Diamine Oxidase Is the Amiloride-binding Protein and Is Inhibited by Amiloride Analogues*  

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Diamine oxidase (histaminase), an enzyme that oxidatively deaminates putrescine and histamine, was purified from human placenta and from pig kidney. Both NH₂-terminal sequences are highly homologous to the human kidney amiloride-binding protein, previously thought to be a component of the amiloride-sensitive Na⁺ channel. Monoclonal antibodies raised against the pig kidney amiloride-binding protein immunoprecipitate a polypeptide with the same M₇ (105,000) as that of pig kidney diamine oxidase. That polypeptide has both diamine oxidase activity and the capacity to bind [3H]phenamil, a tritiated amiloride derivative. Cells stably transfected with human kidney amiloride-binding protein cDNA express a high diamine oxidase activity. In transfected cells as well as with the purified enzyme, this activity was inhibited by amiloride and by some of its derivatives, such as phenamil and ethypropilamylamide. Amiloride inhibition seems to be due to drug binding at the active site of the enzyme. These data indicate that human placental diamine oxidase is identical to the human kidney amiloride-binding protein and that amiloride analogues may have wider physiological effects than those on epithelial ion transport.  

Diamine oxidase catalyzes the oxidative deamination of putrescine and histamine to aminosaldehydes, hydrogen peroxide, and ammonia (1):  

\[ RCH₂NH₂ + O₂ + H₂O \rightarrow RCHO + NH₃ + H₂O₂. \]  

(Eq 3)  

Diamine oxidase activity is found in a wide range of bacteria, plants, and animals (1). Although the exact physiological functions of diamine oxidases are unknown, there are at least three theories for the mammalian enzyme. First, diamine oxidase may serve to regulate the actions of ingested or endogenous histamine on smooth muscle or other target sites (2). Second, diamine oxidase may regulate cell growth by decreasing intracellular levels of the growth-promoting diamine, putrescine (3-5). Third, the production of H₂O₂ that occurs during catalysis of polyamines by diamine oxidase may regulate cell mass during embryonic development (6-8).  

Diamine oxidase is a member of a family of amine oxidases (EC 1.4.3.6.) that are homodimers of 60-105-kDa subunits and contain tightly bound copper, and a carbonyl cofactor (9). Recent studies have shown that the carbonyl cofactor for several members of this family is TOPA, the quinone of 2,4,5-trihydroxyphenylalanine (9, 10). TOPA is thought to be created by direct modification of a tyrosine residue, and a consensus sequence, NY(D or E), has been proposed as required for the TOPA modification of a tyrosine residue (9).  

This work began with the goal of purifying human placental diamine oxidase in order to study its physiological function. Porcine kidney diamine oxidase was purified simultaneously in order to compare the two enzymes. Unexpectedly it was found that the NH₂-terminal sequence of the human placental diamine oxidase is identical to that of the human amiloride-binding protein, a protein initially proposed as being involved in epithelial sodium transport (11). Amiloride and some of its derivatives have been used to inhibit several Na⁺ transporting systems, i.e. epithelial Na⁺ channel, Na⁺/H⁺ antiporter, and Na⁺/Ca⁺ exchanger (12). That property makes amiloride and its analogues useful for in vitro and in vivo studies on the mechanism and physiology of sodium transport, and clinically, as a diuretic and antihypertensive agent (via the blocking of the Na⁺ channel). This work indicates that amiloride and its analogues inhibit diamine oxidase, suggesting that putrescine and histamine metabolism may also be inhibited during action of these agents.  

**EXPERIMENTAL PROCEDURES**  

**Purification of Porcine Kidney Diamine Oxidase**—5 g of lyophilized porcine kidney diamine oxidase (Sigma) were resuspended in 100 ml of 0.01 M Hapes-Cl, pH 7.4, 0.07 M NaCl (buffer A) and dialyzed extensively at 4 °C against the same buffer A, 5000 x g supernatant was loaded onto a column of Q-Sepharose Fast Flow (3 x 23 cm) (Pharmacia LKB Biotechnology Inc.), and active diamine oxidase fractions were eluted with a linear gradient from 0 to 250 mM NaCl. After dialysis, these fractions were loaded onto an heparin-Sepharose column (2 x 20 cm). The heparin-agarose was eluted with a linear gradient from 70 to 700 mM NaCl. Fractons containing peak diamine oxidase activity were pooled, concentrated in a Centricon 30 to a final volume of 5 ml, and stored in aliquots at -70 °C.  

**Purification of Human Placental Diamine Oxidase**—Human diamine oxidase was purified according to Baylin (13) with some modifications. After homogenization in the presence of heparin (10 units/ml), diisopropylfluorophosphate (1 mM), and EDTA (5 mM) and centrifugation, the soluble placental extract was loaded onto a cadaverine-Sepharose column (3 x 10 cm) (13). Fractions eluted with 100 units/ml heparin in buffer A were

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1 The abbreviations used are: TOPA, 2,4,5-trihydroxyphenylalanine; PAGE, polyacrylamide gel electrophoresis; Chaps, 3/3-cholamidopropyldimethylammonio]-1-propanesulfonic acid.
pooled and loaded onto a column of concanavalin A-Sepharose (1.5 x 5 cm). Fractions eluted with 0.6 M mannose were pooled, dialyzed extensively against 140 mM NaCl, 20 mM Hepes, pH 7.4 at 4 °C, and concentrated to 5 ml in a Centricon 30. This purified diamine oxidase was used to immunize a rabbit according to (14). Subsequent purifications of human placental diamine oxidase were performed by immunoaffinity chromatography using the anti-diamine oxidase IgG (3 x 10 cm) (15) and eluted with 1 M sodium thiocyanate, 20 mM Hepes, pH 7.4. The eluted diamine oxidase preparations were dialyzed extensively against 140 mM NaCl, 20 mM Hepes, pH 7.4 at 4 °C, and eventually further purified by concanavalin A-Sepharose chromatography (as above).

**Amino Acid Sequencing**—After SDS-PAGE analyses and transfer of the gel to Immobilon-P membrane (Millipore Corp., Bedford, MA) the 100–105-kDa protein bands of human placental and porcine kidney diamine oxidases were sequenced using an Applied Biosystems model 470A sequencer equipped with a "blot" cartridge and employing the O2CBLT program supplied by the manufacturer. The sequences obtained for both porcine kidney and human placental diamine oxidases are shown in Figure 1. **A**, alignment between human kidney amiloride-binding protein (HK-ABP), human placenta diamine oxidase (HP-DAO), and pig kidney diamine oxidase (PK-DAO) NH2-terminal sequences. **B**, alignment between internal sequences of human kidney amiloride-binding protein and pig kidney diamine oxidase, around the active site of the enzyme. The second X residue from the pig kidney sequence is due to the modified tyrosine; the second residue to the tryptophan. **C**, alignment between human kidney amiloride-binding protein, rat colon amiloride-binding protein (RC-ABP), Hansenula polymorpha amine oxidase (HP-AO), and lentil seedling amine oxidase (LAO). Alignment was generated with standard parameters using a clustal algorithm (32). Black boxes indicate identical residues in all four amine oxidases; dark gray boxes indicate identical residues to the human kidney amiloride-binding protein, and light gray boxes indicate similar residues to the kidney amiloride-binding protein. The consensus TOPA sequence is shown, as well as conserved cysteine or histidine residues (*), possibly involved in copper binding.
were unambiguous, and in each case only one sequence was detected. 

Enzyme Assays and Inhibition by Amiloride and Amiloride Analogs—One-ml samples of conditioned media from 293 cells transfected with human kidney amiloride-binding protein cDNA (11) were preincubated for 15 min with increasing concentrations of phenamil, ethylpropylamiloride, and amiloride at 37°C. Diamine oxidase activity was assayed using [14C]putrescine (106 pCi/mmol) as the substrate essentially as described by Okuyama and Kobayashi (16). The assay was also carried out on culture medium supplemented with 20 mM Tris/Cl buffer, pH 8.2, on cell homogenate in 20 mM Tris/Cl buffer, pH 8.2, and on pure placental diamine oxidase. The reaction was usually carried out at 37°C and terminated by the addition of 10 mM aminoguanidine. 300 μl was extracted with 3 ml of toluene containing 0.5% 2-(4-t-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole. 2 ml of the toluene phase was counted to quantify the formation of Δ¹-pyrroline, the product of the reaction. Initial rates were measured within the first 60 s of reaction.

Antibodies—2 × 50 μg of highly purified human placenta diamine oxidase were used to immunize a rabbit according to standard protocols (14).

Monoclonal antibodies were raised against pig kidney amiloride-binding protein by injection of highly purified amiloride-binding protein into mice, according to standard protocols (14). Several positive monoclonal antibodies were recovered after screening by dot blot against purified pig kidney amiloride-binding protein. These antibodies were further characterized by immunoprecipitation of [3H]phenamil binding. Partially purified amiloride-binding protein (corresponding to the first or the second step of the purification described in Ref. 17) was incubated into 200 μl of 15 mM triethanolamine/Cl, pH 7.4, 1 mM EDTA, 0.1% Chaps in the presence of 10 μM [3H]phenamil. The mixture was incubated with antibodies, precipitated with 7 μl of Pansorbin A (Calbiochem). [3H]Phenamil binding was measured after rapid filtration through Whatman GF/C glass filters soaked 2 h in 140 mM NaCl, 20 mM Tris/Cl, pH 7.4, 3% BSA. Filters were rinsed twice with 50 mM triethanolamine/Cl, pH 7.4. Nonspecific binding was measured in parallel experiments in the presence of an excess (10 μM) of unlabeled phenamil. For protein analyses and diamine oxidase measurements, amiloride-binding protein was precipitated with the same antibodies by protein A-Sepharose CL-4B (Sigma) according to Ref. 11. For diamine oxidase measurements of the precipitated protein, protein A-Sepharose beads were incubated overnight at 25°C with 1[14C]putrescine with shaking. Precipitated proteins were also analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Amino Acid Sequences of Porcine Kidney and Human Placental Diamine Oxidases—Pig kidney and human placental diamine oxidases were purified and found to have molecular weights of around 105,000 on SDS-PAGE under reducing conditions, as described previously (1). These preparations were homogenous and permitted the determination of the NH₂ terminus sequences. Fig. 1A shows the homologies observed between these sequences and the published amino-terminal sequence of the human kidney amiloride-binding protein (11). The amino-terminal sequence of the human placental diamine oxidase differs by only 1 amino acid from the human kidney amiloride-binding protein sequence. Several residues of the pig kidney diamine oxidase are conserved between this sequence and the two others, the differences observed between human and pig being probably due to species variation.

An internal peptide of the porcine kidney diamine oxidase, around the active site of the enzyme, has been sequenced previously (9). A 95% identity is observed between that sequence and the corresponding human kidney amiloride-binding protein (Fig. 1B).

The cDNA sequences coding for Hansenula polymorpha (18) and lentil seedling (19) amine oxidases have been established previously. These two proteins belong to the copper and TOPA containing amine oxidase family but have different substrate specificities from mammalian diamine oxidase. The protein sequences were compared with those of known amiloride-binding proteins, i.e. from human kidney (11) and from rat colon (20) (Fig. 1C), in order to identify important conserved residues. The overall percent similarity between human kidney amiloride-binding protein and H. polymorpha or lentil seedling amine oxidases is not very high (~15%), but potentially important residues are conserved. All sequences contain the NYD/E consensus sequence for TOPA modification. Moreover, several conserved cysteine or histidine residues (i.e. Cys²⁰⁷, Cys²⁴⁷, and His²⁵⁰) around the topaquino monoglutamate participate in the copper binding site, as reported for others copper enzymes (21). In addition, pairwise sequence comparisons revealed that the human amiloride-binding protein, lentil amine oxidase, and yeast amine oxidase have regions of 23–30% identical residues after insertions of gaps (not shown). The lengths of similarity varied from 200 residues for human and lentil, to 385 residues from human and yeast, to 570 residues for yeast and lentil. We quantified the similarity between the segments using the ALIGN program (22). The comparison scores for the human amiloride-binding protein with lentil and yeast diamine oxidases are 10.75 and 13.3 standard deviations, respectively, above that for comparison of randomized sequences of these proteins. The probability of getting a score over 10 standard deviations by chance is less than 10⁻²⁵, which indicates these proteins originated from a common ancestor.
Immunopurified Pig Kidney Amiloride-binding Protein Contains a Diamine Oxidase Activity—Several monoclonal antibodies called 348C, 452B, and 452H are able to immunoprecipitate pig kidney amiloride-binding protein. Fig. 2A shows the capacity of the antibodies to precipitate [3H]phenamil binding activity from solubilized pig kidney membranes. Fig. 2B shows that diamine oxidase activity was also precipitated by these antibodies; immunoprecipitated material was incubated with [14C]putrescine, in order to measure the diamine oxidase activity. This activity was high in samples incubated with the monoclonal antibodies 348C, 452B, and 452H, whereas no activity was measured when the material was incubated with a control monoclonal antibody. SDS-PAGE analysis of the immunoprecipitated material demonstrates the presence of a 105-kDa polypeptide under reducing conditions, corresponding to the pig kidney amiloride-binding protein, after precipitation by 348C, 452B, and 452H. No such band was detected in the control (Fig. 2C). Taken together, these results show that the 105-kDa protein from pig kidney has both a [3H]phenamil binding site and diamine oxidase activity.

Transfected Cells Expressing the Human Amiloride-binding Protein Contain an Amiloride-sensitive Diamine Oxidase Activity—Fig. 3 shows the presence of high diamine oxidase activity in the culture medium from transfected 293 cells expressing the human amiloride-binding protein, whereas no activity was detected in control nontransfected 293 cells. Cells transfected with amiloride-binding protein express binding sites for [3H]phenamil that also recognize benzamil, ethylpropylamiloride, and amiloride (11, 20). The [3H]phenamil binding was characterized in cell membranes (11), but it is also present in conditioned culture medium (not shown). A high diamine oxidase activity was actually measured in both fractions. For convenience, subsequent work was carried out with the activity which is present in the conditioned culture medium. Enzyme activity was characterized by measuring the initial rates of Δ1-pyrroline formation at increasing concentrations of putrescine (Fig. 4A). In conditioned culture medium, the K_m for putrescine was 8 μM. No activity was measured with control nontransfected cells (not shown). Amiloride (Fig. 4A) or phenamil (not shown) behaved as competitive inhibitors of putrescine degradation. A truncated form of the amiloride-binding protein, corresponding to the COOH terminus part of the protein is expressed in some rat tissues such as lung or spleen (20). This truncated protein begins at Met534 and binds amiloride and its derivatives, although with lower affinities than the long form (20). It is thus possible that a conserved negatively charged hydrophobic sequence (LVAW) might participate to the amiloride binding site, as reported previously for the Na+/H+ antiporter (23). Fig. 4B shows the inhibition of diamine oxidase activity by increasing concentrations of phenamil (K_i = 47 μM), ethylpropylamiloride (K_i = 1.75 μM), and amiloride (K_i = 5.1 μM). Equivalent inhibition by amiloride analogues is seen when pure placental diamine oxidase is substituted for the conditioned medium from transfected cells (not shown).

Biological Implications—All these data taken together show that the mammalian amiloride-binding protein is a diamine oxidase and is most probably not involved in epithelial sodium transport. Previous work by Goldstein et al. (24) also supports the conclusion that the amiloride-binding protein is not the epithelial sodium channel. This interpretation is strengthened by the recent cloning of a rat colon Na^+ channel cDNA, corresponding to a protein of 76 kDa. Injection of the corresponding complementary RNA into oocytes leads to expression of an amiloride-sensitive Na^+ channel (25, 26). A multiprotein complex which binds amiloride and its derivatives has been purified from amphibian kidney A6 cells and proposed to be associated with Na^+ channel activity (27). We have found that cultured A6 cells also contain a small amiloride-sensitive diamine oxidase activity (not shown).

Amiloride-binding protein was initially purified from pig kidney membranes (17), and evidence demonstrates that diamine oxidase is also associated with the cell surface. Diamine oxidase binds to the endothelial cell surface by attachment to glycosaminoglycans (28) and is released into the flowing blood in vivo following the intravenous injection of heparin (29). The attachment of amiloride-binding protein/diamine oxidase to glycosaminoglycans may be mediated by a consensus heparin binding domain (30), RFKRLPK, located between amino acids 569 and 576.

These results first suggest that amiloride and its analogues might be useful tools for the study of polyamine metabolism. They also raise several questions: do clinically used doses of amiloride inhibit diamine oxidase in vivo? If so, could some of the antihypertensive effects of amiloride be due to inhibition of diamine oxidase and elevated levels of histamine (2)? Does amiloride therapy make patients more sensitive to the effects of ingested histamine? Are adverse drug reactions observed in the case of amiloride treatment (31), such as pruritus or drug rash, due to diamine oxidase inhibition?

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REFERENCES
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