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The Amino Acid Sequence of the Fluorescein-labeled Peptides of Electric Ray and Brine Shrimp (Na,K)-ATPase

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SUMMARY: (Na,K)-ATPase from Torpedo californica (electric ray) and Artemia salina (brine shrimp) was labeled with flourescein 5'-isothiocyanate (FITC) with concomitant loss of activity. Both inactivation and binding were inhibited in the presence of ATP. The sequence of the peptide resulting from tryptic digest containing labeled lysine from both enzymes is Tyr-Leu-Leu-Val-Met-Lys*-Gly-Ala-Pro-Glu-Arg. Thus the primary structure of this region is shown to be conserved in the enzymes of a nonvertebrate and a vertebrate.

It has been known that fluorescein 5'-isothiocyanate (FITC) covalently modifies a lysine residue of the α subunit of the (Na,K)- ATPase and inactivates enzyme. ATP protects the enzyme from inactivation and modification and, therefore, the binding site of FITC is assumed to be located at or near the ATP binding site (1-2). Recently two groups determined the sequences around the modified lysine residue of the enzymes from dog, rat, and lamb kidney (3,4). The sequences were the same for these three mammals. In this communication, we report homology of the sequence of FITC binding site is also conserved down to the electric ray and the brine shrimp.

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EXPERIMENTAL PROCEDURES

Preparation of Membrane-bound (Na,K)-ATPase: About 280 g of electric organs of T.californica were minced with scissors and homogenized by a polytron for 30 seconds at dial number 7 in the homogenizing medium containing 50 mM imidazole/HCl, pH 7.5, 1 mM EDTA, 0.1 mM phenylmethanesulfonylfluoride(PMSF), 0.1 mM dithiothreitol(DTT), 1 mM ATP and 0.25 M sucrose. The homogenate was centrifuged at 4,000 x g for 10 min at 4°C and the supernatant was decanted and saved. The pellet was resuspended in the homogenizing medium and centrifuged again. The resulting supernatant was combined with the previous one and the solution was filtered through cheesecloth. The filtrate was centrifuged at 125,000 x g for 60 min at 4°C. The pellet containing membranes was stored at -20°C in the homogenizing medium. The crude membranes thus obtained were further purified by centrifugation on a discontinuous sucrose gradient after SDS treatment as follows: Crude membranes were incubated in 50 mM imidazole/HCl, pH 7.5, 2 mM EDTA(Tris), 1 mM DTT, 0.1 mM PMSF, 3 mM ATP, 30 % glycerol, and 0.45 mg/ml of SDS, for 30 min at room temperature with stirring. The mixture was centrifuged at 125,000 x g for 3 hrs at 4°C on the discontinuous sucrose gradient, that is , 10 ml of 30 % (W/V)sucrose as upper layer and 10 ml of 42 % sucrose as lower layer containing 25 mM imidazole/HCl, pH 7.5, 1mM EDTA(Tris), 0.1 mM DTT. The interphase between 30 % and 42 % sucrose was collected and, after dilution with 25 mM imidazole/HCl, pH 7.5, 1 mM EDTA(Tris), 0.1 mM DTT, it was centrifuged at 125,000 xg for 30 min. The pellet was suspended in the same buffer and centrifuged again. The enzyme thus obtained had a specific activity of 400-450 μ mol Pi/mg/hr (25°C).
The (Na,K)-ATPase was also prepared from A.salina as described previously (5).

Labeling of (Na,K)-ATPase with FITC: To label the (Na,K)-ATPase of T.californica FITC was added to 10 mg of the enzyme in 10 ml of 50 mM Tris/HCl, pH 9.2, containing 5 mM EDTA and 100 mM NaCl. The final concentration of FITC was 10 μM. The reaction mixture was stirred for 60 min at 25°C at which time it was diluted to 50 volumes with 25 mM imidazole/HCl, pH 7.5, containing 1 mM EDTA and 5 mM DTT. Labeled enzyme was removed from excess FITC by centrifugation. Labeling of (Na,K)-ATPase (8 mg) of A.salina with FITC was carried out in two steps as describted in RESULTS & DISCUSSION.

Proteolysis: The solution for trypsin-treatment of the FITC-labeled (Na,K)-ATPase from both sources contained 2.5 mg/ml of protein, 50 mM imidazole/HCl, pH 7.5, and 1 mM $CaCl_2$. TPCK-trypsin was added two times in the 1.5 hrs interval and final ratios of TPCK-trypsin to the enzymes from T.californica and A.salina were 1:25 and 1:20, respectively. After 3 hrs incubation at 37°C, the reaction was stopped by adding 1 mM PMSF and centrifuged at 150,000 xg for 30 min. The supernatant was collected and the peptides contained in the supernatant were applied to HPLC.

Isolation of FITC-labeled peptide: Waters HPLC system was used to purify the FITC labeled peptide from the tryptic supernatant. The solvent gradient was formed by mixing 0.1 % trifluoroacetic acid in Milli-Q water (solvent A) and 0.1 % TFA containing 95 % acetonitrile (solvent B) at flow rate of 1 ml/min through Bio-Rad

Highpore^{TM} C-4 reverse phase column. Two variable wavelength spectromonitors, Waters Model 510 and ISCO V-4, were used and monitored the absorbance at indicated wavelength. Fluorescence was monitored with FS-970 Fluoromonitor with a 480 nm excitation filter and 520 nm emission filter.

Amino acid Analysis: The amino acid compositions of the peptides were determined as described by Hodgin (6). Samples were hydrolyzed in evaculated tubes for 24 hr at 110°C with constant boiling 6 N HCl.

Sequence of FITC labeled peptide: The purified peptides were sequenced on a gas phase sequenator, Applied Biosystem Protein Sequenator 470 A, and PTH-derivatives were identified by using a Specra Physics SP 8100 HPLC system with reverse-phase C-8 column and analyzed for 15 cycles. The absolute initial yield was 50 % and repetitive yields of 88-96 % were obtained.

RESULTS & DISCUSSION

Labelling of the Enzymes with FITC: Incubation of (Na,K)-ATPase of T.californica and A.salina with 10 μM FITC for 1.5 hrs at 37°C resulted in incorporation of 1.0 mol and 0.3 mol of FITC per mol of the enzyme, respectively, assuming a molecular weight of 136,000 and the respective residual activities were 19 % and 10 %. The addition of 3 mM ATP to reaction mixtures completely protected both enzymes against inactivation and the incorporation of label (data not shown). The modified enzymes were washed twice by centrifugation to remove unbound FITC. A small fraction of the washed enzymes was analyzed by gel permeation HPLC in the presence of SDS. All of the label was bound to the α subunits (Fig.1). Labeled T.californica enzyme thus obtained was used for peptide isolation but the enzyme of A.salina was further treated to improve the yield of labelling.

Since the enzyme of A.salina has higher optimum NaCl concentration than the ATPases from other sources (5), inactivation at higher NaCl concentration was tried. The partialy labeled A.salina enzyme obtained as above was suspended and incativated in the FITC solution containing 160 mM NaCl instead of 100 mM NaCl. The other conditons of labelling were

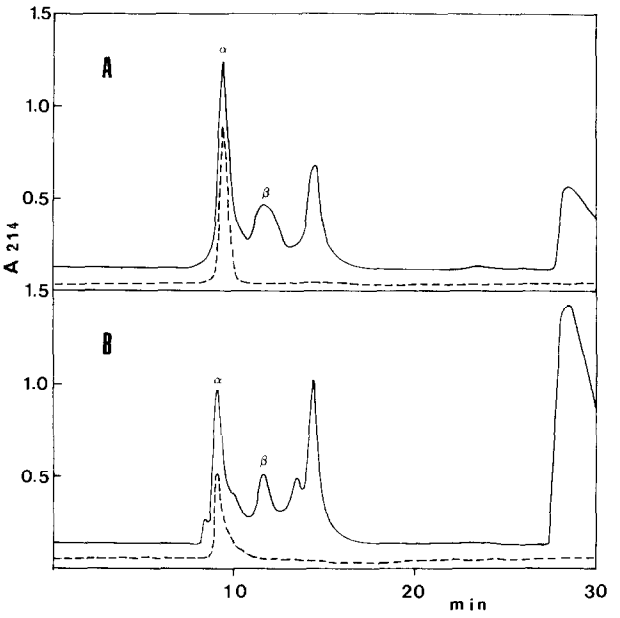

Fig. 1. High performance gel permeation chromatography on a SW 3,000 G column of fluorescein-labeled (Na,K)-ATPase. A: 50 µgs of FITC labeled enzyme of T.californica was solubilized in 1 % SDS, 50 mM Pi (pH 6.0). B: 50 µgs of FITC labeled enzyme of A.salina was solubilized in the same manner. The solublized enzymes were applied to the column equilibrated in 0.1 % SDS and 50 mM Pi (pH 6.0) and eluted at flow rate of 1 ml/min. A_{214}(——) and fluorescence (---) were monitored.

not changed. After 1.5 hrs incubation at 37°C, the enzyme was inactivated completely and 1.8 mol of label per mol of the enzyme were incorporated. The enzyme was washed twice by centrifugation and used for isolation of the labeled peptide. Gel permeation HPLC analysis of an aliquot of the washed enzyme in the presence of SDS showed that 1.0 mol and 0.2 mol of label was bound to the α and the β subunit, respectively (data not shown). The rest of label which eluted at 14.2 min is presumably phospholipid and proteins which contaminated the enzyme preparation.

Purification of labeled peptides: The procedures used to isolate pure labeled peptide from digests of both labeled enzymes were the same except the non-adsorbed fraction of A.salina enzyme was fractionated as described below. After the labeled enzymes were digested with trypsin, the solutions were subjected to

centrifugation. Approximately 50% (T.californica) and 70% (A.salina) of total absorbance at 495 nm of labeled enzyme were recovered in supernatant fractions. The water soluble peptide mixtures were applied to a reverse phase column equilibrated with 9.5 % acetonitrile in 0.1 % trifluoroacetic acid(starting solution). The absorbance at 280 nm and 440 nm and fluorescence of the effluent were monitored. No fluorescence appeared in the non-adsorbed fractions for T.californica. Some amounts of material having both 440 nm absorbance and fluorescence were recovered in the non-adsorbed fractions in the case of A.salina, however, they may be non-peptide derivatives of FITC since two fluorescent components obtained from the non-adsorbed fractions did not yield any PTH-amino acid after Edman degradation (data not shown). After the column was washed thoroughly with a starting solution a linear gradient of acetonitrile was applied. A single peak of fluorescence eluted at 31 % acetonitrile for both enzymes (Fig.2). This peak also showed absorbance at 440 nm. A peak fraction was further purified using a less steep gradient of acetonitrile (data not shown). Approximately 12 nmol and 2.2 nmol of pure labeled peptides, estimated from amino acid analysis, were obtained from 10 mg of T.californica enzyme and 8 mg of A.salina enzyme, respectively. Based on absorbance at 440 nm it was estimated that pure peptides obtained contained 34 % (T.californica) and 19 % (A.salina) of the label incorporated into the (Na,K)-ATPase.

Sequence of Labeled Peptides: Results of 11 cycles of automatic Edman degradation of the purified peptides are shown in Table 1. No peak corresponding to a PTH-derivative of an amino acid present in proteins was found at cycle 6. Only a small amount of PTH-arginine was found at cycle 11 and no PTH-amino acid was found at cycle 12(Table 1). The amino acid analysis was

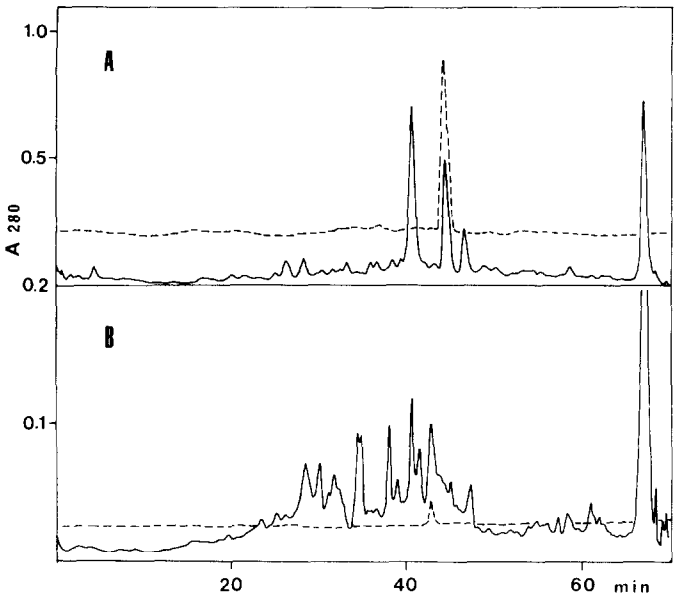

Fig.2. HPLC chromatography of the FITC-labeled Tryptic peptide from (Na,K)-ATPase. A: 10 mg of fluorescein-labeled (Na,K)-ATPase of T.Californica were digested with TPCK-trypsin, and the soluble tryptic peptides were separated on a C_4 reverse-phase column as described in EXPEREIMENTAL PROCEDURES. B: 8 mg of fluorescein-labeled (Na,K)-ATPase of A.salina were digested with TPCK-Trypsin and the FITC labeled peptide was separated as T.californica. A linear gradient from 10 to 40 % solvent B was delivered at 1 ml/min for 60 min. A_{280} (——) and fluorescence (---) were monitored.

consistent with the results of Edman degradation. Both peptides had the same amino acid composition and contained one lysine, one arginine, in addition to the other amino acids found in the

Table 1. Sequence of FITC peptides derived by tryptic cleavage of (Na,K)-ATPase

Source	Sequence
Electric ray	Tyr-Leu-Leu-Val-Met-Lys*-Gly-Ala-Pro-Glu-Arg
Brine shrimp	Tyr-Leu-Leu-Val-Met-Lys*-Gly-Ala-Pro-Glu-Arg
Dog kidney[a]	His-Leu-Leu-Val-Met-Lys*-Gly-Ala-Pro-Glu-Arg
Lamb kidney[b]	His-Leu-Leu-Val-Met-Lys*-Gly-Ala-Pro-Glu-Arg
Rat kidney[b]	His-Leu-Leu-Val-Met-Lys*-Gly-Ala-Pro-Glu-Arg

a) Sequence from Farley et al (3)
b) Sequence from Kirley et al (4)
Lys* represents FITC labeled lysine.

Table II. Automatic Edman Degradation of the FITC Peptides of (Na,K)-ATPase

Cycle	Residue	T.californica[a] (p moles)	A.salina[b] (p moles)
1	Tyr	703	255
2	Leu	1004	489
3	Leu	1078	431
4	Val	1042	268
5	Met	818	371
6	(Lys*)	-	-
7	Gly	560	123
8	Ala	813	231
9	Pro	644	244
10	Glu	420	78
11	Arg	50	+

a) 2 n moles of the FITC peptide were applied.
b) 900 p moles of the FITC peptide were applied.

Edman degradation. Combining the results of Edman degradation and amino acid analysis, and taking into account that lysine is the only amino acid to which FITC can bind in the peptide, we conclude that the sequence of the peptides containing labeled lysine, Lys*, from both enzymes is: Tyr- Leu-Leu-Val-Met-Lys*-Gly-Ala-Pro-Glu-Arg. Thus, the sequences of labeled peptides obtained from (Na,K)-ATPases of T.californica and A.salina are the same and they are very homologous to (Na,K)-ATPases from mammals (Table 2) (3,4). The only difference is that tyrosine is substituted for the histidine found in the mammalian enzymes. The sequence:Lys*-Gly-Ala-Pro-Glu is conserved in the FITC peptide obtained from the rabbit skeletal muscle Ca^{2+}-ATPase (3). It is clear from this work that the FITC binding site, which is presumably the ATP binding site of the (Na,K)-ATPase, is highly conserved. These results indicate that each amino acid residue in this sequence must be important for the function of the enzyme. It has been shown that A. salina has two isoforms of (Na,K)-ATPase (7.8). Since we could not find the FITC-peptide other than that mentioned above, the two forms may have the identical sequence around the FITC binding site.

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