Erythrosin 5′-isothiocyanate labels Cys^{549} as part of the low-affinity ATP binding site of Na^+/K^+-ATPase

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Abstract The high-affinity E_1ATP site of Na^+/K^+-ATPase labeled with fluorescein 5′-isothiocyanate and its E_2ATP site labeled with erythrosin 5′-isothiocyanate (ErITC), as was shown recently [Linnertz et al. (1998) J. Biol. Chem. 273, 28813–28821], reside on separate and adjacent catalytic α subunits. This paper provides evidence that specific labeling of the E_2ATP binding site with ErITC resulted in a modification of the Cys^{549} residue in the tryptic fragment with the sequence Val^{545}-Leu-Gly-Phe-Cys^{549}-His^{550}. Hence, Cys^{549} is part of or close to the low-affinity E_2ATP binding site of Na^+/K^+-ATPase.

Key words: Na^+/K^+-ATPase; Erythrosin 5′-isothiocyanate; Low-affinity ATP binding site; E_2ATP site labeling; Amino acid sequencing

1. Introduction

Na^+/K^+-ATPase is an integral membrane protein which transports sodium and potassium ions against an electrochemical gradient. The transport of Na^+ and K^+ is presumably connected with the oscillation of the enzyme between two major conformational states, designated E_1Na^+ and E_2K^+. The E_1 and E_2 states have different affinities for ATP [1]. A reaction mechanism assuming consecutive changes of a single ATP site during the catalytic process, the so-called Albers-Post model, is inconsistent with the recent kinetic demonstration of simultaneously existing and cooperating ATP sites [2]. Consistent therewith is the observation that specific labeling of the high-affinity E_1ATP [3] or the low-affinity E_2ATP sites does not block labeling and partial activity of the other empty site [4,5]. The recent demonstration of a 'superphosphorylation', i.e. that at least 2 mol of phosphate can be incorporated into the catalytic α subunit per mol of ouabain binding sites [6], is consistent with the observation of a phosphorylation from inorganic phosphate (P_i) during Na^+-ATPase activity [7] and with the possibility that Na^+/K^+-ATPase is phosphorylated from both ATP sites [8].

There are a large number of experiments favoring the idea that the two ATP sites reside on different α subunits [9,10]. Recently information on the location of the E_1ATP and E_2ATP sites was obtained by affinity labeling of the E_1ATP site with the fluorescent pseudo-ATP analog fluorescein 5′-isothiocyanate (FITC) and of the E_2ATP site with the fluorescent pseudo-ATP analog erythrosin 5′-isothiocyanate (ErITC) [11]. Förster energy transfer measurements gave information on the distance between the E_2ATP site and the E_1ATP site. The distance of the low-affinity ATP binding site to the high-affinity site was estimated to be $R = 6.5$ nm, too large to localize both ATP binding sites on to the same catalytic α subunit [11]. The amino acid sequence of the site binding ATP with low affinity and reacting with ErITC in an E_1ATP-protected enzyme (by FITC) is not yet known but amino acids contributing to the recognition and catalysis of ATP at the high-affinity E_1ATP site have been defined by affinity labeling of this site [12,13] with protein-reactive ATP derivatives and the pseudo-ATP analog FITC [14,15]. Hence, information on the amino acid sequence forming the low-affinity E_2ATP binding site should be obtainable by a similar procedure. Therefore, affinity labeling of the low-affinity E_2ATP site was done with ErITC in an enzyme whose E_1ATP site had been blocked by FITC. Subsequently, the amino acid sequence was determined after isolation of the erythrosin-labeled tryptic peptide. This paper shows that ErITC labels Cys^{549} within the cytosolic H_4/H_5 loop. This amino acid has not been shown before to be a part of the ATP sites of Na^+/K^+-ATPase.

2. Materials and methods

All chemicals were of the highest purity available 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).

2.1. Enzyme assays

Na^+/K^+-ATPase from pig kidney, with specific enzyme activity of 25–27 U/mg protein, was isolated by a modification of Jørgensen's procedure [16] and measured by a coupled spectrophotometric assay [17]. One enzyme unit (U) is defined as the amount of enzyme hydrolyzing 1 μmol ATP per minute at 37°C. Protein was determined by the method of Lowry et al. [18] using Lab-Trol as protein standard. Lab-Trol is a mixture of proteins and enzymes used for the calibration of assays in clinical chemical analysis. All buffers used were made up to their respective pH value at room temperature.

K^+-activated *p*-nitrophenylphosphatase activity was measured on a multititer plate by incubating the Na^+/K^+-ATPase at 37°C in a total

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Abbreviations: FITC, fluorescein 5′-isothiocyanate; ErITC, erythrosin 5′-isothiocyanate; rp-HPLC, reversed phase high performance liquid chromatography; 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; H_2-DIDS, dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonate

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Table 1
Effect of FITC and ErITC on the activities of Na^+/K^+-ATPase

Na^+/K^+-ATPase preparation	Na^+/K^+-ATPase activity (%)	*p*-Nitrophenylphosphatase (%)	Mol label per mol α subunit
Native	100	100	–
+10 μM FITC	≤1	~80	0.6
+2 μM ErITC	≤1	≤1	0.5

Native enzyme (6 U) was incubated in a total volume of 1 ml with 10 μM FITC and additionally with 2 μM ErITC. Inactivation of Na^+/K^+-ATPase activity and *p*-nitrophenylphosphatase was measured as described in Section 2. Determination of the binding ratio was done according to Linnertz et al. [11].

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. The reaction was stopped after 15 min by the addition of 200 μl 3 N NaOH. The *p*-nitrophenolate formed was measured at 405 nm by an ELISA reader [3].

2.2. Inactivation of Na^+/K^+-ATPase with FITC and ErITC

Na^+/K^+-ATPase (6 U, 300 μg) was incubated in a total volume of 1 ml overnight at 37°C in a solution containing 20 mM Tris-HCl pH 7.25, 15 mM NaCl and 10 μM FITC. This inactivated enzyme was centrifuged in Eppendorf tubes for 30 min at 100 000×*g*. The pellet was resuspended in 1 ml of a solution containing 20 mM Tris-HCl pH 7.25, 15 mM KCl and 2 μM ErITC. This preparation was incubated additionally for 3 h at 37°C, centrifuged and washed twice in 20 mM Tris-HCl (pH 7.25). After the last centrifugation step, the protein was resuspended in 0.3 ml of 20 mM Tris-HCl buffer of pH 7.25 (final concentration 1.8 mg/ml). The partial activities and specificity of the labeled compounds were measured as described previously [2,3,11,19]. Notably, 5′-isothiocyanates like FITC or ErITC are reportedly potent labels of both Cys and Lys residues at pH 7.25 [20].

2.3. Tryptic digestion

FITC-ErITC double-labeled Na^+/K^+-ATPase (166 mg) was suspended in 8 M urea buffered with $(NH_4)_2HPO_4$ to pH 8. The final protein concentration was 4 mg/ml. This solution was heated for 2 h to 50°C to solubilize the membrane bound enzyme. After dilution with water in a ratio of 1:4 (final urea concentration 2 M, protein concentration 1 mg/ml) trypsin (sequencing grade) was added in a ratio of 1:100 related to the protein concentration. After 24 h standing at room temperature, the same amount of trypsin was added a second time. After additional 48 h of reaction, the membrane fragments and the undigested protein was centrifuged for 30 min at 100 000×*g*. The supernatant was concentrated by evaporation in a Speed-Vac centrifuge. The ErITC-labeled peptides were purified by rp-HPLC.

A reversed phase column Merck 250-3 Lichrospher WP300 PR-18 (5 μm) was used to purify the ErITC-labeled tryptic fragments. The peptides were eluted at 25°C and a flow rate of 300 μl/min applying a non-linear gradient of 2–90% B in A (buffer A: 25 mM KH_2PO_4-pH 7.0; buffer B: CH_3CN). Unlabeled peptides were recorded at 210 nm, peptides labeled with erythrosin were detected at 530 nm. The fraction with the highest absorbance at 530 nm was collected and rechromatographed isocratically on the same column in 50% water and 50% CH_3CN to clean the ErITC-labeled tryptic peptide.

2.4. Amino acid sequencing

The dried labeled peptide sample was dissolved in 90 μl of 0.1% trifluoroacetic acid, 10% CH_3CN, and 15 μl of this solution was applied to the Protein Support (Beckman). The sample was inserted into the cartridge of the Protein Sequencer LF3600 (Beckman) and analyzed by Edman degradation [21].

3. Results and discussion

In agreement with previous results [3], treatment of the pig kidney enzyme with 10 μM FITC at pH 7.25 resulted in a decrease of the ATP hydrolyzing activity of Na^+/K^+-ATPase and left the E_2ATP-site-specific activity of K^+-dependent hydrolysis of *p*-nitrophenylphosphate almost unaltered (Table 1). This labeling resulted in 0.6 mol specifically bound FITC per mol α subunit; non-specific labeling was detected and corrected by the use of anti-fluorescein antibodies as described previously [22]. In agreement with a previous report [11], addition of 2 μM ErITC modified the E_2ATP site and inactivated the remaining K^+-activated *p*-nitrophenylphosphatase with a binding ratio of 0.5 mol erythrosin bound per mol α subunit (Table 1).

Tryptic hydrolysis of the labeled enzyme and peptide purification by the rp-HPLC procedure (Fig. 1) yielded an erythrosin-labeled peptide. Amino acid sequencing of the ErITC-labeled peptides obtained by two independent isolations resulted in the same sequence, viz. Val-Leu-Gly-Phe-Xxa-His. Xxa stands for ErITC-modified amino acid residue, most probably Cys^{549} which is due to its low yield in the Edman degradation hard to detect and after derivatization virtually undetectable. Comparison of this sequence with the α_1 sub-

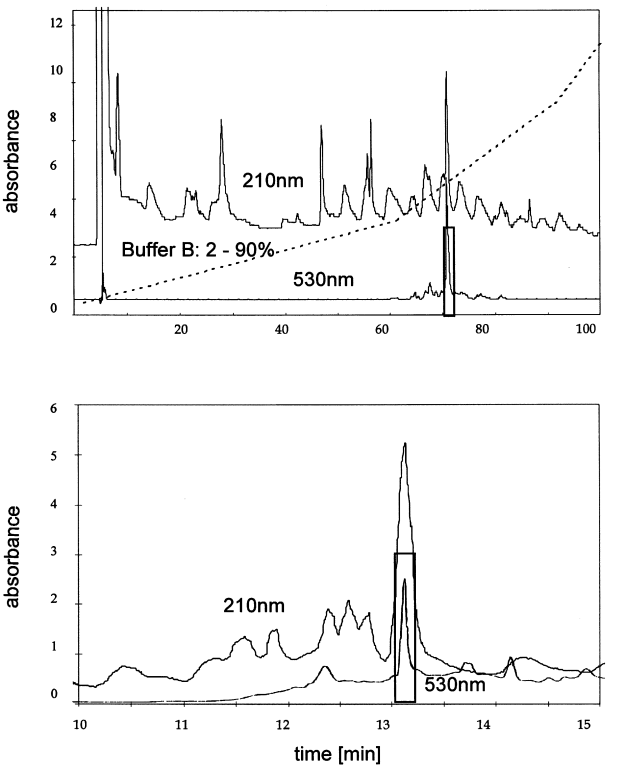

Fig. 1. Separation of tryptic peptides by rp-HPLC on a Merck 250-3 Lichrospher WP300 RP-18 (5 μm) column. Absorbance of the eluate was recorded at 210 nm to detect all peptides and at 530 nm to detect erythrosin-labeled peptides. The upper chromatogram shows the peptide separation with a gradient of 2–90% acetonitrile in 25 mM KH_2PO_4 at pH 7.0. The lower chromatogram shows the rechromatography of the fraction with a retention time of 70–73 min (the bar in the upper graph) in the first step under isocratic conditions in a mixture of equal volumes of acetonitrile and 25 mM KH_2PO_4 pH 7.0. The peak at 13.0–13.3 min (the bar in the lower graph) was used for amino acid sequence analysis.

unit of pig kidney Na^+/K^+-ATPase [23] showed identity with the sequence Val^{545}-Leu-Gly-Phe-Cys^{549}-His^{550}. The reason why the peptide was digested between His^{550} and Leu^{551} by sequence-grade trypsin is not clear. One may speculate that the ErITC label residing at Cys^{549} is involved. SDS gel electrophoresis did not show peptides larger than 5 kDa (data not shown).

As follows from the amino acid analysis of the ErITC-labeled peptide, Cys^{549} is the only possible amino acid residue in this peptide that can form a covalent link with ErITC. It was shown previously that cysteine can be modified by ErITC at pH 7.25 [20], i.e. under conditions used in this work. Interestingly, Cys^{549} (and the peptide starting from Val^{545}) was suggested as a part of the ATP binding site [24,25] in labeling experiments with dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonate (H_2-DIDS) which was used to modify the E_1ATP binding site of Na^+/K^+-ATPase. This compound labels one or two amino acids of the enzyme in dependence on pH and was also able to block its *p*-nitrophenylphosphatase activity. Similar results were obtained with ErITC in this work (Table 1). ErITC binds specifically to the E_2ATP site only at pH 7.25 (and shows no effect at pH 9) in a E_1ATP site-blocked (FITC-labeled) enzyme [11]. The present finding of Cys^{549} as an amino acid of the specific E_2ATP label and the previous reports with H_2-DIDS [24,25] show that this region of the large cytosolic H_4/H_5 loop is involved in the E_2ATP binding site.

In conclusion, the low-affinity E_2ATP binding site has a different specificity for ATP analogs [2,10] and it has a more open structure [19]. Obviously, the conformation of the ATP binding site differs in both main conformations (E_1 and E_2) and Cys^{549} participates in the conformation of the low-affinity E_2ATP binding site. It will be interesting to see how a mutation of this residue affects ATP binding and catalysis.

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References

[1] Lingrel, J.B. and Kuntzweiler, T. (1994) J. Biol. Chem. 269, 19659–19662.
[2] Thoenges, D. and Schoner, W. (1997) J. Biol. Chem. 272, 16315–16321.
[3] Linnertz, H., Thönges, D. and Schoner, W. (1995) Eur. J. Biochem. 232, 420–424.
[4] Scheiner-Bobis, G., Antonipillai, J. and Farley, R.A. (1993) Biochemistry 32, 9592–9599.
[5] Ward, D.G. and Cavieres, J.D. (1996) J. Biol. Chem. 271, 12317–12321.
[6] Peluffo, R.D., Garrahan, P.J. and Rega, A.F. (1992) J. Biol. Chem. 267, 6596–6601.
[7] Campos, M. and Beaugé, L. (1994) J. Biol. Chem. 269, 18028–18036.
[8] Yamazaki, A., Kaya, S., Tsuda, T., Araki, Y., Hayashi, Y. and Taniguchi, K. (1994) J. Biochem. 116, 1360–1369.
[9] Askari, A. (1988) Progr. Clin. Biol. Res. 268A, 149–165.
[10] Schoner, W., Thönges, D., Hamer, E., Antolovic, R., Buxbaum, E., Willeke, M., Serpersu, E.H. and Scheiner-Bobis, G. (1994) in: The Sodium Pump (Bamberg, E. and Schoner, W., Eds.), pp. 332–341, Springer, New York.
[11] Linnertz, H., Urbanova, P., Obsil, T., Herman, P., Amler, E. and Schoner, W. (1998) J. Biol. Chem. 273, 28813–28821.
[12] Tran, C.M., Scheiner-Bobis, G., Schoner, W. and Farley, R.A. (1994) Biochemistry 33, 4140–4147.
[13] Tran, C.M., Houston, E.E. and Farley, R.A. (1994) J. Biol. Chem. 269, 6558–6565.
[14] Farley, R.A., Tran, C.M., Carilli, C.T., Hawke, D. and Shiveley, J.E. (1984) J. Biol. Chem. 259, 9532–9535.
[15] Hinz, H.R. and Kirley, T.L. (1990) J. Biol. Chem. 265, 10260–10265.
[16] Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52.
[17] Schoner, W., von Ilberg, C., Kramer, R. and Seubert, W. (1967) Eur. J. Biochem. 1, 334–343.
[18] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 264–275.
[19] Linnertz, H., Urbanova, P. and Amler, E. (1997) FEBS Lett. 419, 227–230.
[20] Podharsky, D., Drobnica, L. and Kristian, P. (1979) Experientia 35, 154–155.
[21] Edman, P. (1967) Eur. J. Biochem. 1, 80–91.
[22] Amler, E., Abbot, A. and Ball, W.J. (1992) Biophys. J. 61, 553–568.
[23] Ovchinnikov, Y.A., Modyanov, N.N., Broude, N.E., Petrujkhin, K.E., Grishin, A.V., Arzamazova, N.A., Monastyrskaya, G.S. and Sverdlov, E.D. (1986) FEBS Lett. 201, 237–245.
[24] Pedemonte, C.H. and Kaplan, J.H. (1988) Biochemistry 27, 7966–7973.
[25] Pedemonte, C.H. and Kaplan, J.H. (1990) Am. J. Physiol. 258, C1–C23.