Properties

Homogeneity. The purified enzyme with a specific activity of 849 units/mg is homogeneous by ultracentrifugation, by disc gel electrophoresis, by sodium dodecyl sulfate gel electrophoresis, and by gel isoelectric focusing.

Stability and pH Stability. The purified enzyme is stored at −20° in 0.1 *M* phosphate buffer, pH 6.5–7.6, or in sodium acetate buffers, pH 5.0–5.6, containing enough glycerol (30–50%) to prevent freezing.[7] No decrease in enzyme activity could be observed within 18 months.

The enzyme is stable in the range from pH 5.0–9.0.[7]

Structure. D-Galactose dehydrogenase from *P. fluorescens* has a molecular weight of 64,000. The enzyme is composed of two identical subunits and has two binding sites.[7,10]

For a further description of the properties of D-galactose dehydrogenase from *P. fluorescens,* see Blachnitzky *et al.*[7], for its application for the determination of D-galactose, see Kurz and Wallenfels.[11,12]

[10] E. Maier and G. Kurz, unpublished observation.

[11] G. Kurz and K. Wallenfels, *in* "Methoden der enzymatischen Analyse" (H. U. Bergmeyer, ed.), 3rd German ed., p. 1324. Verlag Chemie, Weinheim/Bergstr., F.R.G., 1974.

[12] G. Kurz and K. Wallenfels, *in* "Methoden der enzymatischen Analyse" (H. U. Bergmeyer, ed.), 3rd German ed., p. 1225. Verlag Chemie, Weinheim/Bergstr., F.R.G., 1974.

[30] Aldose Reductase from Human Tissues

By BENDICHT WERMUTH and JEAN-PIERRE VON WARTBURG

$$R - CHO + NADPH + H^+ \rightleftarrows R - CH_2OH + NADP^+$$

Aldose reductase (alditol : $NADP^+$ 1-oxidoreductase, EC 1.1.1.21) and polyol dehydrogenase (L-iditol : NAD^+ 5-oxidoreductase, EC 1.1.1.14) constitute the sorbitol (polyol) pathway converting glucose to fructose in extrahepatic tissues. In addition to glucose, aldose reductase catalyzes the reduction of other sugar aldehydes and of several aliphatic and aromatic aldehydes. Another enzyme, aldehyde reductase[1] (alcohol : NADP oxidoreductase, EC 1.1.1.2), shows a similar substrate specificity. The two enzymes are distinguishable by relative substrate specificities and inhibitor sensitivities, by column chromatography, and immunologically.[2]

[1] J. P. von Wartburg and B. Wermuth, this volume [85].

[2] P. L. Hoffman, B. Wermuth, and J. P. von Wartburg, *J. Neurochem.* **35**, 354 (1980).

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Assay Method

Principle. Aldose reductase is assayed spectrophotometrically by recording the decrease in NADPH absorbance at 340 nm. With crude enzyme solutions, blank reactions may occur with NADPH in the absence of any aldehyde substrate; consequently the rate of this reaction must be recorded before addition of exogenous aldehyde. Depending on the substrate and on the tissue analyzed, other dehydrogenases, notably aldehyde reductase, may interfere. Interference with aldehyde reductase is diminished by the addition of diphenylhydantoin or phenobarbitone to the assay medium. Moreover, the ratio of activities measured with D-xylose, DL-glyceraldehyde, and D-glucuronate may be used to distinguish between the two enzymes.[2]

Reagents

Sodium phosphate buffer, 0.1 *M*, pH 7.0
NADPH, 1.6 m*M*; made up daily in deionized water and kept on ice
D-Xylose, 1 *M*
DL-Glyceraldehyde, 0.1 *M*, dedimerized at 85° for 10 min
D-Glucuronate, 0.1 *M*
Diphenylhydantoin, 0.01 *M*, dissolved in 0.01 *M* NaOH

Procedure. To a 1-ml cuvette add 730 μl of assay buffer, 50 μl of NADPH, 20 μl of diphenylhydantoin (which inhibits aldehyde reductase by approximately 90%), 5–100 μl of enzyme solution, and deionized water to a total volume of 0.9 ml. Place cuvette in photometer and record blank reaction if necessary. Start enzyme reaction by the addition of 100 μl of substrate solution. If the absence of aldehyde reductase has been ascertained, diphenylhydantoin can be omitted.

Definition of Enzyme Unit and Specific Activity. One unit of enzyme is defined as the amount of protein catalyzing the oxidation of 1 μmol of NADPH per minute. The specific activity is expressed as milliunits of enzyme per milligram of protein. The concentration of protein is estimated from the absorbance at 260 and 280 nm.[3]

Preparation of Cibacron Blue-Sepharose. Sepharose containing a spacer arm with a free carboxyl group is formed by covalent linkage of 6-aminohexanoic acid to Sepharose 4B[4] using the cyanogen bromide method.[5] One liter of the derived Sepharose is suspended in an equal volume of water, and 4 g each of Cibacron Blue F3 G-A and *N*-cy-

[3] E. Layne, this series, Vol. 3 [73].
[4] Sepharose 4B containing covalently linked 6-aminohexanoic acid is commercially available from Pharmacia, Uppsala, Sweden.
[5] P. Cuatrecasas and C. B. Anfinsen, this series, Vol. 22 [31].

clohexyl-*N'*-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate are added. The pH is adjusted to and maintained at 5 by the addition of NaOH or HCl, and the slurry is agitated for 20 hr. The Sepharose is then thoroughly washed on a sintered-glass funnel with 0.1 *N* NaOH, 0.1 *N* HCl, 2 *M* NaCl, and H_2O until the dye is undetectable in the effluent. Upon coupling, the dye changes its color from blue to purple.

Purification Procedure

A general procedure has been worked out in our laboratory to isolate and purify carbonyl reducing enzymes from human tissues, and the preparation of brain aldose reductase will be described in detail. Brains, either whole or without cortex and cerebellum, were obtained from legal medical autopsies. The brains were frozen 6–20 hr postmortem and stored at −20°. The whole purification is carried out at 4°, and all buffers contain 50 μM EDTA and 0.5 m*M* 2-mercaptoethanol to stabilize the enzyme and 0.02% sodium azide to prevent bacterial growth.

Step 1. Extraction. Approximately 300 g of brain are homogenized with an equal volume (v/w) of 0.1 *M* sodium phosphate buffer, pH 7.4, in a Waring blender for 2–3 min. The homogenate is centrifuged for 2 hr at 45,000 *g*. The precipitate is reextracted with the same volume of buffer and recentrifuged. The precipitate is discarded. The pooled supernatants are dialyzed for 24 hr against three changes of 10 liters of 5 m*M* sodium phosphate buffer, pH 7.4, and centrifuged for 1 hr at 23,000 *g*.

Step 2. DEAE-Cellulose Chromatography. The supernatant fluid is applied to a column (2 × 40 cm) of DEAE-cellulose equilibrated with dialysis buffer. The column is washed with 500 ml of the same buffer before a linear gradient (2 × 300 ml) of 5 to 100 m*M* sodium phosphate buffer, pH 7.4, is applied. Aldose reductase activity emerges from the column in two fractions immediately before and after aldehyde reductase (Fig. 1). Both fractions are combined for further purification.

Step 3. Gel Filtration. The pooled enzyme (maximum 150 ml/run) is applied to a column (4 × 180 cm) of Sephadex G-100 equilibrated with 10 m*M* sodium phosphate buffer, pH 6.2. The column is washed at a flow rate of 90 ml/hr, and tubes containing aldose reductase activity are pooled.

Step 4. Affinity Chromatography. The pool of aldose reductase activity is applied to a column (1 × 25 cm) of Cibacron Blue-Sepharose equilibrated with 10 m*M* sodium phosphate buffer, pH 6.2. The column is washed with 50 ml of 100 m*M* sodium phosphate buffer, pH 6.2; elution of aldose reductase activity is accomplished by a linear gradient (2 × 12 ml) of 0 to 1 m*M* NADPH in the original column buffer. Fractions containing aldose

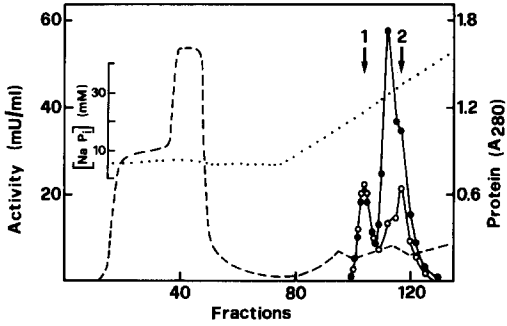

FIG. 1. Elution pattern of human brain aldose and aldehyde reductase activities from DEAE-cellulose. Brain extract was applied to a column equilibrated with 5 m*M* sodium phosphate, pH 7.4. Enzyme activities were eluted with a linear gradient of 5 to 100 m*M* phosphate buffer (· · · ·). Protein (---) was recorded with a Uvicord II spectrophotometer. Activity was assayed using either 0.1 *M* xylose (○——○) or 0.01 *M* DL-glyceraldehyde (●——●) as substrate. No diphenylhydantoin was included in the assay mixture. The elution positions of aldose reductase activity are indicated by arrows.

reductase activity are pooled, dialyzed against 10 m*M* sodium phosphate buffer, pH 7.0, and stored at 4°.

Table I summarizes typical purification data.

Alternative Procedure. An alternative purification procedure[6] including treatment with calcium phosphate gel, ammonium sulfate precipitation, chromatography over DEAE-cellulose and Sephadex G-100, and isoelectric focusing has been described for the enzyme from placenta. The purified enzyme had a specific activity of 1.68 units per milligram of protein with 400 m*M* D-xylose and 0.2 m*M* NADPH as substrates at pH 6.2.

Properties

Purity and Stability. The outlined purification procedure yields a homogeneous enzyme that is stable for several days in the pH range 6–9 at 4°. The activity rapidly decreases below pH 5.0.

Molecular Forms. DEAE-cellulose chromatography of brain extract yields two forms of aldose reductase. Polyacrylamide gel electrophoresis of the purified enzyme forms in the presence and absence of 2-mercaptoethanol indicates that they exist in a thiol-dependent equilibrium. The mechanism of the conversion, however, is not clear.

Physicochemical Properties. Aldose reductase consists of a single polypeptide chain with a molecular weight of 35,000–40,000. The isoelectric point has been reported to be 5.8–6.0.[2,6] The amino acid composition

[6] R. S. Clements, Jr. and A. I. Winegrad, *Biochem. Biophys. Res. Commun.* **47**, 1473 (1972).

TABLE I
PURIFICATION OF HUMAN BRAIN ALDOSE REDUCTASE

Fraction	Activity[a] (μmol min^{-1})	Protein (mg)	Specific activity (nmol min^{-1} mg^{-1})	Recovery (%)
Extract	7.25	5878	1.2	100
DEAE-cellulose	3.00	210	14	41
Sephadex G-100	2.70	25	108	37
Cibacron Blue-Sepharose	1.52	3.2[b]	475	21

[a] Activity was measured using D-xylose (0.1 *M*) as substrate.
[b] The enzyme contains tightly bound material absorbing at 260 nm that may interfere with the protein determination.

is shown in comparison with aldehyde reductase in Table III of this volume [85].

pH Optimum. The optimum pH for aldehyde reduction is between 6 and 7.

Substrate Specificity. Aldose reductase catalyzes the reduction of a number of physiological and xenobiotic aldehydes (Table II). Glycol- and polyolaldehydes are preferred substrates. The Michaelis constants for aldoses increase with increasing chain length, whereas the maximal rate of reduction is less dependent on the size of the substrate. Isocorticosteroids, intermediates in an alternative pathway of corticosteroid metabolism, in which the 17-ketol is replaced by a 17-aldol side chain, are the most efficiently reduced substrates known to date. The 20 α- and β-epimers are reduced at comparable rates. The reduction of biogenic aldehydes, derived from the biogenic amines by oxidative deamination, is little investigated but may be of some importance in view of the finding that in rat brain aldose reductase is responsible for the reduction of these aldehydes.[7]

The reverse reaction, oxidation of polyols to polyolaldehydes by $NADP^+$, proceeds very slowly. It can be used to detect reductase activity in polyacrylamide gels.[8]

Aldose reductase is specific for NADPH as coenzyme. With NADH (0.12 m*M*) about 10% of the NADPH-dependent activity is obtained. During catalysis the pro-4R hydrogen atom is transferred from the nicotinamide ring of the coenzyme to the substrate.[2,9]

[7] S. R. Whittle and A. J. Turner, *Biochim. Biophys. Acta* **657**, 94 (1981).
[8] M. M. O'Brien and P. J. Schofield, *Biochem. J.* **187**, 21 (1980).
[9] H. B. Feldman, P. A. Szczepanik, P. Havre, R. J. M. Corrall, L. C. Yu, H. M. Rodman, B. A. Rosner, P. D. Klein, and B. R. Landau, *Biochim. Biophys. Acta* **480**, 14 (1977).

TABLE II
SUBSTRATE SPECIFICITY OF HUMAN BRAIN ALDOSE REDUCTASE

Substrate	Concentration (m*M*)	Michaelis constant (m*M*)	Relative velocity (%)
DL-Glyceraldehyde	∞	0.06	100
D-Xylose	∞	16	78
D-Glucose	∞	90	45
D-Galactose	∞	110	45
D-Glucuronate	∞	4	75
11-Deoxyisocorticosterone[a]	∞	<0.001	150
Isocortisol[a]	∞	<0.001	150
Indole-3-acetaldehyde	1.2	—	77
4-Hydroxyphenylacetaldehyde[b]	0.4	—	113
4-Hydroxyphenylglycolaldehyde[b]	0.02	—	48
4-Nitrobenzaldehyde	∞	0.015	110
Caprinaldehyde	0.5	—	20
Butyraldehyde	0.5	—	12
NADPH	—	0.004	

[a] Isocorticosteroids are synthesized either enzymically [V. Lippman and C. Monder, *J. Steroid. Biochem.* **7**, 719 (1976)] or chemically [S. Oh and C. Monder, *J. Org. Chem.* **41**, 2477 (1976)]. The presented data are part of unpublished results of B. Wermuth and C. Monder (1981).

[b] Biogenic aldehydes are prepared by incubation of the appropriate amine with monoamine oxidase [B. Tabakoff, R. Anderson, and S. G. A. Alivisatos, *Mol. Pharmacol.* **9**, 428 (1973)].

Activators and Inhibitors. Ammonium sulfate activates aldose reductase, whereas chloride ions are inhibitory. Flavonoids, e.g., quercetin, quercitrin, and rutin, 3,3-tetramethyleneglutaric acid, Alrestatin (1,3-dioxo-1*H*-benz[*de*]isoquinoline-2-(3*H*)acetic acid), Sorbinil [*S*-6-fluorospiro(chroman-4,4′-imidazolidine)-2″,5″-dione], and the chromone, 7-hydroxy-4-oxo-4*H*-chromen-2-carboxylic acid, known inhibitors of animal lens aldose reductase, are also potent inhibitors of the human enzyme.[2,6,10] Differences exist in the susceptibility of the enzyme from various tissues. Aldose reductase from lens, for example, is about 10 times as susceptible to inhibition by quercetin (IC_{50} = 0.6 μM) as the enzymes from placenta (IC_{50} = 7 μM) or brain (IC_{50} = approximately 10 μM).

Tissue Distribution. Aldose reductase activity has been demonstrated in brain, kidney, placenta, testis, lens, lung, heart, and pancreas, but appears to be absent from liver. The enzyme is localized in the cytoplasm of the cell.

[10] P. F. Kador, J. H. Kinoshita, W. H. Tung, and L. T. Chylack, Jr., *Invest. Ophthalmol.* **19**, 980 (1980).