Quenching of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole-modified Na^+/K^+-ATPase reveals a higher accessibility of the low-affinity ATP-binding site

Holger Linnertz[1,a,b], Petra Urbanova[a], Evzen Amler[a,*]

[a] *Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, Cz-142 20 Prague 4, Czech Republic*
[b] *Institute of Biochemistry and Endocrinology, Justus-Liebig-University Giessen, Frankfurter Str. 100, D-35392 Giessen, Germany*

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Abstract 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) labeled Na^+/K^+-ATPase covalently with two different inactivation constants ($K_i = 2.5$ μM; $K_i' = 10$ μM). It apparently modified the two different ATP-binding sites of the enzyme since it decreased the activity of the E_2ATP site, i.e. the K^+-activated para-nitrophenylphosphatase activity, in an enzyme whose high-affinity E_1ATP site had been blocked by fluorescein 5′isothiocyanate (FITC). It also reduced the activity of the E_1ATP site, i.e. the Na^+-activated protein phosphorylation, in an enzyme whose low-affinity E_2ATP site had been blocked by $Co(NH_3)_4PO_4$. Fluorescence quenching experiments with KI, CsCl and $MnCl_2$ of the NBD-Cl-labeled Na^+/K^+-ATPase revealed two differently accessible types of fluorophores depending on the ATP site: The E_2ATP site apparently differs from the E_1ATP site in that it is more open because the fluorophore labeling in the E_2ATP site was sterically better accessible for quenchers.

Key words: Na^+/K^+-ATPase; NBD-Cl; ATP-binding site; ATP analog; Fluorescence anisotropy; Fluorescence quenching

1. Introduction

Na^+/K^+-ATPase (EC 3.6.1.37) is an integral membrane protein which transports sodium and potassium ions against an electrochemical potential gradient. The process of cation transport has been described by the so-called Albers-Post model in which a single ATP site per catalytic α subunit exists in two main conformations which differ in their affinities for ATP: The high-affinity E_1ATP site is associated with Na^+ export while the low-affinity E_2ATP site is implicated in K^+ import [1]. This model, however, is inconsistent with the observations of backdoor phosphorylation and of [^{86}Rb] occlusion in an E_1ATP site blocked enzyme [2,3] and with the phenomena of superphosphorylation from [γ-^{32}P]ATP [4], double labeling with ATP analogs [5] or double phosphorylation from para-nitrophenylphosphate [6]. All these observations, including the observation of a positive cooperative effect of 2′-O-DANS-8-N_3-ATP in the inactivation of Na^+/K^+-ATPase [7] led to the conclusion that two simultaneously existing ATP sites need to cooperate during ATP-driven Na^+/K^+ transport [7,8]. Unfortunately, the available data cannot differentiate between the possibility that two ATP-binding sites exist on a single α subunit and the possibility that the sodium pump works as a functional dimer $(\alpha\beta)_2$ with two cooperating ATP sites. To answer this question, covalent and preferably fluorescent labeling of the E_2ATP-binding site is needed.

To reach this goal, 7-chloro-4-nitrobenzo-1,3-diazole (NBD-Cl) was used to label the ATP sites in Na^+/K^+-ATPase. Cantley et al. hypothesized from their data that two different tyrosine residues within the ATP sites may be modified [9], but Repke's group concluded that ATP may protect the enzyme against modification of sulphydryl groups [10].

It was of interest to learn whether modification of these two types of amino acid residues could underlie the inactivation of the partial activities of the enzyme, i.e. Na^+-dependent phosphorylation at the E_1ATP site [11] and K^+-activated phosphatase activity at the E_2ATP site [12,13]. Therefore, we performed several experiments to study the effect of NBD-Cl on the enzyme with specifically blocked E_1ATP- (FITC-pretreated) or E_2ATP-binding site ($Co(NH_3)_4PO_4$-pretreated). In addition, NBD-Cl is a suitable label to obtain key information on the accessibility or 'depth' of the two ATP-binding sites and, hence, fluorescence quenching by iodide, cesium and manganese(II) ions of NBD-Cl-labeled Na^+/K^+-ATPase was studied in an enzyme preparation where the E_1ATP site had been protected with FITC or $Cr(H_2O)_4$Ado*PP*[CH_2]*P* and whose E_2ATP site had been protected with $Co(NH_3)_4$ATP or $Co(NH_3)_4PO_4$. The differences in the accessibility of the NBD-Cl label to all these quenchers is consistent with the assumption that the E_2ATP site is better accessible to quenchers than the E_1ATP site.

2. 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) and Molecular Probes (Eugene, OR, USA). Lab-Trol protein standard is a product of Merz and Dade (Munich, Germany). [γ-^{32}P]ATP was from Amersham Buchler (Braunschweig, Germany). Calculation and presentation of data were done with the program GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA)

*Corresponding author. Fax: +42 (2) 475/2249.
E-mail: AMLER@BIOMED.CAS.CZ

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Abbreviations: $Cr(H_2O)_4$Ado*PP*[CH_2]*P*, β,γ bidentate complex of chromium(III)-tetraaqua-adenylyl [β,γ-methylene] diphosphonate; $Co(NH_3)_4$ATP, β,γ bidentate complex of cobalt(III)-tetramino-adenosine-5′-triphosphate; $Co(NH_3)_4PO_4$, tetramine cobalt(III)phosphate; FITC, fluorescein 5′isothiocyanate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, 7-chloro-4-nitro-benzofurazane; 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

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2.1. Enzyme and assays

Na^+/K^+-ATPase in the range of 20–25 units/mg protein was isolated by modification of Jørgensen's procedure from pig kidney [14] and measured by a coupled spectrometric assay [15]. One enzyme unit (U) is defined as the hydrolysis of 1 μmole ATP per min at 37°C. Protein was determined by Lowry's method [16] 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.

2.2. Inactivation of Na^+/K^+-ATPase by NBD-Cl

Na^+/K^+-ATPase was inactivated with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) as follows: 1 U of purified Na^+/K^+-ATPase was incubated in a total volume of 1 ml with different concentrations of NBD-Cl in 20 mM Tris/HCl (pH 7.25) and 15 mM NaCl at 37°C. The time course of inactivation of Na^+/K^+-ATPase was followed by transferring aliquots of 50 μl to the coupled optical test [15]. The initial rates of inactivation were calculated and analyzed according to the method of Piszkiewics et al. [7,17].

2.3. Inactivation of Na^+/K^+-ATPase by FITC

Na^+/K^+-ATPase in a final concentration of 1 U/ml (65 mg/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 spun down, 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 various concentrations (0–500 μM) NBD-Cl. After incubation for 1 h at 37°C the enzyme was spun down, washed twice with 20 mM Tris/HCl and K^+-activated para-nitrophenylphosphatase was estimated. For details see [2].

2.4. Frontdoor phosphorylation of Na^+/K^+-ATPase

Na^+/K^+-ATPase in a final concentration of 3 U/ml (195 μg/ml) was incubated overnight at 37°C in a solution containing 20 mM Tris/HCl (pH 7.25) and 1 mM $Co(NH_3)_4PO_4$ (a control without $Co(NH_3)_4PO_4$ was run in parallel). The inactivated enzyme was centrifuged at 100 000×*g*, washed in 20 mM Tris/HCl (pH 7.25) and incubated in 20 mM Tris/HCl, 15 mM NaCl with 100 μM or 1 mM NBD-Cl. After different incubation times at 37°C the enzyme was washed twice with 20 mM Tris/HCl (pH 7.25) and frontdoor phosphorylation was estimated according to Thoenges and Schoner [7]. Background labeling was subtracted using the value of a sample where 1 U of Na^+/K^+-ATPase had been quenched with 250 μl of 25% trichloroacetic acid before phosphorylation from [γ-^{32}P]ATP started. Controls were run in parallel with the native enzyme (no $Co(NH_3)_4PO_4$ pretreatment) to detect the influence of 1 mM NBD-Cl.

2.5. Fluorescence measurements

Na^+/K^+-ATPase in a final concentration of 2.5 U/ml (163 mg/ml) was incubated overnight at 37°C in a solution containing 20 mM Tris/HCl (pH 7.5), 15 mM NaCl and 1 mM $Co(NH_3)_4ATP$ or 25 μM $Cr(H_2O)_4AdoPP[CH_2]P$ (residual activity <5%). The enzyme was centrifuged at 100 000×*g* and washed with 20 mM Tris/HCl (pH 7.5) and resuspended in 20 mM Tris/HCl (pH 7.5), 15 mM NaCl and 1 mM NBD-Cl (the native samples contained no $Co(NH_3)_4ATP$ or $Cr(H_2O)_4AdoPP[CH_2]P$). After a 1 h incubation at 37°C the enzyme was centrifuged and washed again. The fluorescence was read at λ_{ex} 475 nm and λ_{em} 520 nm and fluorescence intensity quenching was achieved by adding increasing concentrations (0–800 mM) of the quenchers (KI, CsCl and $MnCl_2$, respectively). A Specol 211 spectrophotometer was used for absorbance measurements. Steady-state fluorescence data were collected in quartz cuvettes in a Perkin-Elmer LS-5 fluorometer equipped with monochromators and analyzed as described in [18]. Two Glan-Thompson polarizers were used for determination of steady-state anisotropy values. All measurements were performed at 37°C.

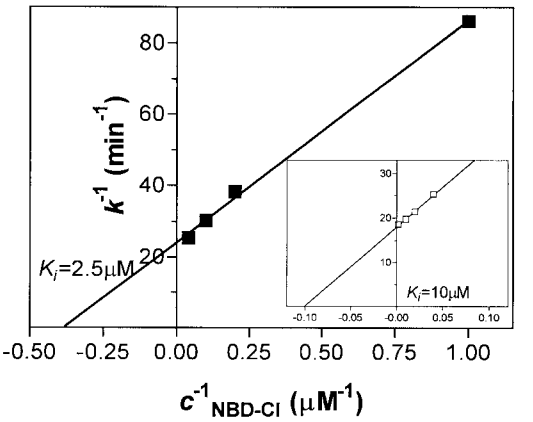

Fig. 1. Inactivation of the Na^+/K^+-ATPase activity by NBD-Cl. Na^+/K^+-ATPase was inactivated with NBD-Cl as follows: 1 U of Na^+/K^+-ATPase was incubated in a total volume of 1 ml with (1, 5, 10, 25, 50, 100 and 500 mM) NBD-Cl in 20 mM Tris/HCl (pH 7.25) and 15 mM NaCl at 37°C. The residual Na^+/K^+-ATPase activity was assayed upon transferring 50 μl aliquots spectrophotometrically [15]. Closed symbols show the residual rate constants of inactivation with 1–25 mM NBD-Cl and the insert with the open symbols the constants of inactivation with 25–500 mM NBD-Cl. Mean values of two experiments are shown.

To detect the protective effect of $Co(NH_3)_4ATP$ against NBD-Cl labeling, FITC-pretreated Na^+/K^+-ATPase was incubated at a final concentration of 2 U/ml for 2 h at 37°C in a solution containing 20 mM Tris/HCl (pH 7.5) and 1 mM $Co(NH_3)_4ATP$ (control without $Co(NH_3)_4ATP$). After washing the enzyme was incubated in 20 mM Tris/HCl buffer (pH 7.5) for 1 h at 37°C with increasing concentrations of NBD-Cl (0–500 μM) and washed again. The fluorescence of these probes was read at λ_{ex} 475 nm and λ_{em} 520 nm. A control without NBD-Cl was run in parallel as background to exclude nonspecific effects.

3. Results

Inactivation rate constants of Na^+/K^+-ATPase at 0–500 μM NBD-Cl revealed two different K_i values, viz. 2.5 μM (Fig. 1) and 10 μM (Fig. 1, insert) and implied a two-site modification process. To learn whether NBD-Cl may affect the partial activity of the E_2ATP site we studied the inactivation of K^+-activated para-nitrophenylphosphatase by this reagent (Table 1) in a FITC-prelabeled Na^+/K^+-ATPase where the E_1ATP site was thus blocked [2]. All inactivation effects of

Table 1
Inactivation of K^+-dependent para-nitrophenylphosphatase activity in native and FITC pretreated enzyme

NBD-Cl (mM)	Relative velocity in native enzyme (%)	Relative velocity in FITC-pretreated enzyme (%)
0	100	100
20	69.7	67.6
40	33.3	34.5
60	13.8	16.7
100	6.5	6.3
500	0.7	1.3

Na^+/K^+-ATPase in a final concentration of 1 U/ml (65 mg/ml) was incubated overnight at 37°C in a solution containing 20 mM Tris/HCl (pH 7.25) and 10 μM FITC and a control without FITC was run in parallel. The enzyme was then incubated in 20 mM Tris/HCl (pH 7.25), 15 mM NaCl and various concentrations (0–500 μM) NBD-Cl. After incubation for 1 h at 37°C remaining K^+-activated para-nitotrophenylphosphatase was estimated. Mean values of two experiments are shown.

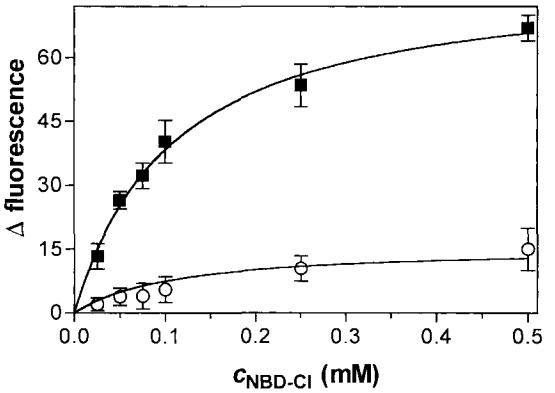

Fig. 2. Protective effect of $Co(NH_3)_4ATP$ towards NBD-Cl labeling. FITC-pretreated Na^+/K^+-ATPase was incubated for 2 h at 37°C in a solution containing 20 mM Tris/HCl (pH 7.5) with 1 mM (○) and without (■) $Co(NH_3)_4ATP$ and then in 20 mM Tris/HCl buffer (pH 7.5) for 1 h at 37°C with increasing concentrations of NBD-Cl (0–500 μM). The fluorescence of these probes was read at λ_{ex} 475 nm and λ_{em} 520 nm. A control without NBD-Cl was run in parallel as background. Mean values and standard deviation of four experiments are shown.

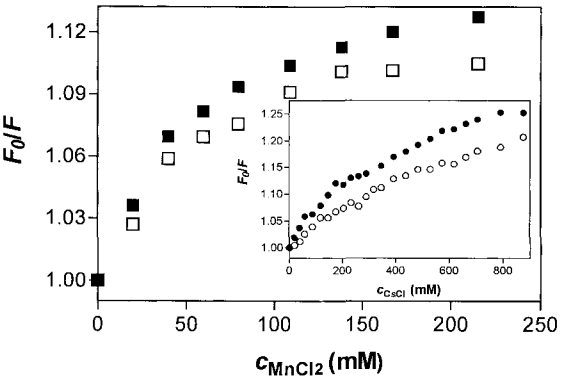

Fig. 3. Fluorescence analysis of fluorescence parameters of NBD-Cl-labeled Na^+/K^+-ATPase. Stern-Volmer plots of quenching of NBD-Cl-labeled Na^+/K^+ATPase by Mn^{2+}-ions (insert shows quenching by Cs^+-ions) bound to enzyme pretreated with 1 mM $Co(NH_3)_4ATP$ (□, ○) and 25 μM $Cr(H_2O)_4AdoPP[CH_2]P$ (■, ●). Mean values of three experiments are shown.

NBD-Cl on the partial activities could be prevented by millimolar concentrations of ATP (data not shown). The protective effect against NBD-Cl labeling was even more pronounced when the FITC-inactivated enzyme was additionally treated by the E_2ATP site specific MgATP complex analog $Co(NH_3)_4ATP$ (Fig. 2). The incorporation of the NBD-Cl label was almost completely blocked by this ATP derivative. Apparently, NBD-Cl reacted with the E_2ATP site in the same way independent of whether the E_1ATP site was blocked by FITC or not. To study the effect of NBD-Cl on the high-affinity ATP-binding site (E_1ATP site) of Na^+/K^+-ATPase, its effect on Na^+-dependent phosphorylation from [γ-^{32}P]ATP (frontdoor) in an E_2ATP site blocked enzyme (by $Co(NH_3)_4PO_4$) was measured. As is evident from Table 2, NBD-Cl modified the E_1ATP site in the untreated control enzyme much more efficiently than in the E_2ATP site blocked enzyme. Generally, less NBD-Cl was necessary to modify the E_2ATP site than the E_1ATP site (compare Tables 1 and 2). These experiments clearly showed that NBD-Cl modified both ATP sites and, unlike FITC, was unable to discriminate between these sites.

Besides inactivation, modification of Na^+/K^+-ATPase by NBD-Cl resulted in a spectral shift of the NBD-Cl absorption spectrum from 330 nm to 425 nm and in the appearance of fluorescence with a maximum at 520 nm. Both the spectral shift and the appearance of fluorescence, were a clear indication of NBD-Cl modifications of the enzyme. This was observed when the enzyme was labeled either with or without $Co(NH_3)_4ATP$ or $Cr(H_2O)_4AdoPP[CH_2]P$. The former compound is known as a specific inhibitor of the E_2ATP site [2,5,7] while the latter blocks specifically the E_1ATP site [2,3]. The absorption or fluorescence spectra of enzyme-bound NBD-Cl did not differ significantly if the labeling of the enzyme was done in the presence of $Co(NH_3)_4ATP$ or $Cr(H_2O)_4AdoPP[CH_2]P$ (E_1ATP site or E_2ATP site is blocked). Quenching experiments, however, clearly distinguished between the two types of ATP site blocking. We used both positively and negatively charged quenchers: Cs^+, Mn^{2+} and I^-. In all cases, the Stern-Volmer plot was not monotonic (Fig. 3) and the modified Stern-Volmer plot revealed a fraction of inaccessible fluorophores.

Table 2
Effect of NBD-Cl on Na^+-dependent frontdoor phosphorylation

Time (min)	Phosphorylation of $Co(NH_3)_4PO_4$-pretreated enzyme (%)[a]		Phosphorylation of native enzyme (%)[b]
	+0.1 mM NBD-Cl	+1 mM NBD-Cl	+1 mM NBD-Cl
0	100	100	100
5	n.d.	91	37
15	86	81	30
30	n.d.	74	15
45	72	65	6.5
60	68	50	6.0
90	61	n.d.	n.d.
120	58	n.d.	n.d.

Na^+/K^+-ATPase (3 U/ml; 195 μg/ml) where the E_2ATP site was blocked with 1 mM $Co(NH_3)_4PO_4$ (see Section 2) was incubated in 20 mM Tris/HCl, 15 mM NaCl and 100 μM or 1 mM NBD-Cl. A native control enzyme was treated in the same way with 1 mM NBD-Cl. Frontdoor phoshorylation from [γ-^{32}P] was studied as a function of the exposure time of the enzyme at 37°C with NBD-Cl. For experimental details see Section 2 and [5,7]. Mean values of three experiments are shown.

[a]Control is $Co(NH_3)_4PO_4$-pretreated Na^+/K^+-ATPase without NBD-Cl measured after the same incubation time (100% is 33.3 pmole phosphate/unit).

[b]Control is native Na^+/K^+-ATPase without NBD-Cl measured after the same incubation time (100% is 100 pmole phosphate/unit). n.d., not determinated.

Interestingly, only a minute difference, if any, was detected between fluorescence quenching of Na^+/K^+-ATPase labeled with NBD-Cl in the presence of $Cr(H_2O)_4AdoPP[CH_2]P$ (E_1ATP site is blocked) and in the absence of any analog. The enzyme pretreated with $Co(NH_3)_4$ATP (E_2ATP site is blocked), on the other hand, showed different fluorescence properties as compared with the enzyme labeled without ATP analogs. The presence of $Co(NH_3)_4$ATP clearly changed the bimolecular quenching constant calculated for low concentrations of the quenchers, i.e. as an initial slope. The obtained Stern-Volmer quenching constants were $(9.6 \pm 0.8)\cdot10^{-4}$ for Cs^+, $(3.3 \pm 0.3)\cdot10^{-2}$ for I^- and $(2.0 \pm 0.2)\cdot10^{-3}$ for Mn^{2+} in the case of $Cr(H_2O)_4AdoPP[CH_2]P$-pretreated enzyme. For $Co(NH_3)_4$ATP-pretreated Na^+/K^+-ATPase where the low-affinity ATP-binding site was occupied, the bimolecular quenching constants decreased to $(4.6 \pm 0.4)\cdot10^{-4}$ for Cs^+, $(2.3 \pm 0.2)\cdot10^{-2}$ for I^- and $(1.2 \pm 0.2)\cdot10^{-3}$ for Mn^{2+}. Additionally, we also observed a different profile of anisotropy changes when the fluorescence anisotropy was followed during incubation of the enzyme with NBD-Cl. The anisotropy after a 6 h incubation of the $Cr(H_2O)_4AdoPP[CH_2]P$-pretreated enzyme with 100 μM NBD-Cl at 37°C (virtually a total binding) was 0.128 while in the presence of $Co(NH_3)_4$ATP the anisotropy value increased to 0.137.

4. Discussion

We confirmed in this study earlier reports that NBD-Cl inhibits Na^+/K^+-ATPase with two different affinities [9,10]. This was evident not only from the kinetics of inactivation with K_i values of 2.5 and 10 μM (Fig. 1), but also from the fact that NBD-Cl inactivated both the K^+-activated para-nitrophenylphosphatase activity in an enzyme where the E_1ATP site was blocked by FITC (Table 1) and the Na^+-dependent frontdoor phosphorylation in an enzyme with the E_2ATP site blocked by $Co(NH_3)_4PO_4$ (Table 2). Thus, we can conclude that NBD-Cl is a fluorescent probe labeling both the low- and high-affinity ATP-binding sites. To our knowledge, this is the first study describing a covalent fluorescent label with an affinity for both ATP-binding sites. In combination with site-specific MgATP derivatives or fluorescent pseudo ATP analogs, this probe is a potent tool for clarifying the structure and mechanism of the low- and the high-affinity ATP-binding sites on Na^+/K^+-ATPase.

Fluorescence intensity studies of NBD-Cl bound to Na^+/K^+-ATPase in this work revealed two populations of fluorophores (Fig. 3). The bimolecular quenching constants for all three quenchers (Cs^+, I^- and Mn^{2+}) were significantly higher for the $Cr(H_2O)_4AdoPP[CH_2]P$-Na^+/K^+-ATPase than for the enzyme treated with $Co(NH_3)_4$ATP. Hence, the accessibility of the quenchers to the fluorophore bound to the E_2ATP site was higher than to the fluorophore bound to the E_1ATP site. Modification of either of the two ATP sites resulted in a rotational immobilization of the fluorophore which was reflected in a relatively high fluorescence anisotropy. This anisotropy was higher when the E_2ATP-binding site was blocked than when the blockage affected the E_1ATP-binding site. Consequently, the E_2ATP site seems to be sterically more open as compared with the E_1ATP site. The fact that the high-affinity binding site (E_1ATP site) is sterically poorly accessible was shown previously by FITC labeling [19].

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