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Microenvironment of the High Affinity ATP-Binding Site of Na^+/K^+-ATPase Is Slightly Acidic

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Fluorescein-5′-isothiocyanate (FITC) was used to study the high-affinity ATP-binding site of Na^+/K^+-ATPase. The molar ratio of specifically bound FITC per α-subunit of Na^+/K^+-ATPase was found to be 0.5 as followed from pretreatment experiments with another specific E_1ATP-inhibitor $Cr(H_2O)_4$Ado*PP*[CH_2]*P*. This indicated an existence of one high affinity ATP-binding site (E_1ATP-binding site) in the native $(\alpha\beta)_2$-diprotomer of Na^+/K^+-ATPase. Fluorescence dual-excitation ratio of specifically bound FITC revealed that at external pH 7.5, the pH value inside the E_1ATP-binding site is 6.95 ± 0.18. In addition, FITC fluorescence quenching by anti-fluorescein and by iodide choline indicated the limited access of water into the small pocket of the E_1ATP-binding site. © 1999 Academic Press

Key Words: **Na^+/K^+-ATPase; FITC; anti-fluorescein; pH, fluorescence dual-excitation.**

Na^+/K^+-ATPase (EC 3.6.1.37) is an integral membrane protein which transports sodium and potassium ions against an electrochemical potential gradient (1). ATP-hydrolysis linked to the cation pumping process is often described by the single site Albers-Post-model (2). The observation of a positive cooperativity of 2′-O-dansyl-ATP in the inactivation of Na^+/K^+-ATPase (3) led to the conclusion of two simultaneously existing ATP-binding sites cooperating during the ATP-driven Na^+/K^+ transport (3) in an $(\alpha\beta)_2$-diprotomeric (4) or oligomeric model (5).

There is only few information on the structure of the ATP-binding sites. Fluorescent and nonfluorescent labels of the specific high affinity ATP-binding site provided some information on amino acids located in or close to it: 8-N_3-ATP and FITC were used to label aminoacids Lys_{480}, Lys_{501} and Lys_{618} (6,7,8,9) which are conserved in other P-Type ATPases (10), too. Specific E_1ATP-inhibitors like $Cr(H_2O)_4$Ado*PP*[CH_2]*P*, $Cr(H_2O)_4$ATP, pyrene-isothiocyanate and FITC (11,12,13) as well as specific E_2ATP-inhibitors like $Co(NH_3)_4$ATP and $Co(NH_3)_4PO_4$ (3) provided in contest with protein-reactive fluorescent dyes basic information on size and accessibility of these sites. The use of 7-chloro-4-nitro-benzofurazane, for example, revealed that the E_2ATP-site is more open and accessible to quenchers than the E_1ATP-binding site (14). A fluoresceinyl-ethylenediamino-ouabain has recently been used to show that the ouabain-binding site has a pH of 5.1 ± 0.2 and therefore a more acidic microenvironment than the fluid (15). Hence, to obtain more information on the microenvironment of ATP in the E_1ATP-binding site the pH-sensitive fluorescein has been used after labeling of this site with FITC to get information on the H^+-environment of the E_1ATP-pocket. In combination with the antibodies quenching

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Abbreviations used: $Cr(H_2O)_4$Ado*PP*[CH_2]*P*, β,γ bidentate complex of chromium(III)-tetraaqua-adenylyl [β,γ-methylene] diphosphonate; $Cr(H_2O)_4$ATP, β,γ bidentate complex of chromium(III)-tetraaqua-adenosine-5′-triphosphate; $Co(NH_3)_4$ATP, β,γ bidentate complex of cobalt (III)-tetramino-adenosine-5′-triphosphate; $Co(NH_3)_4PO_4$, tetramine cobalt(III)phosphate; E_1ATP-site, nucleotide-binding site of Na^+/K^+-ATPase with high affinity for ATP; E_2ATP-site, nucleotide-binding site of Na^+/K^+-ATPase with low affinity for ATP; FITC, fluorescein-5′-isothiocyanate; Anti-Fl, antibody against fluorescein (rabbit IgG fraction, Molecular Probes, Oregon, U.S.A.); TEPA, triethanolamine-phthalic acid.

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of unspecific FITC (16), which was reported to create charge transfer complex with tryptophan, tyrosine or methionine in antibody active site (17,18), it was also possible to restrain the unspecific background. This study shows that the microenvironment of the E_1ATP-binding site is slightly more acidic than the pH at the enzymes surface.

MATERIALS AND METHODS

All chemicals were of the highest available purity and were obtained from Bio-Rad (Munich, Germany), Boehringer-Mannheim (Mannheim, Germany), E. Merck (Darmstadt, Germany), Molecular Probes (OR, USA) and Sigma (St. Louis, MO, USA). Lab-Trol protein standard is a product of Merz & Dade (Munich, Germany).

The molar concentration of Na^+/K^+-ATPase and its α-subunit was calculated from the protein concentration using the molecular weight of 113 kDa for the α- and 55 kDa for the β-subunit (2). The quantity of functional α-subunits was estimated by performing back-door phosphorylation (9,13). One unit of Na^+/K^+-ATPase binds 100 pmol phosphate specifically (9). Therefore, the molar concentration of α-subunits can be calculated from this binding ratio and the specific ATPase activity of 25 U/mg (4).

Enzyme and assays. Na^+/K^+-ATPase with a specific activity of 25 units/mg protein was isolated by modification of Jørgensen's procedure from pig kidney (19) whose activity was measured by a coupled spectrometric assay (20). One enzyme unit (U) is defined as the hydrolysis of 1 μmole ATP per min. at 37°C. Protein was determined according to Lowry et al. (21) using Lab-Trol as a protein standard. Lab-Trol is a mixture of proteins and enzymes used for the calibration of assays in clinical chemical analysis. The partial activity of the E_2ATP-site, the K^+-activated p-nitrophenylphosphatase, was measured in a $Cr(H_2O)_4$Ado*PP*$[CH_2]P$ and/or FITC pretreated enzyme at a p-nitrophenylphosphate concentration of 5 mM (11).

Preparation of $Cr(H_2O)_4$AdoPP$[CH_2]P$. $Cr(H_2O)_4$Ado*PP*$[CH_2]P$ was prepared by the aniline procedure of Cleland and coworkers (22,23) with variations described earlier (11).

Inactivation with $Cr(H_2O)_4$AdoPP$[CH_2]P$. Na^+/K^+-ATPase in a final concentration of 3 U/ml was incubated over night at 37°C in a total volume of 1 ml containing 25 μM $Cr(H_2O)_4$Ado*PP*$[CH_2]P$, 20 mM Tris/HCl buffer (pH 7.25) and 15 mM NaCl. The enzyme was washed twice with 20 mM Tris/HCl buffer (pH 7.25). The protein was resuspended in 20 mM Tris/HCl buffer (pH 7.25) in a final concentration of 5 mg protein/ml.

Labeling of Na^+/K^+-ATPase by FITC. $Cr(H_2O)_4$Ado*PP*$[CH_2]P$ and native Na^+/K^+-ATPase in a concentration of 3 U/ml was incubated for 1 h at 37°C in a volume of 1 ml containing 20 μM FITC, 20 mM Tris/HCl buffer (pH 7.25) and 15 mM NaCl. The enzyme was washed twice with 20 mM Tris/HCl buffer (pH 7.25). The protein was resuspended in 20 mM Tris/HCl buffer (pH 7.25) in a final concentration of 5 mg protein/ml.

Absorbance. Absorbance of FITC-labeled Na^+/K^+-ATPase of a $Cr(H_2O)_4$Ado*PP*$[CH_2]P$ and a control enzyme was measured at pH 7.25 (20 mM Tris/HCl-buffer) to determine the number of fluorophore molecules bound per protein molecules and especially to the E_1ATP-binding site. It was performed at a wavelength of 500 nm with a Philips PU 8740 UV/VIS scanning spectrophotometer.

Fluorescence excitation and emission spectra. Samples of FITC-labeled Na^+/K^+-ATPase and of FITC-labeled Na^+/K^+-ATPase after pretreatment with $Cr(H_2O)_4$Ado*PP*$[CH_2]P$ (blocked E_1ATP-binding site) were diluted in 0.1 M triethanolamine-phthalic acid (TEPA) buffers ranging from pH of 5.36 to 8.62 (final protein concentration: 0.65 mg/ml) and fluorescence excitation spectra were recorded in the range from 400 nm to 518 nm using a computer operated spectrofluorimeter Perkin-Elmer LS 50 B. Fluorescence was detected at an emission wavelength of 525 nm, the excitation bandwidth was 2.5 nm; emission bandwidth 5.0 nm, scan speed 150 nm/s. Spectra were measured twice and averaged. Background fluorescence of Na^+/K^+-ATPase was subtracted from all FITC spectra. The pH sensitive probe FITC are known to show at physiological pH two fluorescent forms (dianionic and monoanionic with pK 6.4) differing in their fluorescence excitation spectra and the dual-excitation ratio method was employed to obtain pH values independently of the total concentration of the fluorescent probe. The fluorescence intensity ratio at 490 nm vs. 435 nm was proportional to pH of the immediate neighborhood (24). The ratio (F_{490}/F_{435}) was calculated as integrated fluorescence intensity in the range of (λ-2 nm, λ+2 nm), where λ was 490 nm and 435 nm, respectively.

Fluorescence quenching. Anti-fluorescein antibodies (anti-Fl) (stock $\geq$1 mg/ml) were used to quench fluorescence of FITC-labeled sites outside the E_1ATP-binding site at a protein concentration of 0.65 mg/ml. The sample was diluted in 0.2 M TEPA buffer of different pH-values. Anti-Fl was added in 5 μl aliquots up to a final concentration of 60 μg/ml and fluorescence spectra were recorded. The fluorescence intensity ratio was plotted either against anti-Fl concentration (Stern-Volmer plot) or the reciprocal concentration of anti-Fl using modified Stern-Volmer equation

$$F_0/(F_0 - F) = 1/f_a K_Q[Q] + 1/f_a, \quad [1]$$

where F_0 and F are fluorescence intensities in the absence and presence of the quencher, respectively, $[Q]$ is concentration of anti-Fl, K_Q is modified Stern-Volmer quenching constant and f_a is a fraction of accessible FITC in the solution.

Choline [2-hydroxyethyl]trimethylamonium iodide salt purchased from SIGMA was also used as a collisional quencher of FITC fluorescence. In this case iodide choline, added in 5 or 10 μl aliquots from a 0.1 M stock solution (final concentration was 16 mM), quenched FITC labeled Na^+/K^+-ATPase (0.65 mg/ml). The classical classical Stern-Volmer equation (Eq. 2) was used under these conditions to calculate the Stern-Volmer quenching constant K_I.

$$F_0/F = 1 + K_I[I], \quad [2]$$

where F_0 and F are fluorescence intensities in the absence and presence of the quencher, respectively, [I] is concentration of iodide choline.

RESULTS

$Cr(H_2O)_4$Ado*PP*$[CH_2]P$ was used to block the specific labeling of the high affinity ATP binding site (E_1ATP-binding site) by FITC. While Na^+/K^+-ATPase was almost completely inhibited, the K^+-activated p-nitrophenylphosphatase activity decreased only slightly (as observed previously (4,11)) and independently on the enzyme prelabeling with $Cr(H_2O)_4$Ado*PP*$[CH_2]P$ (Table 1). The binding ratio of FITC/α-subunit in the $Cr(H_2O)_4$Ado*PP*$[CH_2]P$-pretreated enzyme, however, was significantly lower compared to the control enzyme (Table 1). Absorbance of the FITC-labeled control enzyme was A = 0.219 while that of the $Cr(H_2O)_4$Ado*PP*$[CH_2]P$-pretreated enzyme (where FITC was bound only outside the ATP binding site) was A = 0.173. The concentration of the α-subunits was determined as described in meth-

TABLE 1

Effect of Inhibitors on Na^+/K^+-ATPase

	Residual Na^+/K^+-ATPase activity (%)	Residual p-nitrophenyl-phosphatase (%)	Molar binding ratio FITC/ α-subunit
native	100	100	n.a.
FITC-labeled	≤5	75 ± 5	2.30 ± 0.08
$Cr(H_2O)_4AdoPP[CH_2]P$ pretreated and FITC-labeled	≤5	72 ± 8	1.80 ± 0.07

Note. Na^+/K^+-ATPase in a final concentration of 5 mg protein/ml was used to detect the properties of $Cr(H_2O)_4AdoPP[CH_2]P$ and FITC on the enzyme. All used samples were treated in the same way except of the use of inhibitors. FITC labeled Na^+/K^+-ATPase was inactivated for 1 h with 20 μM FITC. The enzyme pretreated with $Cr(H_2O)_4AdoPP[CH_2]P$ was inactivated over night with 25 μM $Cr(H_2O)_4AdoPP[CH_2]P$ and labeling for 1 h with 20 μM FITC followed. For details see "Methods" and (11). Mean values and standard deviations of four experiments are shown, n.a. stands for not applicable.

ods using backdoor phosphorylation for correction (11). Thus, 2.3 molecules of FITC were bound, in average, per one α-subunit of Na^+/K^+-ATPase. When the E_1ATP-site had been blocked by $Cr(H_2O)_4AdoPP[CH_2]P$, only 1.8 molecules of FITC were bound per one α-subunit. Hence, 0.5 FITC molecules were bound specifically per α-subunit to the E_1ATP-site and fluorescence at this ATP-binding site represents about 22% of the fluorescence intensity of all FITC-molecules bound to the protein (Table 1).

Furthermore, fluorescence of FITC-labeled Na^+/K^+-ATPase was examined presuming that FITC bound to the E_1ATP-binding site was inaccessible by anti-Fl antibodies. It has been formerly reported that anti-Fl quenches at pH 8.0 more than 95% of fluorescence intensity of all the accessible fluorophores (25) and, thus we assumed that FITC fluorescence remaining after anti-Fl application to Na^+/K^+-ATPase originated from the FITC bound to the ATP-binding site (for details see Discussion). Two populations of fluorophores at FITC-labeled Na^+/K^+-ATPase also revealed in our preparation when iodide choline (16 mM final concentration) was employed to quench FITC fluorescence. Two samples of FITC labeled Na^+/K^+-ATPase were investigated at pH 8.03, λ_{ex} = 490 nm, one treated with anti-Fl (60 μg/ml) for 15 min, the other untreated. Stern-Volmer plots of iodide quenching (Fig. 1) showed

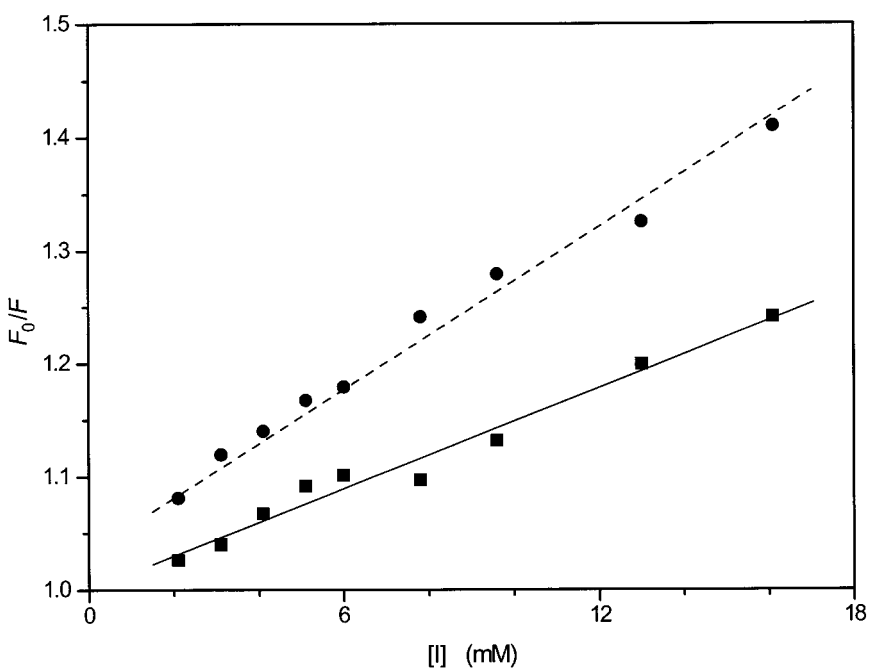

FIG. 1. Stern-Volmer plots of FITC-labeled Na^+/K^+-ATPase. Fluorescence was quenched by different concentrations of iodide choline [I] at pH 8.03, λ_{ex} = 490 nm, λ_{em} = 525 nm. The Stern-Volmer quenching constant was in the absence of anti-Fl (● - - -) K_I = 25 μM^{-1} while in the presence of 60 μl/ml of anti-Fl (■ —), K_I = 14 μM^{-1}.

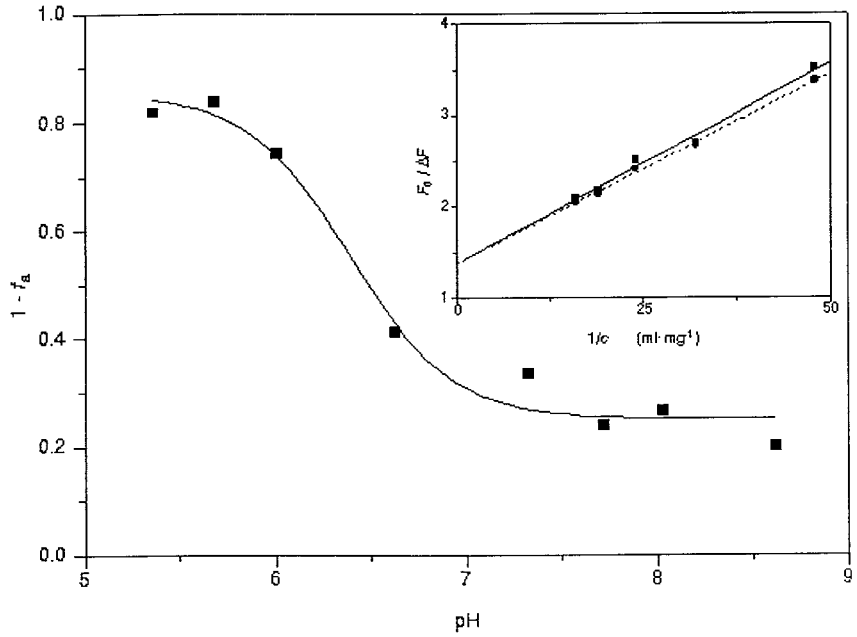

FIG. 2. Dependence of anti-Fl unquenchable fraction of FITC-labeled Na^+/K^+-ATPase derived from the modified Stern-Volmer equation on pH. Fluorescence was excited at λ_{ex} = 490 nm and emission was observed at λ_{em} = 525 nm. Boltzman sigmoidal curve appeared to be the best fit of experimental values. Inset: A typical experiment. The modified Stern-Volmer plots of fluorescence quenching by anti-Fl performed at pH 8.03. Quenching was measured at two different fluorescence excitations λ_{ex} = 435 nm ■ — and λ_{ex} = 490 nm ● - - -. The Stern-Volmer quenching constant K_Q and fraction of accessible fluorophores f_a in this experiment were K_Q = 17 ml/mg, f_a = 0.76 in the former case and K_Q = 18 ml/mg, f_a = 0.76 in the latter case.

different Stern-Volmer quenching constants K_I, in case of anti-Fl untreated protein K_I = 25 μM^{-1}, while in case of anti-Fl presence K_I = 14 μM^{-1}.

First, dependence of accessibility of FITC at the E_1ATP-site on fluid acidity was determined. The fluorescence excitation spectra were measured in the presence of anti-Fl. Fluorescence quenching was, observed at pH 8.03, λ_{ex} = 490 nm and λ_{ex} = 435 nm, respectively, and a modified Stern-Volmer plot (Fig. 2 inset) was constructed to yield the fraction of accessible fluorophores and Stern-Volmer quenching constant (see Methods). The analysis revealed that the fraction of accessible fluorophores, f_a, was f_a = 0.76 and the Stern-Volmer quenching constant was found to be K_Q = 18 ml/mg at λ_{ex} = 490 nm and K_Q = 17 ml/mg at λ_{ex} = 435 nm, respectively. To obtain pH in the ATP binding site, the fraction of accessible fluorophores was similarly detected at different pH-values. Clearly, the fluorescence fraction within the E_1ATP-binding site ($1-f_a$) at λ_{ex} = 490 nm was significantly pH dependent and a Boltzman sigmoidal function was found to be the best fit to describe the pH-dependency (Fig. 2). The fraction of inaccessible fluorophores and unquenchable FITC fluorescence was about 24% at basic pH while at acidic pH it was more than 80%.

FITC bound inside the E_1ATP-binding site was than employed to monitor the acidity of this binding pocket. For determination of pH within the E_1ATP-binding site, there was necessary to obtain fluorescence excitation spectra of FITC specifically bound to the ATP-pocket. This was obtained by two ways:

1) by mathematical subtraction of fluorescence excitation spectra (on a specific wavelength) of the unspecifically attached FITC from the total signal of FITC-labeled Na^+/K^+-ATPase

2) by application of anti-Fl antibodies to quench fluorescence signals of unspecifically bound FITC.

First, fluorescence excitation spectra of FITC labeled Na^+/K^+-ATPase, $\mathbf{F}_{total}$, as well as excitation spectra of Na^+/K^+-ATPase nonspecifically labeled with FITC (ATP-binding site blocked by $Cr(H_2O)_4$Ado*PP*[CH_2]*P*), $\mathbf{F}_{out}$, were observed in a series of eight TEPA buffers (pH 5.36–8.62). The fluorescence intensity ratio F_{490}/F_{435} was plotted against pH of used buffer (Fig 3). In order to get specific FITC fluorescence signal $\mathbf{F}_{in}$ arising from the E_1ATP binding pocket of Na^+/K^+-ATPase, we subtracted the fluorescence excitation spectrum of $\mathbf{F}_{out}$ FITC bound outside the ATP binding site (the E_1ATP-binding site was blocked by $Cr(H_2O)_4$Ado*PP*[CH_2]*P*) from that of the total signal $\mathbf{F}_{total}$.

$$\mathbf{F}_{in} = \mathbf{F}_{total} - \mathbf{F}_{out} \qquad [3]$$

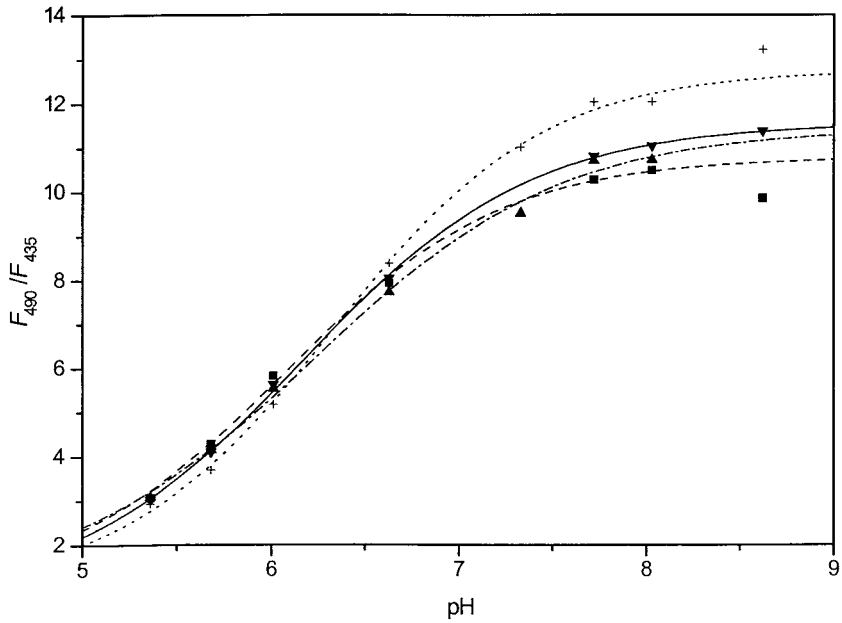

FIG. 3. Dependence of fluorescence ratio (F_{490}/F_{435}) of FITC-labeled Na^+/K^+-ATPase on pH. Fluorescence was excited either at λ_{ex} = 490 nm (F_{490}) or at λ_{ex} = 435 nm (F_{435}), and the emission was observed at λ_{em} = 525 nm in TEPA-buffers with 8 different pH-values ranging from 5.36 to 8.62. Experimental points and best fits: a) ● —, FITC-labeled Na^+/K^+-ATPase (both inside and outside of the E_1ATP-binding pocket, $\mathbf{F}_{total}$); b) + - - -, Na^+/K^+-ATPase nonspecifically labeled by FITC (the binding to the E_1ATP-binding site was blocked, $\mathbf{F}_{out}$). Calculated fluorescence intensity ratio in the E_1ATP-binding site: c) ■ – – –, mathematical subtraction of FITC spectra at each wavelength (see Eq. 3); d) × – – –, application of anti-Fl, the infinitesimal quencher concentration model (see text) yielded the residual fluorescence intensity originating in the E_1ATP-binding site.

Fluorescence intensity ratios F_{490}/F_{435} of spectra obtained in this way were plotted against buffer pH (Fig. 3). The Boltzman sigmoidal curve which provided the best fit, crossed the ratio curve of FITC bound only outside the E_1ATP-binding pocket at external (buffer) pH 6.39 ± 0.18 (standard deviation of approximation method). This cross point indicated the same pH inside and outside the E_1ATP-binding site.

The alternative method based on the quenching of fluorescence of unspecifically bound FITC by anti-Fl. FITC-labeled Na^+/K^+-ATPase was quenched by anti-Fl and a modified Stern-Volmer plot at different pH-values was employed to yield the fractions of inaccessible fluorophores (fluorescence intensity at infinitesimal quencher concentration) or by the other words, the experiment at pH 8.03 shown in Fig. 2 (was repeated for different pH values). The remaining fluorescence signal was considered to originate from FITC bound within the E_1ATP-binding site of Na^+/K^+-ATPase. The fluorescence intensity values at two different wavelengths, namely λ = 435 nm and λ = 490 nm, were plotted as ratio F_{490}/F_{435} against the pH of the TEPA-buffer in which Na^+/K^+-ATPase was suspended (Fig. 3). The best data fit crossed the pH curve obtained for the FITC fluorescence ratio outside the E_1 pocket at the (external) pH of 6.12 ± 0.18 (Fig 3).

DISCUSSION

The same inhibitory effect of both FITC labeled and doubly labeled (FITC and $Cr(H_2O)_4$Ado*PP*$[CH_2]P$) enzyme simultaneously with a significantly lower binding ratio of FITC per α-subunit in the case of $Cr(H_2O)_4$Ado*PP*$[CH_2]P$ pretreated enzyme, was a clear indication of $Cr(H_2O)_4$Ado*PP*$[CH_2]P$ binding to the same binding site as FITC, namely to the E_1ATP-binding site (3,11). Thus, the observed difference between FITC binding ratio of the untreated and $Cr(H_2O)_4$Ado*PP*$[CH_2]P$ pretreated Na^+/K^+-ATPase could provide us with the information how many E_1ATP-binding sites can be presented simultaneously on one α-subunit.

The number of FITC molecules bound to Na^+/K^+-ATPase was obtained by an absorption spectroscopy and confirmed by a quenching method. According to the modified Stern-Volmer plot, anti-Fl quenched approximately 76% of total fluorescence intensity at pH 8.0. This pH and wavelengths λ_{ex} = 490 nm and λ_{em} = 525 nm were chosen because of most often used conditions in studies of anti-Fl fluorescein interaction (16,24,25,27). Under these conditions, anti-Fl IgG quenched more than 95% of all accessible FITC fluorophores (16). Then the quenching of 76% of the total fluorescence means there was 76–80% of FITC fraction

accessible by anti-Fl. Similarly, the remaining fluorescence signal (24%) indicated the inaccessible FITC molecules fraction of 20–24% which was "hidden" in the E_1ATP-binding site of Na^+/K^+-ATPase. This estimation was in a good agreement with the absorption measurement showing that 22% of the total FITC (0.5 mol FITC/mol α-subunit) can be found inside the ATP-binding site. These results and the data about accessibility of 78% of attached FITC clearly showed that FITC labeled specifically only half of the present α-subunits of Na^+/K^+-ATPase. This supports the $(\alpha\beta)_2$-diprotomeric model with two co-operating ATP-binding sites on different α-subunits (3,4) and enables a single molecule imaging of individual ATP turnovers (26) in close future.

Iodide choline quenched fluorescence of FITC bound both outside the E_1ATP-binding site and inside the pocket. Iodide anions are small enough to penetrate to the FITC occupied pocket, accessibility of the pocket was, however, lower even to iodide. This was revealed by the Stern-Volmer quenching constant K_I, which is smaller when fluorescence of nonspecifically FITC bound to the Na^+/K^+-ATPase was prequenched by anti-Fl (Fig. 1). This was a clear confirmation of two populations of FITC-molecules attached to Na^+/K^+-ATPase.

The sigmoidal curves obtained as fluorescence intensity ratio F_{490}/F_{435} (Fig. 3) were very similar to those of fluorescein, free FITC, fluorescein-ethylendiamino-ouabain and FITC bound to other proteins and sugars (15,27). The two curves of FITC bound only inside the pocket based both on the mathematical subtraction of spectra and on the FITC fluorescence quenching by anti-Fl qualitatively corresponded to each other. However, a small discrepancy between them may be due to unpredictable quenching mechanism at higher anti-Fl concentration (from 45 μg/ml per 650 μl/ml of FITC labeled Na^+/K^+-ATPase), especially at lower pH. The pH dependence of the inaccessible and nonquenched FITC fraction was sigmoidal (Fig. 2). Reportedly, that basic form of FITC may be more vulnerable to quenching than the acidic form. However, according to our control experiments with extrapolation to infinite concentration we believe that both forms are completely quenched (27). Therefore, the sigmoidal shape is caused by fluorescence dependence on pH rather than by higher inaccessibility of FITC hidden inside the protein at acidic environment.

The ratio curve of FITC labeled Na^+/K^+-ATPase was different from the curves of FITC covalently bound to the E_1ATP-binding pocket of Na^+/K^+-ATPase especially at alkaline pH. There was no shift of curves along pH axis, thus p*K* of FITC bound outside and inside the pocket was not significantly changed (Fig. 3). This indicated similar chemical environment and binding of the label in both cases. However, an apparent drop of fluorescence ratio of specifically bound FITC was observed in alkaline buffer. On the other hand, the fluorescence ratio in the pocket was slightly higher in acidic environment. The acidity changes inside of the pocket does not follow external pH changes. The sigmoidal curve of the specifically bound FITC differed from the unspecifically attached molecules which could be caused by the buffer capacity of the protein. This could be even more pronounced in the small E_1ATP-binding pocket with a limited water accessibility. The quenching experiments confirmed our presumption that the E_1ATP-binding site occupied by FITC or at least its entrance was too small to allow free access to anti-Fl and, moreover, it restricted even free access of iodide ions. However, water molecules are able to enter the FITC-labeled pocket which is obvious from the pH sensitive fluorescence ratio, but water exchange may be restricted like access of iodide. In conclusion, pH inside the pocket equals to the external pH at values about 6.0–6.5. At physiological pH, let us say pH 7.5, the acidity in the pocket is, as follows from Fig. 3, lower than 7.0.

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