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ISOLATION AND CHARACTERIZATION OF ALDOSE REDUCTASE FROM CALF BRAIN

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Summary

Aldose reductase activity (alditol: $NADP^+$ 1-oxidoreductase, EC 1.1.1.21) from calf brain was separated into two protein fractions by DEAE chromatography. Further purification by molecular sieve chromatography and electrofocusing yielded two distinctive enzymes, which were designated AR I and AR II. AR I was purified 646-fold and found to have an isoelectric point of 6.18. AR I was most active as a monomer with a molecular weight of 29 000 and appeared to be in equilibrium with a less active dimer. AR II was purified 425-fold and found to have an isoelectric point of 4.88. The molecular weight of this enzyme was 30 000. Although both enzymes had specificity for aldoses as substrates, AR I had two to three times larger turnover numbers with aromatic aldehydes and hexonates than did AR II. AR I was activated by sulfhydryl compounds and exhibited biphasic double reciprocal plots. AR I was more sensitive to inhibition by high substrate and phenobarbital concentrations than was AR II. AR I and AR II did not have antigenic similarity as tested by Ouchterlony immunodiffusion and counter immunoelectrophoresis. An immunochemical cross-reaction was observed between AR II and lens aldose reductase.

Introduction

Aldose reductase (EC 1.1.1.21, $NADP^+$ 1-oxidoreductase) is found in a wide variety of mammalian tissues from a number of species [1—4]. Enzymes from non-mammalian sources also have been shown to catalyze the conversion of aldoses to polyols [5,6]. Interest in the properties and function of mammalian aldose reductase has been heightened by the discovery that aldose reductase activity contributes to the development of diabetic neuropathy and sugar cataracts [7—10]. Turner and Tipton [11] have suggested that more than one en-

zyme capable of reducing aldoses occurs in rat brain while recent studies of aldose reductase from this laboratory revealed only the presence of a single form of the enzyme in bovine lens tissues [12].

The purpose of this paper is to describe the isolation of aldose reductase from bovine brain. Unlike aldose reductase activity from bovine lens, two distinct proteins were found to generate polyols in bovine brain. The chemical, physical, and biologic properties of one of the enzymes (AR II) was nearly identical to those of bovine lens aldose reductase. The second enzyme (AR I) was shown to have unique characteristics which clearly distinguish it from AR II and bovine lens aldose reductase.

Experimental procedure

Materials. Chemicals of the highest purity were obtained from the following sources: *p*-nitrobenzaldehyde, 3-pyridine carboxaldehyde, NADPH, dithiothreitol, triethanolamine, calcium phosphate gel, and protamine sulfate from Sigma; DL-glyceraldehyde from K and K Laboratories; 2-mercaptoethanol and DEAE-cellulose from Eastman; sucrose, $(NH_4)_2SO_4$ and molecular weight markers from Schwartz/Mann. Ampholine was from LKB.

Standard assay. The reaction was followed by measuring the decrease in absorbance of NADPH at 340 nm as previously described by Sheys et al. [5]. For the purification procedure, the assay mixture contained 120 nmol of NADPH, 10 mmol of DL-glyceraldehyde, and 100 mM sodium phosphate buffer (pH 7.0, 37°C). Upon addition of the enzyme, the total volume was 1 ml. The initial velocities obtained were reproducible and generally linear for the first 3—5 min. An enzyme unit was defined as that quantity of enzyme which catalyzed the oxidation of 1 nmol of NADPH/min (37°C). The specific activity was defined as the number of enzyme units/mg protein.

Protein determination. Protein determinations were made using either the biuret method (sensitivity 1—10 mg protein/ml) or the Lowry method (sensitivity 10—200 μg protein/ml) as modified by Chaykin [13]. Potential interference of buffers and other substances with the Lowry reaction was circumvented by application of Rej's methods [14]. The ratio of absorbance at 280 and 260 nm was also used to estimate protein concentration in some cases.

Enzyme purification. Calf brains were obtained fresh, kept on ice for 2—3 h after slaughter, and then stored frozen at −55°C. In some experiments, white matter (brainstem, corpus callosum, and centrum semiovale) was separated from grey matter (cortex, cerebellum) and purified. The purification of the enzymes from whole brain is described. In one experiment, enzyme activity from 60 g of rabbit sciatic nerve was partially purified using modifications of Steps 1, 2, 3 and 7. All steps were carried out at 4°C.

Step 1: Preparation of the cell-free extract: Approximately 400 g of brain were minced with scissors and homogenized in 2.3 vols. of 0.5 M sodium phthalate buffer, pH 7.0, with an on-off cycled Waring blendor for a 2 min total grinding time. Insoluble matter was removed by centrifugation at 23 000 × *g* for 40 min; the unbuffered extract was found to be slightly acidic. To avoid loss of enzyme activity at this step, care was taken that the pH not drop below 6.0. However, compared to extraction at higher pH values, extraction

at a pH of from 6.0 to 7.0 allowed the removal of considerable non-enzyme proteins. For these reasons, phthalate (pK 5.41) was a desirable buffer useful at this stage in purification.

Step 2: Protamine sulfate treatment: An aqueous 1% (w/v) solution of protamine sulfate, histone free (Grade I), was added to the cell-free extract so that the proportion of 1 mg of protamine sulfate per 20 mg of protein was achieved at the end of a period of 15 min. The pH was maintained at 7.0 with 0.1 M acetic acid. After stirring for an additional 5 min, the precipitate was removed by centrifugation at 23 000 × g for 15 min and discarded.

Step 3: Ammonium sulfate fractionation: To each 100 ml of supernatant, 24.2 g of solid $(NH_4)_2SO_4$ was added gradually with stirring over a period of 40 min. The resultant solution, 43% saturated $(NH_4)_2SO_4$, was then centrifuged at 23 000 × g for 20 min at 4°C. The precipitate was discarded. Additional $(NH_4)_2SO_4$ (22.3 g/100 ml solution) was added over 50 min and this solution, 80% saturated $(NH_4)_2SO_4$, was stirred for another 10 min. After centrifugation, the fraction precipitated at from 43% to 80% saturation with $(NH_4)_2SO_4$ was dissolved in 20 mM sodium phosphate buffer, pH 6.2, and exhaustively dialyzed versus this buffer.

Step 4: Calcium phosphate gel treatment: The dialyzed enzyme preparation was adjusted to a protein concentration of 10 mg/ml and then 1 mg of calcium phosphate gel per 4 mg of protein was added with stirring over a period of 15 min while maintaining the pH at 6.2 with 1 M acetic acid. The mixture was then centrifuged at 10 000 × g for 5 min. The supernatant was concentrated by precipitation with $(NH_4)_2SO_4$ to 90% saturation. The precipitate was completely dissolved in 10 mM phosphate buffer, pH 7.2, and exhaustively dialyzed against this buffer.

Step 5: DEAE- cellulose chromatography: Ion exchange DEAE-cellulose was washed first with 0.5 M NaOH, then with 0.5 M HCl, and finally with 10 volumes of distilled water. This resin was poured onto a column (1.75 × 30 cm) and equilibrated with 10 mM sodium phosphate buffer at pH 7.2 containing 5 mM 2-mercaptoethanol. The protein solution was pumped onto the column at 40 ml/h with a peristaltic pump and washed with equilibrating buffer until no more protein emerged. The column was then eluted with 500 ml of a linear gradient of from 10 to 60 mM phosphate buffer, pH 7.2. 7-ml fractions were collected. Peak I was pooled as the activity which came through the column directly. Peak II was pooled as the activity obtained upon elution with the gradient. Each peak was concentrated 20-fold by ultrafiltration [1].

In one experiment, each peak from the above DEAE column was rechromatographed at pH 7.6 in 10 mM triethanolamine · HCl buffer. A 10—200 mM buffer gradient was applied to elute each peak from separate columns (Fig. 1).

Step 6: Sephadex G-100 chromatography: The concentrated pools, Peak I and Peak II, were applied to separate Sephadex G-100 columns (2.5 × 100 cm) which were equilibrated and eluted with 10 mM triethanolamine · HCl buffer, pH 7.6. Fractions with the highest specific activity from each column were pooled, concentrated by ultrafiltration and dialyzed briefly against the column buffer.

Step 7: Electrofocusing: Two electrofocusing columns (LKB 8101) were

prepared according to the manufacturer's instructions using pH 4—6 Ampholine in one and pH 5—8 Ampholine in the other. Ampholine in a final concentration of 2% was used in each case. In one experiment, 5 mM 2-mercaptoethanol was included in the columns. A sucrose gradient was prepared and the columns were focused without sample for a minimum of 20 h. Each sample was mixed with sucrose and layered at an isodense position on the columns. The Peak I sample was applied to the pH 5—8 column and the Peak II sample to the pH 4—6 column (Fig. 2). Electrofocusing of the samples was complete in 48 h using 0.45 W. The fractions containing activity were pooled. The viscosity of the samples was reduced by giving each pool a brief dialysis against column buffer before application to an upward flow Sephadex G-100 column (2.5 × 50 cm) equilibrated with 10 mM triethanolamine · HCl buffer, pH 7.6, in 0.5 M NaCl. Almost all the Ampholine was removed in this step. The appropriate fractions were then pooled, concentrated by ultrafiltration, and dialyzed versus 100 mM sodium phosphate buffer, pH 7.0. Purified Peak I, designated AR I, and purified Peak II, designated AR II, were then stored at −55°C.

Polyacrylamide gel electrophoresis. The discontinuous buffer system described by Davis [15] was used in the assessment of enzyme purity and in the vital staining experiments. The gels used may be described by the notation of Hjerten [16] in which the first numeral (*T*) denotes the total weight of monomer (acrylamide plus *N,N'*-methylenebisacrylamide) per 100 ml of solvent, and the second numeral (*C*) denotes the amount of *N,N'*-methylenebisacrylamide expressed as percentage (w/w) of the total amount of monomer. Thus, the upper stacking gel was 3.1 × 20, polymerized in pH 7.2 Tris/phosphate buffer, and the lower running gel was 7.7 × 2.6, polymerized in pH 8.9 Tris/glycine buffer. Sodium dodecyl sulfate gel electrophoresis was performed using the continuous sodium phosphate buffer system of Weber and Osborn [17]. In this case, gels (9.2 × 2.6) were polymerized in pH 7.2 buffer containing 0.1% sodium dodecyl sulfate. Protein samples to be electrophoresed were incubated with 1% sodium dodecyl sulfate plus 10 mM 2-mercaptoethanol for 15 min at 37°C and then dialyzed 16 h against buffer. A maximum of 40 V per cm length of gel was applied until the Bromphenol Blue tracing dye had moved the length of the tube. The gels were stained for protein with Coomassie Blue and destained electrophoretically.

Zymogram vital staining method. The procedure of Korman et al. [18] was modified in the following manner: the polyacrylamide gel system of Davis was used; incubation of the gel in the glycerol-$NADP^+$ solution was done in 63 mM phosphate buffer, pH 7.0, at 37°C for 30 min after which the gel was transferred to a second tube containing 3.5 mg 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride (INT) and 0.2 mg phenazine methosulfate (PMS) per 10 ml buffer at pH 7.0. Tubes were laid horizontally, protected from light for 2 h at 37°C. Enzyme activity correlated with a clear band in the gel which had otherwise turned pink. Controls, not incubated with glycerol were used to demonstrate the specificity of the reaction.

Counter immunoelectrophoresis and Ouchterlony immunodiffusion. Clean 80 × 100-mm glass slides were covered with 12 ml of agarose (1 g/100 ml of 0.01 M Tris/0.1 M NaCl buffer, pH 9.6, containing 0.001 M EDTA). A set of two rows of wells (3 mm in diameter) were cut 4 mm apart. Three such sets

were used, each being cut 6 mm apart. The anodal wells were filled with concentrated antibody preparations to AR I or AR II. The cathodal wells were filled with varying concentrations of the enzyme antigens. The electrode chambers were filled with barbital buffer (I = 0.05, pH 8.6). Electrophoresis was done using constant voltage with 30—35 A current for 60 min. The use of control wells, filled with saline, allowed detection of the occasional arc of nonspecific protein precipitate which appeared around the enzyme or antibody wells. The Ouchterlony immunodiffusion methods used have been described [1,19].

Statistical analysis of kinetic data. The initial velocity data used was obtained by measuring the decrease in absorbance of NADPH at 340 nm such that less than 1% of the NADPH present was consumed. If the observed decrease in absorbance was not within 10% of a duplicate determination, additional samples were run until consistent results were obtained.

Linear regression (computer program BMD 02R) was employed to analyze statistically the data and to determine the K_m values and the standard errors [20].

Thus, for AR I, with glyceraldehyde as substrate, the values which represented one standard error from the K_m of 1.7×10^{-3} M were 9.3×10^{-4} and 4×10^{-3} M. Similarly, for AR II, the values for the K_{m_1} of 1.9×10^{-4} M were 1.6×10^{-4} and 2.1×10^{-4} M, and, for the K_{m_2} of 6.4×10^{-4} M, they were 6.0×10^{-4} and 6.9×10^{-4} M.

TABLE I

PURIFICATION OF CALF BRAIN ALDOSE REDUCTASE

	Step	Total activity (nmol/min)	Protein (mg/ml)	Specific activity (nmol/min per mg protein	Yield (%)	Purification (-fold)
Cell-free extract of whole brain (400 × *g*)	1	12 540	14.0	1.15	(100)	(1)
Protamine sulfate supernatant	2	11 781	9.5	1.47	93.9	1.28
Ammonium sulfate precipitate (after dialysis)	3	9 443	47.0	2.5	75.3	2.19
Calcium phosphate gel supernatant	4	8 869	7.5	3.2	70.7	2.80
DEAE-cellulose eluate:	5					
AR I		6 276	67.0	93.7	50.0	81.6
AR II		2 136	21.0	102.0	17.0	88.6
G-100 eluate:	6					
AR I		5 774	15.5	372.0	46.0	324.0
AR II		2 023	5.0	405.0	166.1	352.0
Isoelectric focusing	7					
pH 5—8 (AR I)		965	1.3	742.0	7.7	646.0
pH 4—6 (AR II)		586	1.2	488.0	4.7	425.0

Results

Purification

The results of a 646-fold purification of AR I and a 425-fold purification of AR II are shown in Table I. The ratio of AR I to AR II was about 3 : 1 as estimated from the results of purification Step 5. Thus, if it is assumed that about three-fourths of the original activity was from AR I and one-fourth from AR II, then the actual recovery of the enzymes may be estimated at 9 and 11%, respectively. As shown in Fig. 1, AR I and AR II were rechromatographed at higher pH using triethanolamine · HCl buffer and eluted as symmetrical peaks.

White and grey matter were purified separately. Extracts of white matter had 10—20% higher specific enzyme activity than grey matter. As estimated by DEAE chromatography, the ratio of AR I to AR II was 2 : 1 in white matter while the total units of AR II in this tissue were nearly equal to that contained in grey matter when compared on a wet weight basis.

Rabbit sciatic nerve aldose reductase was partially purified using modifications of the purification scheme outlined in Table I. After treatment of the extract with protamine sulfate, 70% of the aldose-reducing activity originally present in the extract was precipitated by a 30—70% concentration of $(NH_4)_2SO_4$.

This precipitate was dissolved in and dialyzed against 10 mM triethanolamine · HCl buffer, pH 7.6, and isofocused according to Step 7 using a pH 3—10 gradient. The fractions with enzyme activity were pooled and applied to a second isofocusing column with a pH 4—6 gradient. Rapid loss in activity of enzyme from this second isofocusing column, and interference by Ampholine with the protein determination prevented calculation of the degree of purification

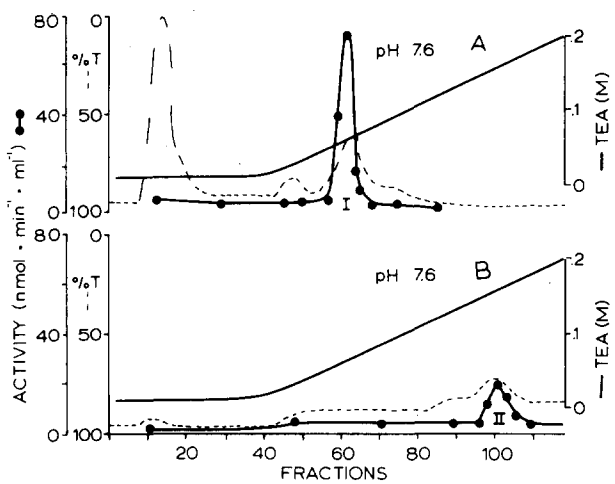

Fig. 1. Rechromatography of AR I and AR II on DEAE ion-exchange resin. Solid lines denote buffer concentration. Broken lines denote protein concentration in percent transmittance (284 nm). Each space and dash represents a 7-ml fraction. Activity determined using 10 mM DL-glyceraldehyde as shown by solid circles (●———●). A. Rechromatography of AR I at pH 7.6 on DEAE-cellulose and elution with a linear gradient of triethanolamine (TEA). B. Rechromatography of AR II at pH 7.6 on DEAE-cellulose and elution with a linear gradient of triethanolamine (TEA).

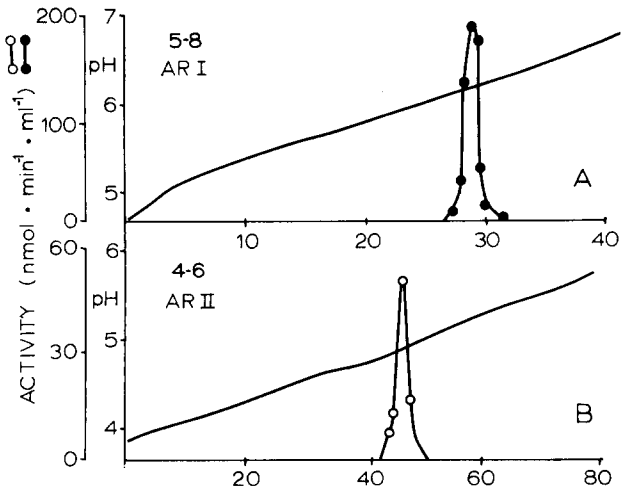

Fig. 2. Isoelectric focusing of aldehyde-reducing enzyme forms on ampholine-sucrose pH gradients. (Solid lines represent pH gradient determination). A. AR I isofocused on pH 5—8 gradient. Activity determined using 1-mM glucuronate (●———●). p*I* = 6.18. B. AR II isofocused on pH 4—6 gradient. Activity determined using 10-mM DL-glyceraldehyde (○———○). p*I* = 4.88.

achieved. The yield was less than 5% after activation with sulfate and incubation with 5 mM 2-mercaptoethanol. Sulfhydryl compounds did not prevent loss of activity of the enzymes. The purified enzymes were not stable upon freeze-thawing greater than twice or prolonged storage at −55° C.

Isoelectric points

AR I had an isoelectric point of 6.18 and AR II had an isoelectric point of 4.88 (Fig. 2). Aldose reductase from rabbit sciatic nerve had an isoelectric point of 5.0. Inclusion of 5 mM 2-mercaptoethanol in the gradient did not change the isoelectric point of either AR I or AR II.

Subunit and molecular weight studies

Upon purification to near homogeneity, AR I was found to have a molecular weight of 29 000 on Sephadex G-100 using standard molecular weight markers for calibration of a gel filtration column equilibrated with 10 mM triethanolamine · HCl, pH 7.6, in 0.5 M NaCl. A value of 10% for the uncertainty in molecular weights reported here was found on replicate determinations. After electrophoresis on polyacrylamide gel, this enzyme was resolved into two distinct bands, both of which had activity as determined by vital staining. The top band, with R_f value of 0.19, was most intensely stained for protein by Coomassie Blue, but had only very weak enzyme activity. The second band with R_F value of 0.27 was only weakly stained by Coomassie Blue, but had the most enzyme activity.

On sodium dodecyl sulfate gels, AR I appeared as one major band with an M_r of 30 000 plus a second much less intensely stained band with M_r of 64 000, presumably a dimer of the enzyme. It is possible that the conditions used in preparing the protein samples for the gels were insufficient to dissociate an en-

zyme aggregate. However, it seems more likely that the strength of association of the sodium dodecyl sulfate with the monomeric form of the enzyme was not as great as the affinity of the monomers for each other in the presence of polyacrylamide gel. In either case, different quaternary forms of AR I appeared to be in equilibrium with each other under the conditions of polyacrylamide gel electrophoresis used. A higher molecular weight form of AR I may not have been detected after gel filtration because of its very low activity or dissociation under aqueous conditions. Determination of special gel electrophoretic conditions which might allow complete separation of AR I to the monomeric form was beyond the scope of this work.

AR II was found to have a molecular weight of 30 000 on Sephadex G-100. After electrophoresis on polyacrylamide gel, this enzyme appeared as a very strong band (R_F 0.44) detectable with both the Coomassie Blue and vital staining techniques.

However, when a sample of AR II in 10-fold increased concentration was applied to a second gel and stained with Coomassie Blue, a faint band (R_F 0.58) appeared below this major band. The lesser band was not enzyme active as shown by zymogram. Since disc electrophoresis can detect at least 20 ng of protein, this other band may be estimated to represent less than 1% of the protein in the AR II preparation.

On sodium dodecyl sulfate gel electrophoresis, AR II appeared as one major band with an M_r of 32 000 and two trace bands with M_r 25 000 and 19 000. Using polyacrylamide gels, the apparent molecular weights were readily determined with a precision of 2%.

Immunologic studies

Counter immunoelectrophoresis and Ouchterlony immunodiffusion were used to help evaluate the immunochemical similarities between AR I, AR II, and purified bovine lens aldose reductase [12]. The antibodies against these enzymes were prepared in rabbits.

No cross-reactivity was found between anti-AR I and AR II enzyme or between anti-AR II and AR I enzyme. Anti-lens aldose reductase formed a single band when reacted against AR II, but had no reaction against purified AR I. In the Ouchterlony plate, this band formed a line of identity with one of the two bands which formed when anti-AR II was reacted against AR II enzyme.

Upon discovery of the two bands which formed when AR II was reacted against anti-AR II in the Ouchterlony plate, immunologic evaluation of the proteins contained in the AR II preparation was done. As determined by polyacrylamide gel electrophoresis and zymogram studies, a non-enzymatic protein representing less than 1% of the AR II preparation was present. This protein could be sliced out of the polyacrylamide gels and thus separated from the enzyme-active protein. When the various polyacrylamide slices were arranged in different wells of an Ouchterlony plate so as to surround a center well filled with antibody to the AR II preparation, precipitin lines of non-identity formed between the slices containing enzyme and those containing non-enzymatic protein.

The enzyme activity of AR I and AR II was inhibited (> 50%) in vitro by

the antibody directed against it. However, incubation with antibody to the other enzyme was no different from incubation with preimmune control.

Effects of pH

Both enzymes were stable over a broad range of pH values. Less than 5% activity was lost upon incubation of the enzymes for 2 h at pH values between 4.8 and 8.6. Activity rapidly declined at a pH of 4.4 or less. Using DL-glyceraldehyde as substrate, both enzymes were found to have a pH optima of 6.6 in 0.1 M imidazole buffer. However, differences in the shapes of the pH optima curves between the enzymes for this and phosphate buffer were noted. The pH optima of both enzymes for the oxidation of glycerol with $NADP^+$ as coenzyme was about pH 10 at a rate 20% that of the forward reaction.

Pyridine nucleotide cosubstrate specificities

NADPH and NADH were compared as coenzymes for the reduction of DL-glyceraldehyde at pH 7. The maximal velocity with NADH was about 10% of that obtained with NADPH for AR I and 20% for AR II.

Substrate profile

Various aldoses, as well as aldehydes, were used as substrates (Table II). Turnover numbers were calculated from using solutions of enzymes which were either 3.4×10^{-6} M in AR I or 5×10^{-6} M in AR II. Lower K_m values were obtained for AR II than for AR I with both aldehydes and aldoses. Greater turnover numbers were apparent when aromatic aldehydes were used as substrates for AR I than when they were used for AR II. At comparable concentrations, glucuronate was reduced by AR I several-fold more rapidly (turnover number 9.3×10^{-5}) than by AR II (turnover number 6×10^{-6}). This is as opposed to galactose which was reduced several-fold more rapidly by AR II (turnover number 7×10^{-6}) than by AR I (turnover number 2.4×10^{-6}). The kinetic parameters for glucose, the substrate of greatest physiologic interest, were not obtained because of the extremely large quantity of enzymes estimated to be required on the basis of preliminary data obtained using the less purified enzymes. These data suggested that the K_m value with glucose as substrate was some 10-fold greater for AR I than for AR II. K_m values with glucose as sub-

TABLE II

MICHAELIS CONSTANTS OF AR I AND AR II FOR ALDOSES AND ALDEHYDES

	AR I		AR II		
	K_m (M)	Turnover number $\times 10^{-6}$	K_{m_1} (M)	K_{m_2} (M)	Turnover number $\times 10^{-6}$
p-Nitrobenzaldehyde	$3.0 \cdot 10^{-4}$	104	$3.0 \cdot 10^{-5}$	$2.0 \cdot 10^{-4}$	32
3-Pyridine carboxaldehyde	$3.0 \cdot 10^{-4}$	96	$6.0 \cdot 10^{-5}$	$2.5 \cdot 10^{-4}$	54
Glyceraldehyde	$1.7 \cdot 10^{-3}$	58	$1.9 \cdot 10^{-4}$	$6.4 \cdot 10^{-4}$	24
Xylose	—	10	$2.0 \cdot 10^{-5}$	$8.0 \cdot 10^{-5}$	24
Galactose	$2.9 \cdot 10^{-1}$	2	$1.2 \cdot 10^{-2}$	$9.8 \cdot 10^{-2}$	7
NADPH	$2.2 \cdot 10^{-5}$	—	$1.2 \cdot 10^{-5}$	$2.2 \cdot 10^{-5}$	—

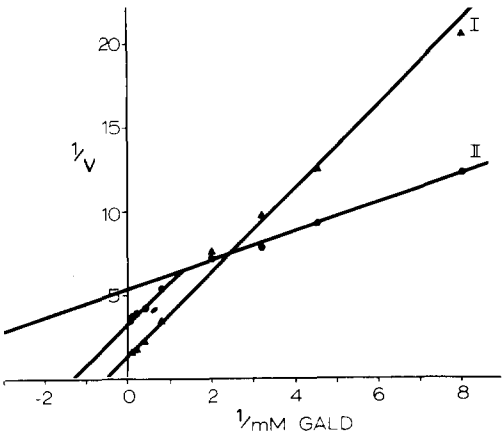

Fig. 3. Double reciprocal plots of velocity against glyceraldehyde (GALD) substrate. Concentrations used ranged from 0.125 to 10 mM DL-glyceraldehyde. [NADPH] was kept constant at 100 μM. The plots for AR I and AR II were designated by the respective Roman numerals. The plots for AR I (▲) were linear and those for AR II (●) were biphasic.

strate for other aldose-reducing enzymes have been reported to be from 2- to 20-fold greater than those determined with xylose [3,21,22].

Double reciprocal plots of initial velocities with DL-glyceraldehyde concentration varied from 10 to 0.125 mM and NADPH held constant at 100 μM showed a marked downward curvature at the high substrate concentrations for AR II. Such plots were linear for AR I (Fig. 3). This phenomena was observed for all the aldose and aromatic aldehyde substrates studied. Double reciprocal plots with DL-glyceraldehyde held constant at 10 mM and NADPH concentrations varied from 5 to 120 μM also showed this downward curvature for AR II, but not AR I. Such biphasic double reciprocal plots have been shown to occur for a single enzyme molecule and do not necessarily imply the presence of two enzyme species [12].

Two apparent K_m values for DL-glyceraldehyde and NADPH were found for AR II using linear regression analysis (Table II). For AR I, the Michaelis constant for NADPH was 2.2×10^{-5} M and that for DL-glyceraldehyde was 1.7×10^{-3} M.

When NADPH was held constant at 100 μM and DL-glyceraldehyde was varied, the inclusion of 5 mM 2-mercaptoethanol in the reaction mixture resulted in biphasic double reciprocal plots for AR II in which the turnover number increased about 2-fold. However, no change in the 'breakpoint' (1 mM DL-glyceraldehyde) of the biphasic curve was noted. The K_{m_1} was shifted to a higher value of 3.3×10^{-4} M and the K_{m_2} to a lower value of 6.6×10^{-5} M for AR II using DL-glyceraldehyde was the varied substrate.

Activators and inhibitors

When included in the reaction mixture at 2 mM concentration at 37°C, 2-mercaptoethanol, dithiothreitol, glutathione, and cysteine activated AR II from Step 5 or 6 prior to electrofocusing; 2-mercaptoethanol had the greatest effect and cysteine the least. After electrofocusing, concentrations of 2-mercapto-

ethanol greater than 2 mM, which had activated the less purified AR II, were found to inhibit both enzymes; AR I was inhibited to a greater degree than AR II. Sulfhydryl reagents inhibited AR I at all stages of purification of this enzyme. In these experiments, incubation of the aldehyde substrate with the sulfhydryl reagent was used as control. Variation in time of incubation of these reagents had no effect on the reaction making the possibility of mercaptal formation unlikely.

Both Na_2SO_4 and $(NH_4)_2SO_4$ in 0.1 M concentration activated AR I, but had no effect on AR II. This sulfate effect was found at all stages of purification. Sodium phenobarbital at 1 mM concentration did not affect AR II but inhibited AR I by 64% (K_i = 9.5 × 10^{-5} M). At 2 mM concentration phenobarbital completely inhibited AR I, but inhibited AR II only 15% (K_i = 2.8 10^{-3} M). DL-Glyceraldehyde concentrations from 10 to 50 mM were found to inhibit AR I (K_i = 2.5 × 10^{-2} M) but not AR II, while 3-pyridine carboxaldehyde at 10 mM or greater inhibited both enzymes. One might speculate that this substrate inhibition was isomer dependent. This possibility was not investigated. In the study of a similar enzyme from liver, Attwood and Doughty [1] found slightly different K_m values for the separated isomers of glyceraldehyde, but no isomer-dependent inhibition.

Discussion

Two distinct NADPH-linked aldose-reducing enzymes exist in calf brain. These enzymes, designated AR I and AR II, have similar molecular weights as shown by the results of gel filtration and sodium dodecyl sulfate gel electrophoresis using calibrated columns and molecular weight markers. AR I appears to be a monomer in equilibrium with a less active dimer as indicated by the zymogram vital stains and sodium dodecyl sulfate gels.

Polyacrylamide gel zymogram, and Ouchterlony immunodiffusion studies indicated that the AR II enzyme was monomeric. The AR II preparation was nearly homogeneous (> 99% pure). The trace contaminant was not antigenically related to the enzyme-active protein.

Substrate profiles for AR I and AR II revealed that both had a general specificity for aldehydes. However, AR II demonstrated significantly higher conversion rates of the sugars, xylose and galactose to their respective sugar alcohols. The relative activity was found to be highest in AR I for several non-aldose aldehydes, particularly 3-pyridine carboxaldehyde and *p*-nitrobenzaldehyde. Substrate profiles similar to AR I have been found for a number of enzymes from calf tissues. These enzymes have been named aldehyde reductases [23—25] or hexonate dehydrogenases [26—28].

A number of additional properties which clearly distinguished AR I from AR II were defined. Sulfate anions, phenobarbital, and sulfhydryl reagents affected AR I and AR II in a manner unique to each enzyme.Both enzymes were inhibited by high substrate concentrations. However, when the same substrate was compared, AR II was affected by a relatively higher concentration than was AR I.

Differences in K_m values and the biphasic nature of the double reciprocal

plots for AR II compared to the linear double reciprocal plots for AR I further establish each enzyme as a separate entity.

A wide disparity in the isoelectric points of AR I and AR II was found. This finding plus the fact that the enzymes did not have antigenic similarity, eliminated the possibility that the separate activities isolated might be isoenzymes or one enzyme in two stable or conformational states. Similarly, this data showed that AR I could not have been formed from AR II as an artefact due to proteolysis during extraction and purification. Lens aldose reductase and AR II were found to have similar isoelectric points and immunochemical properties. An aldose-reducing enzyme in rabbit sciatic nerve was found to have an isoelectric point of 5, also similar to that of lens and AR II aldose reductases.

Upon comparing the physical and biologic properties of the aldose reductases from calf lens, brain and rabbit sciatic nerve, AR II appears to be the enzyme important in the pathogenesis of diabetic neuropathy.

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References

1 Attwood, M.A. and Doughty, C.C. (1974) Biochim. Biophys. Acta 370, 358—368
2 Hastein, T. and Velle, W. (1969) Biochim. Biophys. Acta 178, 1—10
3 Hayman, S. and Kinoshita, J. (1965) J. Biol. Chem. 240, 877—882
4 Gabbay, K. and Wah, J.T. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1435—1439
5 Sheys, G.H., Arnold, W.J., Watson, J.A., Hayashi, J.A. and Doughty, C.C. (1971) J. Biol. Chem. 246, 3824—3827
6 Faulkner, P. (1958) Biochim. J. 68, 374—380
7 Ward, J.D., Baker, R.W.R. and Davis, B.H. (1972) Diabetes 21, 1173—1178
8 Gabbay, K., Merola, L.O. and Field, R.A. (1966) Science 151, 209—210
9 Kinoshita, J.H. (1965) Invest. Ophthalmol. 10, 786—798
10 Varma, S.D. and Kinoshita, J.H. (1974) Biochim. Biophys. Acta 338, 632—640
11 Turner, A. and Tipton, K.F. (1972) Biochem. J. 130, 765—772
12 Sheaff, C.M. and Doughty, C.C. (1976) J. Biol. Chem., 251, 2696—2702
13 Chaykin, S. (1966) Biochemistry Laboratory Techniques, pp. 17—20, John Wiley and Sons, New York.
14 Rej, R. and Richards, A.H. (1974) Anal. Biochem. 62, 240—247
15 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
16 Hjerten, S. (1962) Arch. Biochem. Biophys. Suppl. 1, 147—151
17 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
18 Korman, A.W., Hurst, R.O. and Flynn, T.G. (1972) Biochim. Biophys. Acta 258, 40—55
19 Dray, S., Young, G. and Gerald, L. (1963) J. Immunol. 91, 403—415
20 Dixon, W.J. (1969) Biomedical Computer Programs, pp. 233—257, University of California Press, Berkeley
21 Clements, Jr., R. Morrison, A. and Winegrad, A. (1969) Science 166, 1007—1008
22 Moonsammy, G.I. and Stewart, M.A. (1967) J. Neurochem. 14, 1187—1193
23 Turner, A. and Tipton, K. (1972) Eur. J. Biochem. 30, 361—368
24 Bosron, W.F. and Prairie, R.L. (1972) J. Biol. Chem. 247, 4480—4485
25 Bosron, W.F. and Prairie, R.L. (1973) Arch. Biochem. Biophys. 154, 166—172
26 Mano, Y., Suzuki, K., Yamada, K. and Shimanzono, N. (1961) J. Biochem. Tokyo 49, 618—634
27 Gabbay, K. and O'Sullivan, J.B. (1968) Diabetes 17, 300
28 Clements, Jr., R.S. Weaver, J.P. and Winegrad, A. (1969) Biochem. Biophys. Res. Commun. 37, 347—353