Characterization and Purification of a Mammalian Osmoregulatory Protein, Aldose Reductase, Induced in Renal Medullary Cells by High Extracellular NaCl*

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GRB-PAP1 is a continuous line of epithelial cells derived from a rabbit renal inner medulla. Elevation of the NaCl concentration in the medium bathing these cells strongly induced the expression of a soluble protein with an apparent molecular mass of 39 kDa. The protein, purified by affinity chromatography with Amicon Maxisep Gel Orange A, had enzyme activity characteristic of aldose reductase (alditol:NADPH+ oxidoreductase, EC 1.1.1.21). Goat antiserum against this purified aldose reductase selected the 39-kDa band from immunoblots of cells grown in a medium containing high NaCl. When the osmolality of the medium was increased by adding NaCl, the amount of aldose reductase protein and the aldose reductase activity increased together from very low to sustained high levels over several days. The aldose reductase protein was more than 10% of the soluble cell protein when cells were propagated in medium made hyperosmotic by adding NaCl to increase medium osmolality to 600 mosm·kg⁻¹.

Osmoregulation is a fundamental and widespread attribute of cells. In bacteria and plants a number of proteins have been identified whose expression depends on the osmolality of the medium (Comeaw et al., 1985; Epstein, 1978; Singh et al., 1987), but such osmoregulatory proteins had not previously been identified in animals. The osmoregulatory proteins identified in bacteria were the porins (Comeaw et al., 1985), potassium transporters (Epstein, 1978), and regulators of their expression (Comeaw et al., 1985; Epstein, 1978). No function was reported for the 26-kDa protein induced in plants by high NaCl (Singh et al., 1987).

We report here on the characterization, purification, and analysis of an osmoregulatory protein in rabbit renal medullary cells in tissue culture. The protein, aldose reductase, was strongly induced by elevation of extracellular NaCl. Aldose reductase (alditol:NADPH+ oxidoreductase, EC 1.1.1.21) catalyzes the conversion of glucose to sorbitol. Sorbitol accumulates in renal medullary cells exposed to high extracellular NaCl both in vivo (Bagnasco et al., 1986a) and in tissue culture (Bagnasco et al., 1987). The sorbitol is a non-perturbing "osmolyte" (Yancey et al., 1982) which helps maintain cell volume and intracellular milieu during hyperosmotic stress.

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EXPERIMENTAL PROCEDURES

Cells—GRB-PAP1 is a line of epithelial cells, derived from the surface of the rabbit renal papilla and grown in an isosmotic medium (200 mosm kg⁻¹) with a normal NaCl concentration past 7 years and 70 passages (Uchida et al., 1986). The medium contained 90% Coon's modification of Ham's F-12 with 10% Liebowitz's L-15, selenium (10 ng/ml), transferrin (5 μg/ml), progesterone (2 μg/ml), hydrocortisone (50 ng/ml), penicillin (100 units·ml⁻¹), streptomycin (100 μg·ml⁻¹), 1% heat-inactivated calf serum and was adjusted to 300 mosmol·kg⁻¹ (isosmotic) or 600 mosmol·kg⁻¹ (hyperosmotic) with NaCl. The PAP-HT25 strain of these cells resulted from switching GRB-PAP1 to hyperosmotic medium. The PAP-HT25 strain has grown in hyperosmotic medium for more than 3 years and 45 passages since it was derived from passage 25 of GRB-PAP1. The cultured cells were studied at passages 70-80.

Chemicals—All chemicals used were of reagent grade unless otherwise stated. Dimethyl sulfoxide (Spectragrade) was obtained from Mallinckrodt Inc.; N-potassium phosphate (Aldrich) was recrystallized from ethylene glycol; NADPH (Type 1) was obtained from Sigma or Boehringer Mannheim; DL-glyceraldehyde, D-xylulose, D-gulonate, and L-glucuronic acid was from Sigma; L-gulonate was prepared from L-gulotic acid (Yoshitake et al., 1961); and valproic acid was obtained from Schar Labs, Morton Grove, IL.

Enzyme Analysis—Aldose reductase activity was determined spectrophotometrically by monitoring the decrease in NADPH absorbance at 340 nm using DL-glyceraldehyde as a substrate (Hermann et al., 1985). Results were expressed as a change in optical density per min per mg of protein. Kinetic analyses were made on the PROPHET computer system using the BINKIN2 public procedure as previously described (Kador et al., 1981b).

Protein Determination—Protein determinations were made using the microassay of Bradford (Bradford, 1976) with bovine γ-globulin (Bio-Rad) as a standard.

Enzyme Purification—Culture media were removed from confluent cultures, containing approximately 2 × 10⁶ cells/15-cm Petri dish, by washing four times at room temperature with a hyperosmotic (600 mosmol kg⁻¹) phosphate-buffered saline (302 mM Na, 9.6 mM phosphate, pH 7.4). The collected cells were trypsinized at 20-30 ml of phosphate-buffered saline and centrifuged at 800-1000 g for 5 min. The supernatant was removed, and the cell pellet was frozen (−20°C) until required.

Frozen pellets (0.5 g, 3 × 10⁶ cells) were thawed, suspended in 10 ml of 20 mM sodium potassium phosphate buffer, pH 7.5, containing 0.05% glycerol, 0.025 mM EDTA, and 5 mM mercaptanethanol, homogenized at 4°C in a Kontes Dounce glass tissue homogenizer (20-30 strokes using a tight-fitting pestle), and centrifuged at 12,300 × g for 10 min. The supernatant was discarded, and the aliquot of the supernatant retained for protein determination and measurement of the activity of aldose reductase.

The supernatant was placed on an Econo column (2.5 × 20 cm) (Bio-Rad) containing 80-90 ml of Amicon Maxisep Gel Orange A (Amicon Inc., Danvers, MA) previously equilibrated with 1 liter of the same buffer, pH 7.5 (Shiono et al., 1986). The column was washed with 600 ml of the buffer, and column eluant was collected in 15-ml fractions. 450 ml of a 0.1 mM NADPH solution in 20 mM sodium potassium phosphate buffer, pH 7.5, was then used to elute the
enzyme from the column. Finally the column was washed with 1 M KC1 in 20 mM potassium phosphate buffer, pH 7.5, to remove any remaining protein from the column. Both protein concentration and aldose reductase activity (using 100-μl samples) were monitored in all the fractions.

Fractions with aldose reductase activity were combined and concentrated on an Amicon CEC-1 column eluant concentrator with a YM-10 filter. The concentrated samples were then heated for 2 min at 100°C with buffer (60 mM Tris/HCl, pH 6.5, 10% SDS, 12% glycerol, 1.2% mercaptoethanol, 0.006% bromphenol blue) in the ratio of 1:3 (sample:buffer). After electrophoresis, the gels were fixed in methanol/water/acetic acid (8:9:1), stained with either Coomassie Brilliant Blue R-250 (Schwarz/Mann) or silver (Bio-Rad silver stain kit) (Singh et al., 1987). The Coomassie Brilliant Blue R-250 was applied in the fixation solution, and gels were destained in the same solution with the proportions of 10:10:1. Molecular weights were estimated from the mobilities of the following standard proteins (Pharmacia Biotechnology Inc.): phosphorylase b, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400.

Antibodies were partially purified by precipitation with 27% ammonium sulfate (enzyme grade, Schwarz/Mann) followed by DEAE-cellulose (Bio-Rad) chromatography.

SDS-Polyacrylamide Gel Electrophoresis—Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Laemmli, 1970) of the protein-containing samples was performed using a 12% separating gel with a monomer/bis ratio of 30:0.8 (w/w). Samples were concentrated and desalted by centrifugation in Amicon Centricon 10 filters, with an exclusion limit of M, = 12,000. The concentrated samples were then heated for 2 min at 100°C with buffer (60 mM Tris/HCl, pH 6.5, 10% SDS, 12% glycerol, 1.2% mercaptoethanol, 0.006% bromphenol blue) in the ratio of 1:3 (sample:buffer). After electrophoresis, the gels were fixed in methanol/water/acetic acid (8:9:1), stained with either Coomassie Brilliant Blue R-250 (Schwarz/Mann) or silver (Bio-Rad silver stain kit) (Singh et al., 1987). The Coomassie Brilliant Blue R-250 was applied in the fixation solution, and gels were destained in the same solution with the proportions of 10:10:1. Molecular weights were estimated from the mobilities of the following standard proteins (Pharmacia Biotechnology Inc.): phosphorylase b, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400.

Antibody Production—Antiserum against the purified aldose reductase from PAP-HT25 cells was produced in a goat. After preimmune serum was obtained, the goat at 2 weekly intervals was given 3 intradermal injections, each containing approximately 100 μg of the purified fraction mixed with an equal volume of complete Freund’s adjuvant (Miles Scientific, Naperville, IL). Reactivity of the antisera was determined with immunoblotting techniques (see below) and with Ouchterlony plates.

Purification of Aldose Reductase Activity from PAP-HT25 Cells by Affinity Chromatography—The supernatant from crude homogenates of PAP-HT25 cells was applied to the affinity column after centrifugation at 12,300 × g, and the NADPH-dependent enzyme was removed from the Matrix Gel Orange A by elution with 0.1 mM NADPH (Fig. 4) to give

RESULTS

SDS-Polyacrylamide Gel Electrophoresis of Soluble Proteins of Cells Grown under Different Osmotic Conditions—SDS-polyacrylamide gel electrophoresis of the soluble protein fractions from supernatants of crude homogenates of cells grown in hyperosmotic (600 mosm kg⁻¹) or isosmotic (300 mosm kg⁻¹) medium are shown in Fig. 1. A conspicuous band corresponding to a molecular mass of about 39 kDa was present in the preparation from hyperosmotic medium (Fig. 1, left lane), but this band appeared much reduced in the cells grown in isosmotic medium (Fig. 1, right lane). This 39-kDa protein band was only observed in the soluble protein fraction. When PAP-HT25 cells, grown in an isosmotic medium were switched to a hyperosmotic medium, the 39-kDa band was observed to increase between 1 and 5 days (Fig. 2). Likewise when these same cells (PAP-HT25) were grown in a hyperosmotic medium and then placed in a medium where the osmolality was decreased to 300 mosm kg⁻¹, there was a decrease in the staining intensity of this band (data not shown).

Moreover this protein co-migrated with aldose reductase purified from rat lens (Fig. 3). These observations correlate with previously noted changes in intracellular sorbitol concentration and aldose reductase activity under similar conditions (Bagnasco et al., 1987). We, therefore, investigated the possibility that this 39-kDa protein band might be aldose reductase.

FIG. 1. SDS-polyacrylamide gel electrophoresis of 75 μg of soluble protein from PAP-HT25 cells grown in medium with high NaCl (600 mosm kg⁻¹) (left) and 120 μg of soluble protein from PAP-HT25 cells grown in isosmotic medium (300 mosm kg⁻¹) (right). An arrow marks the conspicuous band later shown to be aldose reductase (AR). SDS-polyacrylamide gel electrophoresis was carried out as described under “Experimental Procedures.” Standard molecular weights are indicated with arrows.

FIG. 2. SDS-polyacrylamide gel electrophoresis pattern of soluble proteins from PAP-HT25 cells grown in an isosmotic medium (300 mosm kg⁻¹) and after switching to hyperosmotic medium (600 mosm kg⁻¹). Cells were monitored for up to 2 weeks. The medium was made hyperosmotic with the addition of NaCl. Equal amounts of protein (100 μg) were loaded onto the gel. An arrow marks the conspicuous band later shown to be aldose reductase (AR).
an 84% recovery of aldose reductase activity (n = 4) (Table I) with an 8.1-fold increase in specific activity. The purified enzyme appeared as a single band on SDS-polyacrylamide gel electrophoresis with an apparent molecular mass of 39 kDa (range 37–40 kDa) (Fig. 3). Aldose reductase protein represented more than 10% of the soluble cell protein and had a specific activity of 543 nmol min⁻¹ mg⁻¹ (Table I). Activity of the purified enzyme appeared relatively stable in 20 mM sodium potassium phosphate buffer, pH 7.5, containing 10% glycerol, with two-thirds of the initial activity remaining after 6 weeks of storage at 4°C; however, all activity of the purified enzyme was destroyed upon freezing.

**Inhibitors and Substrate Preferences of the Purified Enzyme**—The substrate profile of the purified enzyme is similar to that of aldose reductases isolated from rat lens (Hermann et al., 1983) and the human placenta (Kador et al., 1981a) (Table II). In general, the enzyme preferred aromatic or aliphatic aldehydes to the aldose sugars. Substrate specificity, as shown by increasing $K_m$, was (millimolar) $p$-nitrobenzaldehyde (0.0007) > glyceraldehyde (0.054) > glucurionate (4.9) > xylose (8.1) > galactose (50.8) > glucose (285.0). No activity was detected with galonic acid.

With 10 mM DL-glyceraldehyde as a substrate enzyme activity was stimulated by the addition of sulfate ions (0.2 M LiSO₄) and inhibited by chloride ions (0.1 M NaCl) (Table II). The purified enzyme was also inhibited by the aldose reductase inhibitors Statil (3-(4-bromo-2-fluorobenzyl)-4-oxo-3H-phthalazine-1-yl-acetic), tolrestat (N-(5-(trifluoromethyl)-6-methoxy-1-naphthalenylthioxomethyl)-N-methylglycine), sorbinil (S-6-fluoro-spirochroman-4-4'-imidazolidine-2',5'-dione), and less so by chrome (7-hydroxychromone-2-carboxylic acid) (Table III). Only slight inhibition was observed with valproic acid (Table III). Similar observations have been reported with other aldose reductases.

![Fig. 3. Separation and identification of aldose reductase by SDS-polyacrylamide gel electrophoresis stained with silver. A, pure aldose reductase (20 μg of protein) from PAP-HT25 cells grown in a hypotonic medium deliberately overloaded; B, soluble proteins (100 μg of protein) from PAP-HT25 cells grown in a hypotonic medium (600 mosm kg⁻¹); C, aldose reductase (5 μg of protein) from rat lens. Molecular weight standards are identified by arrows.](image)

![Fig. 4. Elution pattern from Amicon Orange A Matrex Gel column eluted with 20 mM sodium potassium phosphate buffer, pH 7.5, with 10% glycerol (v/v) and 5 mM mercaptoethanol. Solid lines, protein (mg/ml); dotted lines, aldose reductase activity with 10 mM DL-glyceraldehyde as a substrate expressed as absorbance units min⁻¹ ml⁻¹. At fraction 40, pH 7.5, phosphate buffer containing 0.1 mM NADPH was added, and at fraction 70, 1 mM KCl in the same buffer but without NADPH, was added. Each fraction was 15 ml. No further protein was seen with the KCl elution.](image)

![TABLE I](image)

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<th>Fraction</th>
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<td>Total Specific Activity</td>
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![TABLE II](image)

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<td>No activity at 0.2 M</td>
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* ND, not determined.

![TABLE III](image)

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<td>Tolrestat</td>
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<td>Chromone</td>
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<tr>
<td>Valproic acid</td>
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</table>
were similar to results previously obtained using crude homogenates of these cells grown under the same conditions (Bagnasco et al., 1986b, 1987).

Antibody Production—Goat antiserum against the fraction prepared from PAP-HT25 cells and eluted by NADPH from the affinity column specifically reacted with a 39-kDa band on Western blots prepared both from homogenates of PAP-HT25 cells grown in hyperosmotic medium and from the purified protein (Fig. 5). Slight nonspecific binding with the preimmune serum was observed. However, this did not correspond to the band detected with the antibody (Fig. 5). On Ouchterlony plates the antiserum formed a single line of identity with either the aldose reductase purified from the PAP-HT25 cells or with supernatants of the crude tissue homogenate of these cells (Fig. 6). A faint, partial line of identity was obtained with aldose reductase from rat lens but no reaction was observed with aldehyde reductase purified from rat kidney (Fig. 6).

Time Course of Induction of Aldose Reductase—PAP-HT25 cells that had been growing in isosmotic medium were switched to hyperosmotic medium. Changes in Western blots probed with antibody against the 39-kDa protein and in aldose reductase activity were determined over several days (Fig. 7). The levels of immunoreactive protein and enzyme activity increased in parallel, reaching a maximum after 4 days. This correlation supports both the identity of the immunoreactive 39-kDa protein as aldose reductase and the conclusion that increased enzyme protein accounts for the increase in aldose reductase activity.

DISCUSSION

Previously, cultured epithelial cells, derived from the papilla of a rabbit kidney (PAP-HT25) (Uchida et al., 1986), were found to accumulate large amounts of sorbitol (Bagnasco et al., 1987) when grown in a medium made hyperosmotic (600 mosm kg\(^{-1}\)) by the addition of NaCl. However, when these same cells were grown in an isosmotic medium (300 mosm kg\(^{-1}\)) only trace amounts of sorbitol were detected. The increase in intracellular sorbitol followed induction of aldose reductase activity.

Identification of Aldose Reductase Protein—The principal protein band seen by SDS-polyacrylamide gel electrophoresis of the soluble fraction of the PAP-HT25 cells grown in medium with high NaCl was eluted by affinity chromatography. This resulted in the purification of the protein in one step to apparent homogeneity as determined by SDS-polyacrylamide gel electrophoresis. The kidney enzyme is similar with respect to its kinetic and chemical properties, although not identical to, aldose reductases extracted from other tissue such as rat lens (Hermann et al., 1983) and human placenta (Kador et al., 1981a). It has similar substrate preferences, although it does not show as high a preference for p-nitrobenzaldehyde as those aldose reductases extracted from rat lens (Hermann et al., 1983) and human placenta (Kador et al., 1981a). Aldose reductase from PAP-HT25 cells was inhibited by Statil, tolrestat, sorbinil, and 7-hydroxychromone-2-carboxylic acid (Table III), similar to the results with other aldose reductases (Kador et al., 1985). On the other hand,
antibodies against aldose reductase from PAP-HT25 cells formed only a partial line of identity to rat lens aldose reductase compared to the very strong line with PAP-HT25 aldose reductase itself (Fig. 6), indicating that the two enzymes are not immunologically identical.

Further proof that the purified enzyme is aldose reductase comes from experiments that indicated gulonate was a poor substrate (Table II). Similarly, this enzyme was not significantly inhibited by 1 mM valproic acid (Towbin et al., 1979) and the antibody raised against this purified enzyme also failed to react with rat kidney aldehyde reductase (Fig. 6).

Increased Expression of Aldose Reductase following Elevation of Extracellular NaCl—Previously, aldose reductase activity was observed to increase in PAP-HT25 rabbit renal inner medullary cells exposed to elevated extracellular NaCl (Bagnasco et al., 1987). In the present studies the amount of aldose reductase protein in the cells also increased under similar conditions. The increase in the amount of the enzyme protein is correlated with and apparently accounts for the increase in enzyme activity following elevation of extracellular NaCl (Fig. 7).

Although the mechanisms that control the expression of aldose reductase remain unknown, there is a clear rationale for the changes in its expression in PAP-HT25 cells (and presumably also in renal inner medullae). Intracellular osmolality generally is equal to that of the extracellular fluid. Changes in external osmolality therefore must be followed by compensating changes in the cellular solutes if cell volume is to be maintained. Solutes which may serve this function in the intact renal inner medulla include glycerolphosphorylcholine, betaine, inositol, and sorbitol, all of which were identified from the inner medulla of a rabbit kidney. Therefore, the changes in aldose reductase and in intracellular sorbitol that they express in response to elevated NaCl may reflect mechanisms normally at work in the renal medulla. This is supported by identification of aldose reductase (Corder et al., 1977) in renal inner medullae.

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REFERENCES