Effect of Peptide Bond Splitting on Ouabain Sensitive Conformational Changes in Na⁺,K⁺-ATPase Treated with N-[p-(2-Benzimidazolyl)phenyl]maleimide

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Abstract—Trypsin treatment of N-[p-(2-benzimidazolyl)phenyl]maleimide modified enzyme caused a marked reduction in Na⁺,K⁺-ATPase activity and in the amount of the α-chain, which contains the phosphorylation and ouabain binding sites. However, these preparations retained nearly 90% of the ouabain binding capacity and showed ouabain sensitive dynamic fluorescence changes accompanying the hydrolysis of ATP. The data showed that the three dimensional structure of Na⁺,K⁺-ATPase, which is important in the dynamic fluorescence change, is little affected in spite of extensive covalent bond splitting in the α-chain of Na⁺,K⁺-ATPase.

Na⁺,K⁺-ATPase consists of phospholipids and two different peptides, a large one and a small one, designated as the α and β chain, respectively (1-4). The α-chain contains the sites of ATP binding, phosphorylation and ouabain binding (2-4). The amino acid sequence deduced from cDNA has been reported recently (5-7). However, information about the three dimensional structure of the enzyme during ouabain sensitive hydrolysis of ATP to transport Na⁺ and K⁺ across the membranes is needed to understand better the mechanism of the Na⁺ pump. Tryptic digestion of the α-chain has been used (2, 8, 9) effectively to distinguish two different conformational states, namely, the Na⁺ form (E₁Na) and K⁺ form enzyme (KE₂) (2-4). On the other hand, conformational changes during the hydrolysis of ATP have been studied extensively with pig kidney Na⁺,K⁺-ATPase modified with N-[p-(2-benzimidazolyl)phenyl]maleimide (BIPM) (10-15). The data demonstrated clearly that ATP hydrolysis occurred (10) through the Mg-Na-ATP enzyme complex, ADP sensitive phosphoenzyme (E₁P), K⁺ sensitive phosphoenzyme (E₂P) and K⁺ bound enzyme; and the data indicated that ouabain almost completely stabilized the breakdown of E₂P to form ouabain bound phosphoenzyme (11). Quite recently it was identified (16) that the conformationally sensitive site is Cys 964 of the α-chain: the microenvironment of the BIPM residue covalently bound to Cys 964 changes accompanying ouabain sensitive hydrolysis of ATP (10-16). To understand better the mechanism of ouabain sensitive conformational change, we investigated the effect of trypsin treatment (2, 8, 9) on the BIPM treated enzyme (BIPM enzyme) to follow the conformational change after covalent bond splitting of the α-chain.

Na⁺,K⁺-ATPase was purified and modified with BIPM as described (10, 11). The specific activity of the enzyme preparation thus obtained was 544±13 nmol Pi/mg protein/hr. The BIPM enzyme was digested by trypsin

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(Sigma type I) at the trypsin/Na\textsuperscript{+},K\textsuperscript{+}-ATPase weight ratio of 1/50 for 4 min at 37°C in the presence of 25 mM imidazole-HCl, 25 mM sucrose, 1 mM EDTA-Tris and 16 mM NaCl (pH 7.4). The reaction was terminated by adding trypsin inhibitor (trypsin/trypsin inhibitor weight ratio of 1/3). The sample was washed twice by centrifugation and suspension (20,000 rpm for 20 min at 0°C) with a solution containing 25 mM imidazole-HCl, 25 mM sucrose, and 0.1 mM EDTA-Tris (pH 7.4), as described (11). The fluorescence measurements were performed at 25°C by using a Shimadzu RF-503 difference spectrophotometer (10, 11). Polyacrylamide SDS gel electrophoresis was carried out as described (17), except that the treatment of the BIPM enzyme with SDS was carried out in a solution containing 0.5 mg protein/ml of the enzyme preparations, 1% SDS, 1% β-mercaptoethanol, 10 mM Tris-HCl (pH 6.8) and 20% glycerol at 25°C for 30 min. Other analytical methods used were the same as described (11).

Trypsin treatment reduced the Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity to nearly 20% (108±10 mol Pi/mg protein/hr) of the control (BIPM enzyme). However, the amount of phosphoenzyme formed was 1.1±0.1 nmol/mg protein (n=4), which was about 70% of the control. The amount of ouabain bound was 1.5±0.3 nmol/mg protein, which was about 90% of the control. The data suggested that the treatment reduced apparently the rate of breakdown of phosphoenzyme, because phosphoenzymes are shown to be intermediates of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase reaction (1, 3, 4, 12-15).

Phosphoenzymes formed in the presence of 16 mM Na\textsuperscript{+} and Mg\textsuperscript{2+} with ATP became more sensitive to ADP and less sensitive to K\textsuperscript{+} by trypsin treatment as already reported with a purified enzyme not treated with BIPM (8).

According to the analysis by SDS gel electrophoresis, the amount of α-chain decreased to below 20% with little change in the amount of β-chain (Fig. 1A and B). Neither 78 nor 58 kdal peptides were detected. These peptides had been shown to be present in the trypsin treated samples in the presence of 150 mM NaCl or KCl, respectively (2, 8): these peptides were already shown to be phosphorylated by ATP or Pi (8). These data indicated that the sites for ouabain binding and the phosphorylation are conserved in spite of extensive splitting of the α-chain.

To investigate the effect of peptide bond splitting on the changes in the microenviorn-
ment of Cys 964 (16) during ouabain sensitive hydrolysis of ATP. Trypsin treated BIPM enzyme preparations were phosphorylated by ATP in the presence of 2000 and 16 mM NaCl with 0.43 mM MgCl$_2$: the equilibrium between $E_1$P and $E_2$P in Na$^+$,K$^+$-ATPase shifted to the former ($K_{0.5}$=0.6 M) in the presence of high concentrations of Na$^+$ (11). Addition of ATP decreased the fluorescence intensity (−0.9%) in the former condition and further addition of ouabain increased the fluorescence intensity slowly to give +3.3% level (Fig. 2A$_1$). Similar fluorescence changes, except for the extent of changes, were observed by ATP (−1.3%) and ouabain (+3.7%) in nontrypsinized BIPM enzyme (Fig. 2A$_2$) as already reported (11). The decrease and the increase in the presence of 2000 mM Na$^+$ with Mg$^{2+}$ were already shown to be due to the accumulation of $E_1$P and ouabain $E_2$P, respectively (11).

Similar experiments in the presence of 16 mM Na$^+$ with 0.43 mM MgCl$_2$ were performed with trypsin treated enzyme (Fig. 2B$_1$). Addition of ATP increased the fluorescence (+2.4%). The fluorescence intensity slowly decreased with time ($t_{0.5}$=70 min) to the lowest level. Readdition of ATP increased the fluorescence and the addition of ouabain completely stabilized the fluorescence intensity at the maximum level. Similar fluorescence changes (11) were observed in the non-trypsin treated BIPM enzyme (Fig. 2B$_2$), except that the time

**Fig. 2.** Ouabain sensitive dynamic fluorescence change during the hydrolysis of ATP. BIPM enzyme protein treated with (A$_1$ and B$_1$) or without (A$_2$ and B$_2$) trypsin (140 μg) was suspended in 7 ml of a solution containing 25 mM imidazole-HCl (pH 7.4), 0.1 mM EDTA-Tris, 25 mM sucrose with 2000 (A$_1$ and A$_2$) or 16 (B$_1$ and B$_2$) mM NaCl. The sample and reference cells contained 3.2 ml of suspension. When added, the legands were 1 μl of 85.1 mM ATP-Tris (pH 7.4) and 20 μl of 15 mM ouabain. The same volume of water or 86.4 mM ADP-Tris were added to the reference cell to keep the sample volume of both cells constant. The data shown are percentage values: 100% values of the fluorescence intensity were taken from the difference between the fluorescence intensity at 365 nm and that at 520 nm of the reference sample. The time scale shown in A$_2$ is the same in A$_1$ and that in B$_1$ is the same in B$_2$.
required for the decrease in the fluorescence intensity to the original level decreased to nearly a fifth. This was because the trypsin treatment reduced the ATPase activity to nearly 20% that of non-treated BIPM enzyme as described above.

It has been shown recently that the BIPM enzyme reversibly changes the light scattering intensity which suggests the sequential appearance of species of $E_1P$ that precede $E_2P$ formation (18). Trypsin treated BIPM enzyme also showed a similar ouabain sensitive light scattering increase following formation of $E_1P$, except the extent was reduced to half that observed in the BIPM enzyme not treated with trypsin.

ATP binding sites and phosphorylation sites are known to contain Lys 501 and Asp 369 of the $\alpha$-chain, respectively (5). A derivative of ouabain, $^3$H-nitrophenyltriazene ouabain, bound covalently to 41 kdal peptides (19), which are situated at the amino terminal part of the sequence of the $\alpha$-chain and do not contain Lys 501 (5). Ouabain binding sites are known to be localized outside the membrane, while ATP binding (Lys 501) and phosphorylation sites (Asp 369) are localized inside (1-4). The binding of ATP followed by phosphorylation and the binding of ouabain occur on the opposite side of the membranes. The binding of both ligands influenced the microenvironment of BIPM residues covalently attached to Cys 964 of Na+,K+-ATPase irrespective of the trypsin treatment (Fig. 2): phosphorylation induced dynamic fluorescence change and $E_2P$ formation induced ouabain binding, which stabilized the fluorescence change. These findings indicated that the three dimensional structure of Na*,K*-ATPase is conserved in such a way as to show dynamic ouabain sensitive ATP-dependent conformational changes in spite of extensive splitting of the $\alpha$-chain: nicking of the $\alpha$-chain little affects the gross structure required for the conformational change, but reduces the rate of ATP-hydrolysis considerably.

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References
1 Schwartz, A., Lindenmayer, G.E. and Allen, J.C.: The sodium potassium adenosine triphosphatase, pharmacological, physiological and biochemical aspects. Physiol. Rev. 27, 3-134 (1975)
2 Jorgensen, P.L.: Mechanism of the Na*,K*-pump, protein structure and conformations of the pure (Na+K*)-ATPase. Biochim. Biophys. Acta 694, 27-68 (1982)
3 Glynn, I.M.: The Na*,K*-transporting adenosine triphosphatase. In The Enzymes of Biological Membranes, 2nd ed., Edited by Martonosi, A.N., p. 35-114, Plenum Press, New York and London (1985)
4 Taniguchi, K.: Mechanism of energy transduction in Na*,K*-ATPase. Folia Pharmacol. Japon. 83, 193-206 (1984) (Abs. in English)
5 Shull, G.E., Schwartz, A. and Lingrel, J.B.: Amino acid sequence of the catalytic subunit of the (Na*+K*)ATPase deduced from a complementary DNA. Nature 216, 691-695 (1985)
6 Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nogima, H., Nagano, K., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. and Numa, S.: Primary structure of the $\alpha$-subunit of Torpedo californica (Na*+K*)-ATPase deduced from cDNA sequence, Nature 316, 733-736 (1985)
7 Shull, G.E., Lane, L.K. and Lingrel, J.B.: Amino acid sequence of the $\beta$-subunit of the (Na*+K*)-ATPase deduced from cDNA. Nature 321, 429-431 (1986)
8 Jorgensen, P.L.: Purification and characterization of (Na*+K*)-ATPase. V. Conformational changes in the enzyme. Transition between the Na-form and the K-form studied with tryptic digestion as a tool. Biochim. Biophys. Acta 401, 399-415 (1975)
9 Jorgensen, P.L.: Purification and characterization of (Na*+K*)-ATPase. VI. Differential tryptic modification of catalytic functions of the purified enzyme in presence of NaCl and KCl. Biochim. Biophys. Acta 486, 97-108 (1977)
10 Taniguchi, K., Suzuki, K., Shimizu, J. and Iida, S.: ATP dependent reversible conformational change of Na*,K*-ATPase modified with N-{p-(2-benzimidazolyl)phenyl}maleimide. J. Biochem. 88, 609-612 (1980)
11 Taniguchi, K., Suzuki, K. and Iida, S.: Conformational change accompanying transition of ADP-sensitive phosphoenzyme to potassium-sensitive phosphoenzyme of (Na*,K*)-ATPase modified with N-{p-(2-benzimidazolyl)phenyl}maleimide. J. Biol. Chem. 257, 10659-10667 (1982)
12 Taniguchi, K., Suzuki, K. and Iida, S.: Stopped flow measurement of conformational change induced by phosphorylation in (Na⁺,K⁺)-ATPase modified with N-[p-(2-benzimidazolyl)phenyl]maleimide. J. Biol. Chem. 258, 6927–6931 (1983)

13 Taniguchi, K., Suzuki, K., Kai, D., Matsuoka, I., Tomita, K. and Iida, S.: Conformational change of sodium- and potassium-dependent adenosine triphosphatase: Conformational evidence for the Post-Albers mechanism in Na⁺- and K⁺-dependent hydrolysis of ATP. J. Biol. Chem. 259, 15228–15233 (1984)

14 Taniguchi, K., Suzuki, K., Kai, D., Kudo, M., Tomita, K. and Iida, S.: ATP hydrolysis in the presence of Na⁺ with or without K⁺ occurs accompanying conformational changes via E₁ATP, E₁P, E₂P and E₂ in sequence. In The Na⁺ Pump, Edited by Glynn, I. and Ellory, J.C., p. 383–387. Company of Biologists, Cambridge (1985)

15 Taniguchi, K., Suzuki, K., Sasaki, T., Shimokobe, H. and Iida, S.: Acceleration of the rate of fluorescence decrease by high concentrations of ATP under the condition of accumulation of ADP-sensitive phosphoenzyme in Na⁺,K⁺-ATPase. J. Biochem. 100, 1231–1239 (1986)

16 Nagai, M., Taniguchi, K., Kangawa, K., Matsu, H., Nakamura, S. and Iida, S.: Identification of N-[p-(2-benzimidazolyl)phenyl]maleimide modified residue participating in dynamic fluorescence changes accompanying Na⁺,K⁺-dependent ATP hydrolysis. J. Biol. Chem. 261, 13197–13202 (1986)

17 Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. Nature 227, 680–685 (1970)

18 Taniguchi, K., Suzuki, K., Sasaki, T., Shimokobe, H. and Iida, S.: Reversible change in light scattering following formation of ADP-sensitive phosphoenzyme in Na⁺,K⁺-ATPase modified with N-[p-(2-benzimidazolyl)phenyl]maleimide. J. Biol. Chem. 261, 3272–3281 (1986)

19 Rossi, B., Ponzio, G. and Lazdunski, M.: Identification of the segment of the catalytic subunit of (Na⁺,K⁺)ATPase containing the digitoxigen binding site. EMBO J. 7, 859–861 (1982)