even in buffer (50 mM Tris/HCl, 3 mM $MgCl_2$, pH 7.4) an intrinsic lifetime heterogeneity (Table 3). Compared with PITC in solution, however, the fluorescence intensity decay became more heterogeneous when PITC was attached to pig kidney Na^+/K^+-ATPase. This fact was reflected by a different dependence of phase and modulation on light modulation frequencies compared with the free fluorophore (more striking changes in modulation). Only a three-component distribution gave a satisfactory fit for the measured data, i.e. a Lorentzian distribution with two narrow, relatively short-lived peaks and one very broad distribution centered at 43.92 ns (Table 3). Application of the distribution with two components (similarly as for the free label in buffer) led to an unacceptably high $\chi_r^2 = 48$. The average lifetime of

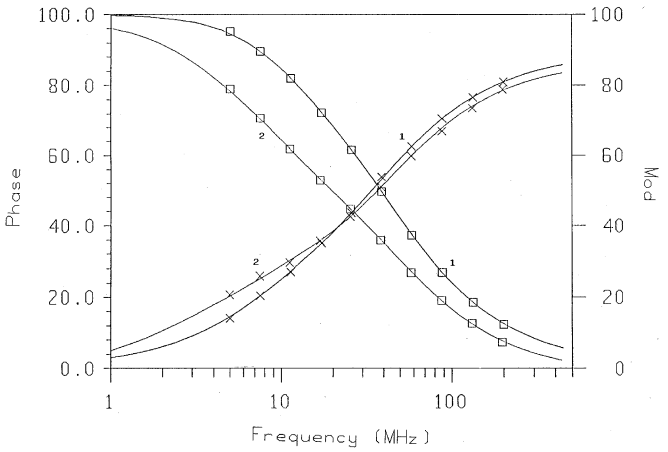

Fig. 5. Frequency response of PITC: the best fits. Frequency response of PITC in buffer (curves 1) and after binding to Na^+/K^+-ATPase (curves 2). Free PITC (20 mM in 100 mM Tris/HCl, 1 mM EGTA, pH 7.4) and bound PITC-Na^+/K^+-ATPase (1 mg protein/ml) were excited at 345 nm over modulation frequencies 5−200 MHz and the fluorescence was isolated using a filter. The fitting curve is the sum of the two-component (free fluorophore) and three-component (bound fluorophore) Lorentzian distribution (see Table 3).

Table 3. The Lorentzian lifetime distribution to fit PITC fluorescence intensity decays. The frequency response of PITC fluorescence was measured using the frequency-domain method (modulation frequencies 5−200 MHz) and analyzed as described in the Materials and Methods section. For each component, τ_i is the lifetime of the ith component, w_i the half-width at the half maximum of the ith component of the lifetime distribution, α_i the respective preexponential factor, and f_i the fractional intensity of the ith component. While in buffer, a two-component Lorentzian distribution appeared to be a sufficient fit; it was necessary to fit the data of the fluorophore bound to the enzyme to a three-component distribution. The background (protein in buffer) was subtracted according to [23].

Condition	Component	τ_i	w_i	α_i	f_i	χ_r^2
PITC in buffer	1	14.57	0.05	0.42	0.70	
	2	4.44	0.05	0.58	0.30	2.1
PITC-Na^+/K^+-ATPase	1	43.92	12.75	0.30	0.68	
	2	16.30	0.13	0.29	0.24	
	3	4.03	0.05	0.41	0.08	4.6

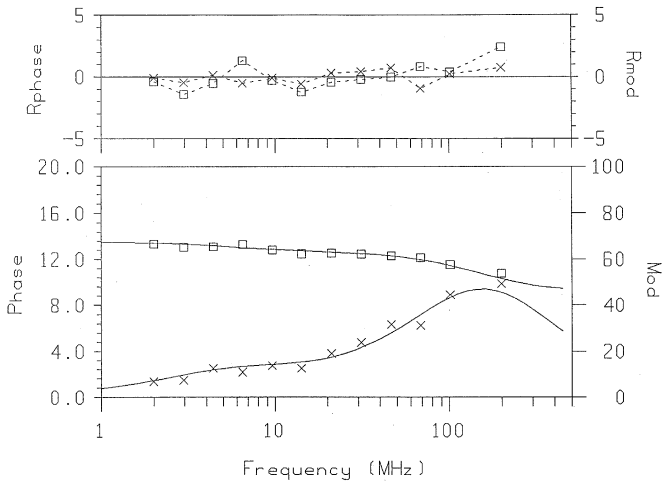

Fig. 6. Demodulation (□) and phase difference (X) of PITC-Na^+/K^+-ATPase fluorescence anisotropy. Fluorescence was excited at 345 nm, modulated over a 5−200 MHz frequency range and the emitted fluorescence was isolated using an appropriate filter. The fitting curve was a sum of two exponential decays of one species with rotational correlation times of 195.0 ns ($r_{01} = 0.15$) and 0.1 ns ($r_{02} = 0.13$); $\chi_r^2 = 5.1$. The upper part shows the differences between the experimental points and the fitting curve.

PITC after binding was prolonged significantly; the average lifetime calculated from the distribution centers increased from 10.0 ns for free PITC in buffer to 34.1 ns for PITC bound to the Na^+/K^+-ATPase. The long-lived component ($\tau = 43.92$ ns) was responsible for more than two-thirds of the steady-state fluorescence intensity (see the f_i parameters) and could be attributed to a specific binding. Two short-lived components could be ascribed either to a complex lifetime decay of the specifically bound fluorophore or to PITC bound nonspecifically to the enzyme. The latter possibility could indicate that for one specifically attached PITC molecule there would be less than one-half of nonspecifically bound PITC (Table 3; see fractional intensities f_i). However, to monitor the rotational mobility of the H_4-H_5 loop, even these molecules, which were bound nonspecifically to the loop, acted in our case as fine-reporting fluorophores.

The fluorescence anisotropy of PITC-Na^+/K^+-ATPase decayed with at least one very long rotational correlation time (Fig. 6). We tried to fit our data with several rotational models and the best model for the observed anisotropy decay data appeared to be a sum of two exponential decays of two species:

$$r(t) = r_{01}\exp(-t/\varrho_1)+r_{02}\exp(-t/\varrho_2),$$

where $r_{01} = 0.15$, $r_1 = 195.0$ ns, $r_{02} = 0.13$, $r_2 = 0.1$ ns.

The first rotational correlation time was in our case obviously much longer than the second one. Hence, the above equation can be rewritten as:

$$r(t) = r_0 g_1\exp(-t/\varrho_1)+r_0 g_2\exp(-t/\varrho_1)\exp(-t/\varrho_2),$$

where $r_0 = 0.4$, or even as:

$$r(t) = r_0[g_2\exp(-t/\varrho_2)+g_1]\exp(-t/\varrho_1)$$

The values of g_i are complex functions of the rates of rotation around the molecular axes of the asymmetric body (a fast rotational relaxation process can be included) and the orientation of the absorption and emission dipoles relative to these axes. The last equation actually describes a simple model of the fast rotational motion of the fluorophore (PITC or FITC) with a rotational correlation time ϱ_2 on the slowly rotating segment (the H_4-H_5 loop) with the rotational correlation time ϱ_1.

Clearly, the rotational correlation time $\varrho_1 = 195$ ns of the H_4-H_5 loop was very long. It was impossible to observe such a slow rotation with the FITC-labeled enzyme because the lifetime of the excited state of FITC bound to the enzyme was shorter by two orders of magnitude. This high ratio of the rotational correlation time and the lifetime of FITC is also an explanation of the high steady-state anisotropy of FITC-Na^+/K^+-ATPase [9, 10].

The H_4-H_5 cytoplasmic loop of the α subunit has a molecular mass of about 30 kDa. Assuming a free globular protein of an equivalent molecular mass, one can predict that the rotational correlation time of such a molecule would be about 8 ns. Even if we take into account the fact that a hydration shell can increase the rotational correlation time of the protein about twofold, it follows from the experiment that not only the ATP-binding site but the whole H_4-H_5 cytoplasmic loop has to be very rigid, rotationally restricted, and maintaining its own firm structure. Such a firm structure of the large cytoplasmic loop, which carries the ATP-binding site but which is far from the ion-transporting domains, could serve as an efficient rigid arm to transmit the conformational change after ATP hydrolysis to the transmembrane domains. This immobilization of the cytoplasmic loop might be associated with the recent identification of two disulfide bonds [22] between Cys452 and Cys456, and between Cys511 and Cys549. On the other hand, there are mutipole-multipole interactions between the segments of the loop that can support the structure. The possibility also exists that, in a dimeric structural unit, the large cytoplasmic loops of both α subunits in the dimer are in contact.

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