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PURIFICATION OF ALDOSE REDUCTASE FROM HUMAN PLACENTA AND STABILIZATION OF THE INHIBITOR BINDING SITE

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Abstract—Aldose reductase from human placenta was purified to homogeneity by a rapid (2 day) and efficient purification scheme involving Red Sepharose affinity chromatography, chromatofocusing and high performance liquid chromatography on a size-exclusion column. Addition of $NADP^+$ at all steps in the purification of aldose reductase and during storage of the enzyme at −20° stabilized both the enzyme active site and the major site for binding of aldose reductase inhibitors such as sorbinil and tolrestat. Aldose reductase is a monomer with a molecular mass of 38 kD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, apparent pI 5.9. Placenta aldose reductase exhibited no cross-reactivity with aldehyde reductase from human liver in an ELISA assay. Aldose reductase showed broad specificity for aldehydes, was specific for NADPH, and was activated by sulfate.

Aldose reductase (alditol:$NADP^+$ oxidoreductase, EC 1.1.1.21) is a member of a family of NADPH-dependent oxidoreductases that catalyze the reduction of a wide variety of aldehydes and ketones. The physiological functions of these enzymes are not clear. However, the wide distribution and the broad, overlapping substrate specificities of these enzymes suggest that one of their functions is to detoxify reactive carbonyl-containing compounds. Aldose reductase also functions with sorbitol dehydrogenase to provide a pathway (the polyol pathway) for the conversion of glucose into fructose by way of the intermediate sugar-alcohol sorbitol (Fig. 1).

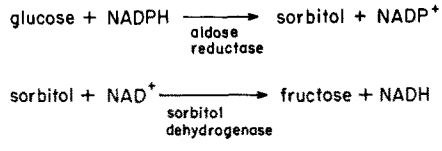

Fig. 1. Polyol pathway for metabolism of glucose.

Aldose reductase and the polyol pathway have been implicated in the development of numerous diabetic complications involving eye, nerve, kidney and joints [1–3]. These are often debilitating late-onset complications of diabetes which affect tissues with insulin-independent glucose transport. Sorbitol levels may become elevated in tissues which exhibit diabetes-associated pathology [4]. The exact role of sorbitol in the development of these pathologies is not clear. One possibility is that sorbitol accumulation leads to a hyperosmotic effect which produces an influx of fluid and subsequent changes in membrane permeability [4]. In spite of uncertainties regarding the pathogenesis of diabetic complications, the polyol pathway does appear to provide a common biochemical link [1–4].

Currently, there is wide interest in the development of aldose reductase inhibitors (ARI) for possible use in the prevention of diabetic complications. A number of ARI are being tested clinically [4]. The development of ARI has generally utilized animal sources of aldose reductase for initial testing of compounds. There are tissue and species differences in the sensitivities of various aldose reductases to ARI. It would be desirable, therefore, to conduct initial tests with an enzyme from human tissues, such as placenta which is a convenient source of human aldose reductase. However, the ARI binding site on placenta aldose reductase is labile, especially in purified preparations of this enzyme [4, 5]. This has prevented the general use of this enzyme for initial screening of ARI.

In the present report, we describe a rapid (2 day) and efficient purification scheme for isolation of aldose reductase from human placenta, and we describe conditions for the long-term storage of the purified enzyme with stabilization of the ARI binding site.

MATERIALS AND METHODS

Fresh human placenta were obtained from the University of New Mexico Hospital. Placentas were perfused with cold phosphate-buffered saline (PBS), pH 7.4, and membranes and connective tissue were removed. The placentas were then either used immediately or they could be stored at −70°. One-half placenta was generally homogenized by blender, using 200 ml of cold PBS, followed by centrifugation at 700 *g* (10 min), 600 *g* (10 min) and 100,000 *g*

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Table 1. Purification of aldose reductase from human placenta

Step	Volume (ml)	Protein (mg/ml)	Sp. act.* (μmol/min/mg)	Purification (step)	% Recovery (step)
Supernatant	810	4.53	0.0018		
Red Sepharose	200	0.67	0.078	43.0	157
Chromatofocusing	7.2	0.71	0.68	8.7	70
HPLC	4.8	0.025	1.13	1.7	88
				Overall 630	

* Activity was determined at pH 7, 25°, with 10 mM D,L-glyceraldehyde and 0.1 mM NADPH.

(30 min). Normally, a mixture of protease inhibitors [chymostatin, pepstatin, antipain, bestatin and leupeptin (Sigma), 1 μg/ml] was added at this point.

Red Sepharose Cl-6B (Pharmacia) was equilibrated by washing a column, 3 × 17 cm, with 4.5 M urea, 200 ml; 0.1 M Tris (pH 8.5) containing 0.5 M NaCl, 200 ml; 0.1 M sodium acetate (pH 4.5) containing 0.5 M NaCl, 200 ml; 0.15 M potassium phosphate (pH 7.4) containing 0.4 M NaCl, 200 ml; 0.15 M potassium phosphate (pH 7.4), 200 ml; and 0.01 M potassium phosphate (pH 7.4), 300 ml.

The 100,000 *g* supernatant material from homogenization and centrifugation of one-half placenta was diluted 4-fold with water before being applied to a column of Red Sepharose. Addition of 810 ml of solution (containing 6.6 units of reductase activity) to a 3 × 17 cm column allowed all of the aldose reductase activity to be adsorbed to the Red Sepharose. The column was washed with 0.15 M potassium phosphate, pH 7.4, 300 ml. Aldose reductase was then eluted by addition of 200 ml of this buffer containing 0.4 M NaCl and 0.5 mM $NADP^+$. The eluent from Red Sepharose chromatography was concentrated to 5 ml by pressure filtration on an Amicon PM-10 membrane. The concentrated material was desalted on a Pharmacia PD-10 desalting column which was equilibrated with 1 mM potassium phosphate, pH 7.4, containing 0.015 M NaCl.

Chromatofocusing was carried out with a 0.8 × 10 cm column of Pharmacia PBE 94 resin equilibrated with 0.025 M imidazole, pH 7.2. One-half of the desalted sample of aldose reductase (3.5 ml) was added to the chromatofocusing column. The column was developed with 50 ml of polybuffer 74, diluted 1:8 with distilled water and containing 0.1 mM $NADP^+$. Aldose reductase was obtained as a single peak of activity in a volume of about 5 ml.

High performance liquid chromatography (HPLC) was carried out with a BioSil TSK-250 size-exclusion column (BioRad) equilibrated with 0.15 M sodium phosphate, pH 6, containing 0.1 M NaCl and 0.1 mM $NADP^+$. A sample of aldose reductase from the chromatofocusing step (0.25 ml) was added to the BioSil column and was eluted with PBS adjusted to pH 6.0 and containing 0.1 mM $NADP^+$. This purification step separates aldose reductase from the polybuffer 74 which is present after the chromatofocusing step.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 20% gels containing Acrylaide (FMC Corp). Gels were polymerized on plates covered with Gel Bond (FMC). Stacking gel (5%) was used. Electrophoresis was carried out overnight at 7 mA [6].

Aldose reductase was assayed by addition of enzyme samples to 1 ml volumes of 0.1 M sodium phosphate, pH 7, containing 0.1 mM NADPH and 10 mM D,L-glyceraldehyde. Reactions were monitored at 340 nm, 25°. Kinetic studies of purified aldose reductase at pH 7 were carried out using this buffer with or without addition of 0.4 M $(NH_4)_2SO_4$. Kinetic studies at pH 6 were carried out with 0.1 M sodium phosphate, pH 6, with or without 0.4 M $(NH_4)_2SO_4$. The units of all specific activities are micromoles per minute per milligram. Inhibition constants for sorbinil (Pfizer) and tolrestat (Ayerst) were determined from Dixon plots. Proteins were determined by the method of Bradford [7].

Antisera to purified aldehyde reductase was raised in rabbits. The antisera was titered against aldose reductase and against aldehyde reductase using an ELISA assay as described previously [8]. The aldehyde reductase was purified from human liver [9] and was homogeneous by SDS-PAGE, with a molecular mass of 40 kD.

RESULTS

Purification of aldose reductase from human placenta. The scheme for purification of aldose reductase is summarized in Table 1. The first step utilized Red Sepharose for rapid extraction of all reductase activity from the 100,000 *g* supernatant fraction. Recoveries of activity for this step were generally greater than 100%, suggesting that some inhibitory substance is removed. In the example given in Table 1, recovery was 157%. This step afforded material with a 43-fold increase in specific activity.

The eluent from Red Sepharose chromatography, 200 ml, was concentrated to 5 ml by pressure filtration on an Amicon PM-10 membrane with 95% recovery of activity. Desalting of the concentrated material by addition of 2 × 2.5 ml fractions to a Pharmacia PD-10 desalting column afforded 7.0 ml of solution with 100% recovery of activity.

Purification of aldose reductase by chromatofocusing (Fig. 2) on Pharmacia PBE 94 resin provided one major peak of reductase activity which was activated by sulfate, a common property of aldose reductase [10]. This major peak represented 70% of the initial activity applied to the chromatofocusing column. This step provided an

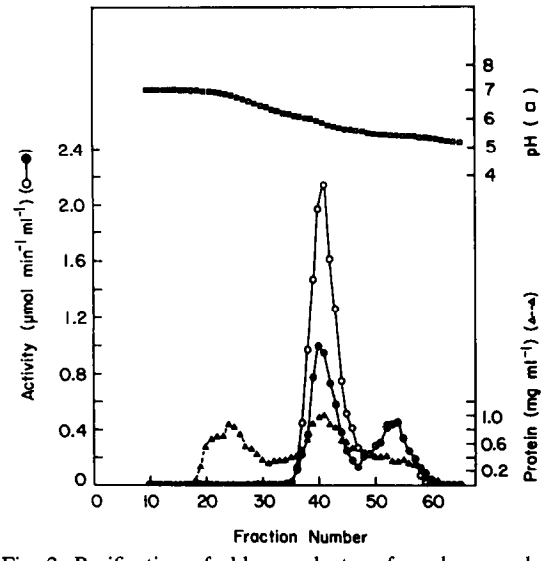

Fig. 2. Purification of aldose reductase from human placenta by chromatofocusing. After desalting (Pharmacia PD 10) the concentrated enzyme from purification on Red Sepharose, a solution of aldose reductase, 3.5 ml, was added to a column of chromatofocusing resin (Pharmacia PBE 94), 0.8 × 10 cm. The column was developed by addition of 1:8 diluted polybuffer 74 containing 0.1 mM $NADP^+$; fractions were 0.6 ml. Activity profiles are in the presence (○) or absence (●) of 0.4 M ammonium sulfate.

additional 8.7-fold purification. The apparent isoelectric point of aldose reductase, as determined by chromatofocusing, was 5.9.

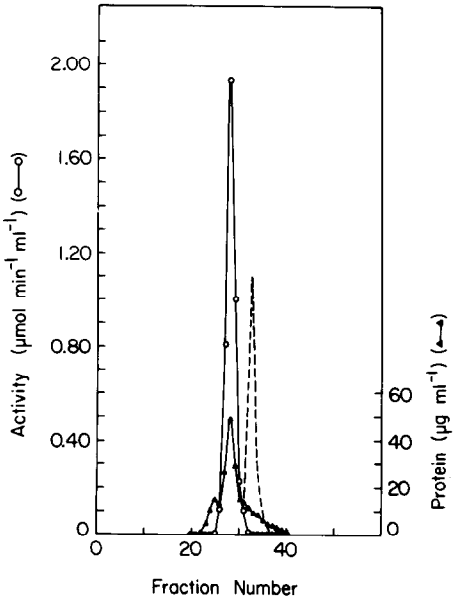

Fig. 3. Purification of aldose reductase by HPLC on a size exclusion column (BioRad BioSil TSK-250, 0.75 × 60 cm). Aldose reductase, 0.25 ml, 0.71 mg/ml, from the chromatofocusing purification step was added; the flow rate was 0.8 ml/min, 0.8-ml fractions. Protein (▲) and activity (○) profiles are shown. Polybuffer elutes after the enzyme (dashed line).

The partially purified enzyme from the chromatofocusing step was purified further by HPLC on a size-exclusion column, as shown in Fig. 3. This step afforded enzyme which was purified an additional 1.7-fold with 88% recovery. In addition, this step separated aldose reductase from polybuffer which was present after the chromatofocusing step. $NADP^+$ was present in the eluting buffer.

The scheme summarized in Table 1 for the purification of placenta aldose reductase provided a rapid (1–2 days) 630-fold purification of this enzyme. The scheme is highly efficient, with excellent recoveries of activity at each step. The purified enzyme was homogeneous on SDS-PAGE with a molecular mass of 38 kD (Fig. 4).

Stability of purified aldose reductase. Addition of $NADP^+$ at all steps in the scheme markedly stabilized aldose reductase. The purified enzyme, either after the chromatofocusing step or after HPLC, can be stored without further concentration of the enzyme. Storage at −20° in the presence of 0.1 mM $NADP^+$

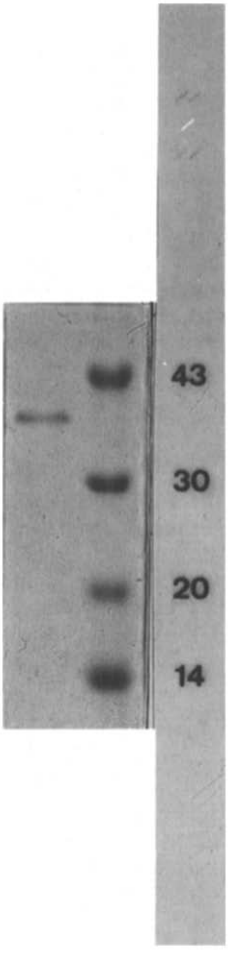

Fig. 4. SDS-PAGE of purified aldose reductase from human placenta. Purified aldose reductase (1.5 μg) was run on a 20% gel with a 5% stacking gel. Standards were ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lactalbumin. Aldose reductase migrated as a 38 kD protein.

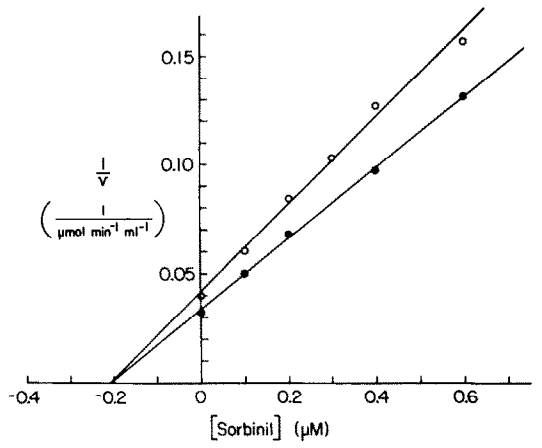

Fig. 5. Dixon plots of the inhibition of aldose reductase from human placenta by sorbinil. Reactions were monitored at pH 7, 25°, in the absence of added sulfate. K_i = 0.21 μM.

provided 100% stability for at least several months. In addition, these conditions provided stability for the inhibitory site on aldose reductase (see below). The presence of polybuffer 74 after the chromatofocusing step did not perturb the kinetic or binding properties of aldose reductase. Consequently, the final HPLC step in the purification scheme is not essential.

Immunochemical comparison of aldose and aldehyde reductases. Rabbit antisera, prepared against liver aldehyde reductase, was titered against placenta reductase and against aldehyde reductase. Liver contains aldehyde reductase but apparently does not express aldose reductase [10]. There was no significant cross-reactivity between these two proteins as determined by an ELISA assay.

Inhibition of aldose reductase. Currently, sorbinil is the benchmark compound against which potential aldose reductase inhibitors are compared. Aldose reductase from placenta, purified as described above, is highly sensitive to sorbinil. This sensitivity did not change during storage of purified enzyme at −20° for up to 6 months. Inhibition of aldose reductase by sorbinil was analyzed by Dixon plots, as shown in Fig. 5. Sorbinil binding was noncompetitive with NADPH binding; K_i = 0.21 μM, pH 7.0, 25°.

Table 2. Effects of pH and sulfate on the specific activity of aldose reductase and on inhibition by sorbinil

Conditions	Sp. act.* (μmol/min/mg)	K_i (sorbinil) (μM)
pH 6	0.89	1.3
pH 6, 0.4 M sulfate	1.88	0.48
pH 7	1.13	0.21
pH 7, 0.4 M sulfate	3.32	0.55

* Activity was determined at 25° with 10 mM D,L-glyceraldehyde and 0.1 mM NADPH.

Most reported studies of various aldose reductases and especially of drug binding to aldose reductase were carried out at pH 6 or 7, with or without sulfate present. The specific activity of aldose reductase was pH and sulfate sensitive, as shown in Table 2. There was a 4-fold range in specific activities, depending upon conditions. Likewise, the binding of sorbinil to aldose reductase was pH and sulfate dependent, as shown in Table 2. Tightest binding was observed at pH 7 in the absence of sulfate.

Tolrestat, another aldose reductase inhibitor, was compared with sorbinil for binding to aldose reductase at pH 7 in the absence of sulfate, with D,L-glyceraldehyde as substrate. The binding of tolrestat was noncompetitive with NADPH binding.

Substrate specificity of aldose reductase. The substrate specificity of purified aldose reductase toward a range of aliphatic and aromatic substrates was determined at pH 7 in the absence of sulfate. The results are shown in Table 3. Aldose reductase showed broad specificity, characterized by k_{cat} values which were insensitive to the nature of the aldehyde. The binding constants, reflected in K_m values, varied markedly. Consequently, the molecular activities (k_{cat}/K_m) showed a large variation with changes in the substrate, spanning a 40,000-fold range between *p*-nitrobenzaldehyde and glucose.

DISCUSSION

Aldose reductase and the polyol pathway were first discovered in seminal vesicles where this pathway functions to produce fructose for sperm [11]. The polyol pathway was subsequently found in many

Table 3. Substrate specificity of placenta aldose reductase

Substrate	Relative V_{max}*	k_{cat} (min^{-1})	K_m (mM)	M.A.‡ ($mM^{-1}min^{-1}$)
D,L-Glyceraldehyde	1.0	43	0.02	2,150
p-Nitrobenzaldehyde	1.3	56	0.003	18,667
Glucose	1.0	43	92	0.47
Glucuronic acid	0.7	30	2.2	13.6

* V_{max} values, 25°, pH 7, in the absence of sulfate are given relative to D,L-glyceraldehyde which shows specific activity 1.13 μmol/min/mg under these experimental conditions.

† k_{cat} was determined from the specific activities with purified enzyme assuming one active site and a molecular mass of 38 kD.

‡ M.A. (molecular activity) = k_{cat}/K_m.

tissues. The possible relationship between the polyol pathway and diabetic complications was suggested by studies of van Heyningen in 1959 [12] in which it was shown that sorbitol accumulates in the lenses of diabetic rats. Demonstration that sorbitol accumulates in a variety of tissues associated with diabetic complications has provided for the idea that aldose reductase and the polyol pathway are an intimate part of the etiology of diabetic complications. Under conditions of normal glycemia, aldose reductase appears not to compete effectively with hexokinase for glucose. Sorbitol production in diabetic complications arises as a consequence of hyperglycemia, insulin-independent uptake of glucose, and the presence of aldose reductase. The extent of sorbitol accumulation depends upon these factors and upon the level of sorbitol dehydrogenase.

Aldose reductases from a variety of species and tissues have been studied [1, 4]. All aldose reductases of animal origin appear to have a major drug binding site which is separate from, or perhaps partially overlaps, the active site. Numerous compounds have been shown to bind to this ARI site. Several of these have been used in animal studies and have been shown to be effective in preventing the development of diabetic-like complications in the galactose-fed rat model of diabetes [1].

The pharmacophor properties of the ARI site on aldose reductase have been estimated by comparison of the molecular properties of numerous ARI [13]. The composite picture explains the inhibitory properties of numerous compounds that represent a wide variety of structures. The ARI site appears to be fairly large, comprising two lipophilic regions with adjacent residues which participate by hydrogen bonding and charge–transfer interactions [4, 13].

The instability of the ARI site is most pronounced with aldose reductase from human tissues. This instability is not seen with all ARI which suggests that a small part of the ARI site is labile [4]. This is especially apparent in studies of the binding of sorbinil to human placenta aldose reductase where sensitivity to this drug decreased several thousand fold during storage of purified enzyme for a period of 8 days [4]. Placenta aldose reductase is a convenient source of human enzyme. In the present study, we have addressed this problem of stabilization of the ARI site of placenta aldose reductase.

Aldose reductase from human placenta has been purified previously [5, 14, 15]. The enzyme was first purified by Clements and Winegrad who used a six-step procedure which included ammonium sulfate fractionation, conventional chromatography on ion-exchange and gel-exclusion columns, and two isoelectric focusing steps. Final recovery of activity was 3% [14]. Kador *et al.* developed a three-step procedure which involved ammonium sulfate fractionation and two affinity chromatography steps. Final recovery of activity was 15% [5]. Maragoudakis *et al.* purified aldose reductase by ammonium sulfate fractionation, ion-exchange chromatography, isoelectric focusing and affinity chromatography. Final recovery of activity was 20% [15].

The procedure described here was designed to avoid any lengthy steps in the purification scheme. The ammonium sulfate fractionation step was avoided in order to eliminate the need for a prolonged dialysis step. Red Sepharose was used for rapid extraction of all reductase activity from a crude homogenate. Red Sepharose exhibits specificity for $NADP^+$/NADPH requiring enzymes. Concentration of the sample after Red Sepharose chromatography by pressure filtration represents the only step where concentration of sample is necessary. The scheme described here can be completed in 1–2 days. The recovery is excellent. Most noteworthy, the addition of $NADP^+$ at all steps in the scheme and during storage of aldose reductase provides a simple method to stabilize the ARI binding site.

Recently, Srivastava and coworkers described the purification of aldehyde reductase from human placenta [16] and described some interrelationships among human aldo-keto reductases [17]. These authors reported that there are two aldehyde reductases, designated aldehyde reductase I and II. Aldehyde reductase I was reported to be a dimer of α, β subunits and aldehyde reductase II is a monomer of δ subunits. Most significant was the suggestion that aldose reductase represents the α subunit of aldehyde reductase I. These authors reported that there is very little aldose reductase in the placenta. They propose that the aldose reductase reported by others [5, 14, 15] represents the formation of free α subunits due to the $(NH_4)_2SO_4$ precipitation step which was used in all of these purification schemes. Several observations from the present study question this conclusion. First, we did not use $(NH_4)_2SO_4$ in our scheme. Aldose reductase was extracted from placenta with Red Sepharose affinity matrix, thus avoiding the initial precipitation step used by other investigators. Second, the profile for purification of aldose reductase by chromatofocusing revealed a major reductase which upon further purification was homogeneous on SDS-PAGE with a single band at 38 kD. Aldehyde reductase I was reported to exhibit two bands at 32.5 and 39 kD [16].

Additional comparisons between the present results and previous studies also support the conclusion that human placenta expresses an aldose reductase which represents a significant amount of the aldo-keto reductase activity in placenta. The enzyme isolated by us is activated by sulfate ion, similar to previous reports of aldose reductase [10]. The broad specificity and the binding constants of placenta aldose reductase (Table 3) are in good agreement with previous reports both of placenta aldose reductase [14] and of aldose reductase from human brain [10]. In addition, the size of aldose reductase as determined by us (38 kD) agrees well with previous reports of human aldose reductase: 39 kD [14], 37 kD [5], and 35–40 kD [10]. It would appear, therefore, that aldose reductase exists as a separate enzyme in human placenta. The purification scheme described here provides a simple procedure for the rapid and efficient isolation and stabilization of this enzyme.

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