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Affinity modification of E_1-form of Na^+,K^+-ATPase revealed Asp-710 in the catalytic site

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An alkylating ATP analogue, γ-[4-(*N*-2-chlorethyl-*N*-methylamino)]benzylamide ATP (ClRATP), covalently binds to the catalytic α-subunit of Na^+,K^+-ATPase yielding a product resistant to hydrolysis by the enzyme and inhibiting the ATP-hydrolysing activity. The Na^+-form of the membrane-bound Na^+,K^+-ATPase modified with ClRATP was hydrolysed by pepsin under conditions providing maximum stability of the modification product (4°C, pH 1.5). The modified peptide was isolated by HPLC and its amino acid sequence was found to involve residues 706–713 of the α-subunit polypeptide chain. This fragment located near the γ-phosphate of ATP is a component of the active site. It is highly homologous with corresponding regions of the catalytic subunits of all the known E_1-E_2 ATPases. In the Na^+-(or E_1-)enzyme form Asp-710 is the target of modification. Evidently E_1- and E_2-enzymes have different targets in ClRATP modification, i.e. the polypeptide chain regions near the ATP γ-phosphate in the enzyme active site differ somewhat in their conformations.

Na^+,K^+-ATPase; E_1-conformation; Affinity modification; Catalytic site structure

1. INTRODUCTION

Na^+,K^+-ATPase is an integral membrane protein providing a nonequilibrium distribution of sodium and potassium ions between the cell and medium coupled with the ATP hydrolysis. Ion-activators, Na^+ and K^+, regulate both the enzyme affinity for ATP [1] and the stability of the phosphorylated product whose formation is an intermediate stage in the enzymatic hydrolysis of ATP [2]. Investigations of the active site topography for the enzyme in E_1 and E_2 conformations and especially the arrangement of the regions neighbouring ATP γ-phosphate are indispensable for understanding the molecular mechanism of the enzyme function. However lack of information on the primary structure of the enzyme subunits hindered such studies. The establishment of the primary structure [3–8] paved the way for research into the enzyme functioning at the molecular level and first of all into the topography of the Na^+,K^+-ATPase active site.

The ATP derivative γ-[4-(*N*-2-chlorethyl-*N*-methylamino)]benzylamide ATP (ClRATP) is a functional analogue of ATP. We studied its interaction with Na^+- and K^+-enzyme forms [9]. The peptide including the 706–718 modification region of the α-subunit of the low-affinity K^+-form was isolated.

This paper describes the isolation of the α-subunit fragment involving the ClRATP modification region of the high-affinity Na^+-form of Na^+,K^+-ATPase. We determine the structure of this fragment, its position in the polypeptide chain, and location of the affinity reagent modification point.

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2. MATERIALS AND METHODS

Isolation of the homogeneous Na^+,K^+-ATPase preparation from pig kidney, determination of the enzyme activity, protein concentration, and amino acid composition were performed as in [10].

The enzyme was modified with [^{14}C]ClRATP, containing a labelled benzyl residue, in 20 mM Tris-HCl buffer (pH 7.5) at 37°C. Final concentrations of the protein and reagent were 5–7 mg/ml and 0.1–0.3 mM, respectively. To enhance the reagent incorporation, non-radioactive ClRATP was added once again in a 30 min interval up to a concentration of 1 mM.

2.1. *Pepsin hydrolysis*

A sample of membrane-bound Na^+,K^+-ATPase was resuspended in distilled water up to a protein concentration of 10–12 mg/ml. Pepsin dissolved in 10% acetic acid was added to the protein suspension, its volume being a half of the suspension volume, pH of the mixture was adjusted to 1.5 by HCl at 20°C, the final pepsin concentration was 1 mg/ml. After incubation of the mixture at 4°C for 48 h the membrane-bound protein was spun down by ultracentrifugation (a L5-50 Beckman instrument, Austria, a SW-50.1 rotor, 2.5 h, 40000 rpm/min). The supernatant was incubated at 4°C for 48 h, divided into portions corresponding to 7–8 mg of the original membrane-bound protein and frozen at −20°C.

2.2. *Isolation and sequencing of the modified peptide*

HPLC was performed on an LKB HPLC system (Sweden) equipped with pumps (model 2150), a variable wavelength monitor, model 2151, and Uvicord SD model 2158 flow-type spectrophotometers. The hydrolysate was primarily separated on a 0.46 × 25 cm column with reverse-phase Bakerbond Wide-Pore C_{18} (J.T. Baker Research Products, USA) in 0.1% trifluoroacetic acid (TFA), triethylamine was added up to pH 2.5 (buffer A), acetonitrile concentration gradient 0–50% (the loading and flow rates 0.2 ml/min). The gradient profile is shown in fig.2. Rechromatography was carried out on a Milichrom microcolumn liquid-phase chromatograph (reverse-phase Nucleosil $5C_{18}$ column 0.2 × 6.4 cm) at pH 4.5 in 0.01 M sodium acetate and then at pH 2.5 in buffer A; in both cases the stepwise acetonitrile gradient was used: step 1, 700 μl of 10% CH_3CN in a corresponding buffer; step 2, 700 μl of 20% solution; and step 3, 500 μl of 30% solution (flow rate 50 μl/min, separation time 20–30 min). The affinity-labelled peptide was identified by radioactivity and by the presence of the characteristic spectrum.

The peptide primary structure was determined either by automated degradation on an Applied Biosystems model 470A gas-phase sequenator (phenylhydantoins identified by HPLC) or by the manual dansyl-Edman procedure.

To synthesise the model compound 200 μl of 3 M CH_3COONa (pH 5.5) were added to 4.25 mg of 4-(*N*-2-chloroethyl-*N*-methylamino)benzylamine (ClR) and incubated for 1 h at 56°C. The product was extracted and stored in butanol. (The model compound and peptides were treated with 1 M NH_4OH, pH 10, at 20°C for 48 h to hydrolyse the ester bond.)

3. RESULTS AND DISCUSSION

The peptide containing covalently and specifically bound ClRATP in the E_1-form of the enzyme was isolated from pig kidney Na^+,K^+-ATPase modified with ClRATP in the presence of sodium ions. Fig.1 shows that the

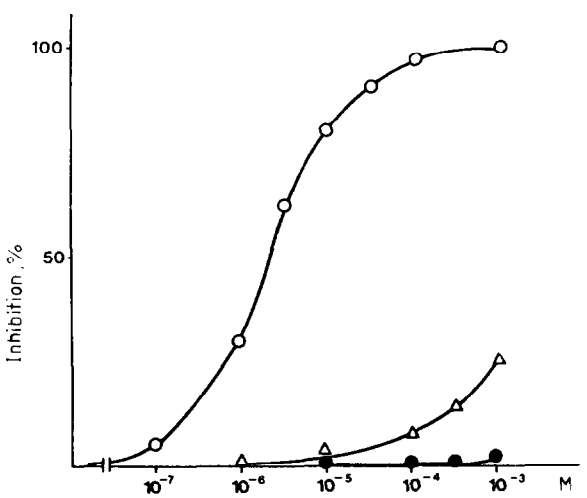

Fig.1. Dependence of inactivation of Na^+,K^+-ATPase on ClRATP concentration in the presence of 150 mM NaCl (○), 150 mM KCl (△), 150 mM NaCl and 10 mM ATP (●). The reaction mixture was incubated for 30 min at 37°C.

Na^+-enzyme (○) form is completely inactivated at considerably smaller reagent concentrations than its K^+-form (△), ATP effectively protects the enzyme from inactivation (●). At first we applied a technique for the differential modification [9] to affect the active site selectively, but now the protein is modified directly with [^{14}C]ClRATP since the level of unspecific insertion is low in the intact protein and peptide material: after separation of the pepsin hydrolysate, a main radioactivity peak was registered and corresponded to the fraction with the absorption maximum at 260 nm.

The incorporated reagent remained unchanged and inactivation of the enzyme was irreversible for at least 1 month if the protein was stored at 4°C, pH 7.2. The labelled modified protein was washed many times by ultracentrifugation or immobilization on filters [11]; still the amount of inserted label was the same, i.e. the covalent bonding inside the enzyme is rather strong. However, after denaturation of the modified protein in the presence of SDS the reagent-protein bond appeared to be thermo- and baselabile as in the case of the K^+-enzyme. After solubilization of the protein with 1% SDS at 100°C for 3 min, the major portion of the label was bound to the protein only at pH 2.0; at pH 9.0 such a short denaturation time liberated 70% of the original amount of the bound [^{14}C]ClRATP (not shown; measurements were carried out as in [9]). The 'Na^+-protein' was hydrolysed with pepsin at pH 1.5, 4°C (see section 2), i.e. under the conditions described in [9]. The conditions were chosen judging from characteristics of the reagent-protein covalent bond (thermolability and resistance to acids) and from comparison of the results obtained for the modified 'K^+-protein'.

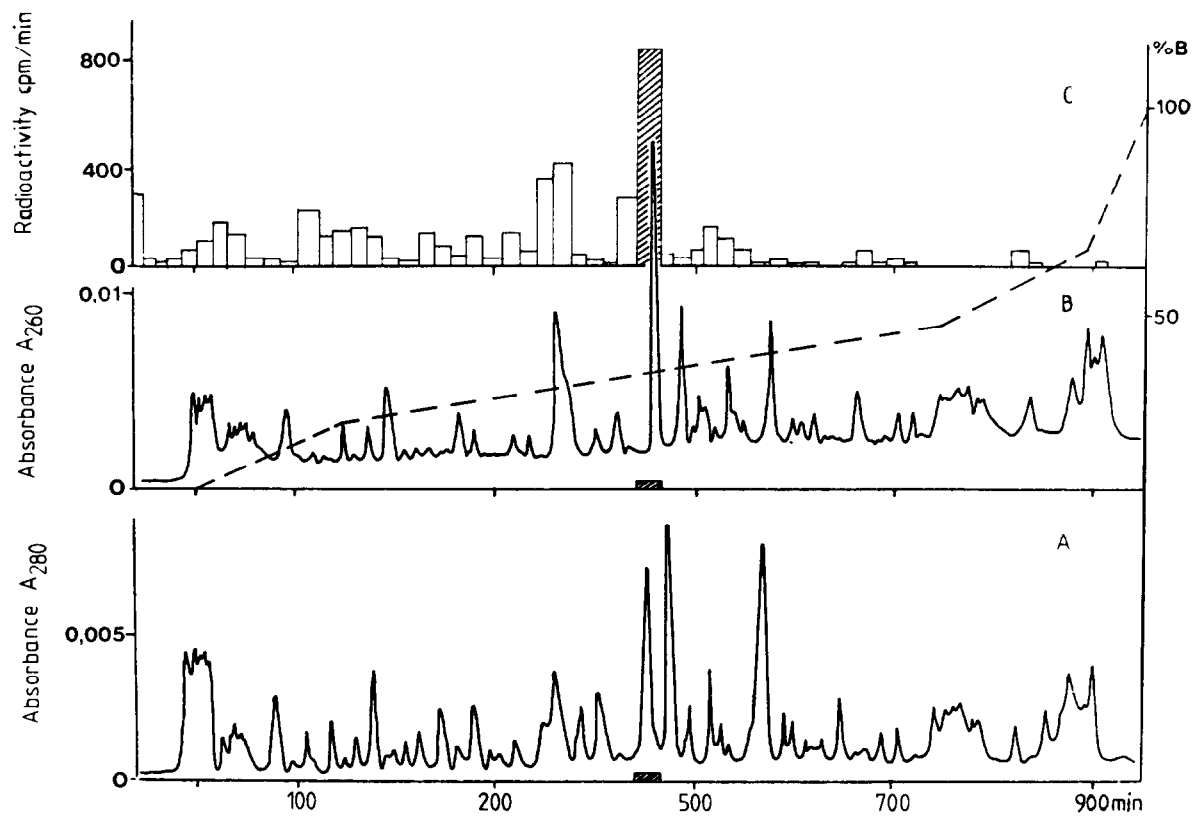

Fig.2. Separation of peptides from the pepsin hydrolysate and distribution of radioactivity along the column 0.46 × 25 cm (support Bakerbond Wide Pore C_{18}). Eluting buffers: A, 0.1% TFA, triethylamine was added up to pH 2.5; B, 50% CH_3CN in buffer A. Flow rate 0.2 ml/min, upwards: A, absorbance at 280 nm; B, at 254 nm; broken line, change in per cent content of buffer B. The height of columns (C) corresponds to radioactivity of the fraction. The fraction subjected to further chromatography is hatched.

The peptide was isolated from the pepsin hydrolysate by HPLC on a Bakerbond Wide Pore C_{18} column, then detected by absorbance (at λ 260 and 280 nm) and radioactivity measurements. One should note that ATP cleaves from the ClRATP molecule because of instability of the phosphoamide bond in the acidic medium. ClR bound to the protein has an absorption maximum at 260 nm and $\epsilon = 10000$. The fraction with the absorption ratio $A_{260}/A_{280} > 1$ was rechromatographed at two different pH values by microcolumn HPLC to decrease irreversible sorption. It contained the largest amount of radioactive label (fig.2). The peptide obtained (fig.3) differed from the earlier isolated K^+-peptide in length (from ratio A_{210}/A_{260}) and in retention time.

Thermo- and baselability indicate the ester bonding of the protein and reagent. To prove this suggestion the model compound (ester of GlR and acetic acid) was synthesised. Under conditions chosen for its complete hydrolysis (1 M NH_4OH, pH 10, 20°C) the label was fully removed from both Na^+- and K^+-peptides for 48 h. In the acidic medium the bond was stable. In such a way the protein-reagent linkage via the covalent ester bond was confirmed.

The structure of the isolated peptide was established by two ways: (i) the label was removed and the peptide was sequenced by the dansyl-Edman method that gave Asx at the fifth step; (ii) the labelled peptide was analysed on a gas-phase sequenator that resulted at the fifth step in a derivative of the amino acid residue differing in chromatographic characteristics as compared to known amino acids. Deciphering the complete peptide structure allowed us to identify it as the fragment of the polypeptide chain of the catalytic α-subunit with coordinates Ala-706–Ala-713. Spectral (fig.3) and sequencing data, lack of aromatic amino acid residues in the peptide structure, ester bonding of the reagent and protein provide evidence that the isolated peptide is modified and consequently represents a component of the enzyme active site. The Asp-710 residue is a target, whose modification with ClRATP causes the complete loss of the enzyme activity.

Comparison of the amino acid sequence of this peptide and the corresponding regions of the α-

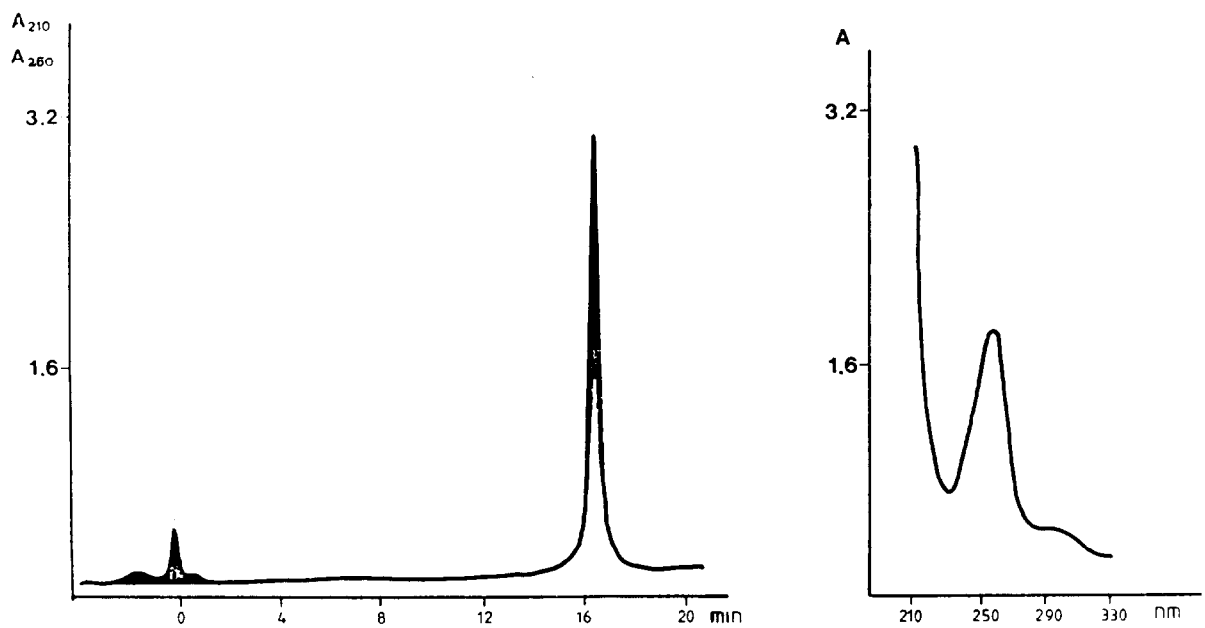

Fig.3. Final stage of purification of the fraction denoted in fig.2 and absorption spectrum of the homogeneous modified peptide. Column, 0.2 × 6.4 cm; support, Nucleosil $5C_{18}$. Flow rate, 50 μl/min. Dark contour, absorbance at 210 nm; light contour, at 260 nm. Stepwise gradient of acetonitrile (700 μl of 10% CH_3CN, 700 μl of 20% CH_3CN, 500 μl of 30% CH_3CN) in 0.01 M CH_3COONa (pH 4.5) was used.

Table 1

Comparison of amino acid sequences of the modified peptide and corresponding regions of other E_1-E_2 ATPases

ATPase	Amino acid sequence
Na^+,K^+-ATPase [3]	^{706}Ala-Val-Thr-Gly-Asp-Gly-Val-Asn^{713}
Ca^{2+}-ATPase [14]	^{698}Ala-Met-Thr-Gly-Asp-Gly-Val-Asn^{705}
Kdp-ATPase [15]	^{512}Ala-Met-Thr-Gly-Asp-Gly-Thr-Asn^{519}
H^+-ATPase [16]	^{630}Ala-Met-Thr-Gly-Asp-Gly-Val-Asn^{637}

subunit of Na^+,K^+-ATPase from different sources: sheep kidney [5], ray electric organ [4], human Na^+,K^+-ATPase [12,13] and from other transport ATPases: Ca^{2+}-ATPases of sarcoplasmic reticulum of fast and twitch [14] muscles, subunit B of Kdp-ATPase from *E. coli* [15], H^+-ATPase from the yeast plasma membrane [16] and plasma membrane of *Neurospora crassa* [17], shows the high sequence homology of these fragments (table 1). The Asp-710 residue is identical in all the structures mentioned above. The homology region including the isolated peptide is rather extended: it embraces also the fragment of the polypeptide chain modified with 5′-(*p*-fluorosulfonyl)benzoyladenosine [18]. Here Lys, being a modification target, is invariant in all the mentioned ATPases except for Kdp-ATPase of *E. coli* [15]. This region begins from Phe-683 and involves the fragment previously identified as a component with a low-affinity active site for binding ClRATP in the K^+-form of the enzyme [9]. This fragment contains two invariant Asp residues for all the structures of the transport ATPases listed in table 1. Each of them can be a target for modification of the enzyme in the K^+-form, at present we try to localise the points of modification. Pepsin hydrolyses of the α-subunit of Na^+,K^+-ATPase modified with ClRATP in the K^+-form, and in the Na^+-form produce different peptides. In the case of the K^+-form the modified peptide is longer and includes the second Asp residue and suggests that Asp-714 is the modified residue. Binding of K^+ or Na^+ stabilises the enzyme in E_1 or E_2 conformations, respectively, and influences the enzyme affinity for ATP. It can induce small translocations of the polypeptide chain around the γ-phosphate binding site in such a way that spatially separated residues Asp-710 or Asp-714 become the target of modification with affinity reagent ClRATP.

REFERENCES

[1] Post, R.L., Küme, S., Tobin, T., Orcutt, B. and Sen, A.K. (1969) J. Gen. Physiol. 54, 306s.

[2] Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116.

[3] Ovchinnikov, Yu.A., Arsenyan, S.G., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Aldanova, N.A., Arzamazova, N.M., Aristarkhova, E.A., Melkov, A.M., Smirnov, Y.V., Guryev, S.O., Monastyrskaya, G.S. and Modyanov, N.N. (1985) Dokl. Akad. Nauk SSSR 285, 1490–1495.

[4] Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, N., Nagano, K., Hirose, T., Inyama, S., Hayashida, H., Miyata, T. and Numa, S. (1985) Nature 316, 733–736.

[5] Shull, G.E., Schwartz, A. and Lingrel, J.B. (1985) Nature 316, 691–695.

[6] Ovchinnikov, Yu.A., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Kiyatkin, N.I., Arzamazova, N.M., Gevondyan, N.M., Smirnov, Y.V., Malyshev, I.V., Monastyrskaya, G.S. and Modyanov, N.N. (1985) Dokl. Akad. Nauk SSSR 287, 1491–1496.

[7] Noguchi, S., Noda, M., Takahashi, H., Kawakami, K., Ohta, T., Nagano, K., Hirose, T., Inayama, S., Kawamura, M. and Numa, S. (1986) FEBS Lett. 196, 315–320.

[8] Ovchinnikov, Yu.A., Modyanov, N.N., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Arzamazova, N.M., Aldanova, N.A., Monastyrskaya, G.S. and Sverdlov, E.D. (1986) FEBS Lett. 201, 237–245.

[9] Dzhandzhugasyan, K.N., Lutsenko, S.V. and Modyanov, N.N. (1986) Biol. Membrany 3, 858–868.

[10] Dzhandzhugasyan, K.N., Modyanov, N.N. and Ovchinnikov, Yu.A. (1981) Bioorg. Khim. 7, 847–857.

[11] Dzhandzhugasyan, K.N., Modyanov, N.N. and Mustayev, A.A. (1984) Biol. Membrany 1, 823–830.

[12] Kawakami, K., Ohta, T., Nojima, H. and Nagano, K. (1986) J. Biochem. 100, 389–397.
[13] Ovchinnikov, Yu.A., Monastyrskaya, G.S., Broude, N.E., Ushkarev, Y.A., Dolganov, G.M., Melkov, A.M., Smirnov, Y.V., Akopyants, N.S., Dulubowa, I.E., Alikmets, R.L., Modyanov, N.N. and Sverdlov, E.D. (1985) Dokl. Akad. Nauk SSSR 287, 1251–1255.
[14] Brandl, C.J., Green, N.M., Korczak, B. and MacLennan, D.H. (1986) Cell 44, 597–607.
[15] Bastido, F., Meissner, G., Fleischer, S. and Post, R.L.J. (1973) J. Biol. Chem. 248, 8385–8391.
[16] Serrano, R., Kielland-Brandt, M.C. and Fink, G.R. (1986) Nature 319, 689–693.
[17] Hager, K.M., Mandala, S.M., Devenport, J.M., Speicher, D.W., Benz, E.J.J. and Slayman, C.W. (1986) Proc. Natl. Acad. Sci. USA 83, 7693–7697.
[18] Nagano, K., Ohta, T. and Yoshida, M. (1986) Proc. Natl. Acad. Sci. USA 83, 2071–2075.