Purification and characterization of an enzymically active cleavage product of pig kidney aldehyde reductase

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(Received 25 June 1982/Accepted 15 October 1982)

During the purification of pig kidney aldehyde reductase by an established procedure [Flynn, Cromlish & Davidson (1982) *Methods Enzymol.* **89**, 501–506] a second enzyme with aldehyde reductase activity may be purified. When the procedure was performed in the presence of 5 mM-EDTA, only traces of the second reductase, pig kidney aldehyde reductase (minor form), were present. By the criterion of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, pig kidney aldehyde reductase (minor form) had M_r 35 000, in comparison with M_r 40 200 found for pig kidney aldehyde reductase. Amino acid analysis of both enzymes and tryptic-peptide-map comparisons indicated differences in primary structure. The *N*-terminus of pig kidney aldehyde reductase (minor form) had the sequence Lys-Val-Leu, in contrast with the blocked (acetylated) *N*-terminus of pig kidney aldehyde reductase. The *C*-terminal sequence of both enzymes was the same. Both reductases were immunologically identical by double immunodiffusion and rocket immunoelectrophoresis. Pig kidney aldehyde reductase (minor form) had 50% of the specific activity of pig kidney aldehyde reductase when tested with a variety of aldehyde substrates. Michaelis constants of both enzymes for these substrates and for NADPH were similar, but values for k_{cat} and k_{cat}/K_m indicated that catalytically pig kidney aldehyde reductase was the more efficient enzyme. Typical aldehyde reductase inhibitors, such as phenobarbital and sodium valproate, had the same effect on both enzymes. It was concluded that pig kidney aldehyde reductase (minor form) is an enzymically active cleavage product of pig kidney aldehyde reductase which is formed when the latter is purified in the absence of the metalloproteinase inhibitor EDTA.

Multiple forms of the enzyme aldehyde reductase have been observed in several mammalian tissues. In pig brain there are two major enzymes, which have been designated the high- K_m and low- K_m forms respectively (Turner & Tipton, 1972). The high- K_m form is identical with the enzyme formerly known as glucuronate reductase (EC 1.1.1.19) or L-hexonate dehydrogenase from pig kidney (Mano & Shimazono, 1970; Bosron & Prairie, 1972) and currently named pig kidney aldehyde reductase. The other major form in brain (Hoffman *et al.*, 1980; Turner & Tipton, 1972) may be identifiable with aldose reductase (EC 1.1.1.21), an enzyme implicated in the etiology of some of the complications of diabetes (Gabbay, 1975). Two additional aldo-

keto reductases (Bachur, 1976) are also present in human brain, one of which is a succinic semi-aldehyde reductase (Hoffman *et al.*, 1980) and the other a ketone or carbonyl reductase (Wermuth, 1981). Two major forms of aldehyde reductase have also been found in the livers of mouse (Tulsiani & Touster, 1977) and rat (Rivett *et al.*, 1981), and in human (Wermuth *et al.*, 1977) and pig (Branlant & Biellman, 1980) liver. The question of the number of forms of aldehyde reductase in a given tissue and relationship of one or more of these forms with an enzyme previously named under a specific name may be even more complex. For example, four aldehyde reductases have been isolated from rabbit liver, two of which may be identical with 3(17) β -hydroxy steroid dehydrogenase and 3 α -hydroxy steroid dehydrogenase respectively (Sawada *et al.*, 1980). Guinea-pig liver contains three aldo-keto

Abbreviations used: SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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reductases, two of which also may be steroid dehydrogenases (Sawada *et al.*, 1979a,b). The major form in both rabbit and guinea-pig liver appears to be identifiable with the high- K_m form of aldehyde reductase, but the substrate specificity of the other enzymes in these tissues suggests that they may be more typical of the ketoreductases or even true aldo-keto reductases.

There is therefore some uncertainty regarding the specific nature and number of aldehyde reductases in tissues, and this, together with the undetermined metabolic roles of these enzymes, has resulted in a confusing and often conflicting nomenclature (Flynn, 1982). In view of this, it is exceedingly important to establish the number of distinct and different aldehyde reductases present in each tissue and to distinguish between expressed gene products and enzymes which have been subjected to some kind of post-translational processing. The present paper describes the isolation and purification of a lower-molecular-weight form of pig kidney aldehyde reductase which co-purifies with the major form. This enzyme appears to be an enzymically active cleavage product of pig kidney aldehyde reductase and not a separate and distinct aldehyde reductase.

Materials and methods

Materials

Iodoacetic acid, diphenylhydantoin, pyrazole, chlorpromazine, quercetin, DL-glyceraldehyde, pyridine-3-aldehyde, NADH, NADP⁺, *p*-iodotetrazolium Violet, phenazine methosulphate, glycerol, polyethylene glycol (average M_r 200), trypsin (diphenylcarbamoyl chloride-treated), D-glucuronate, succinic semialdehyde, indolylacetaldehyde bisulphite and D-xylose were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Blue Sepharose CL-6B and DEAE-Sephacel were purchased from Pharmacia (Canada) Ltd., Toronto, Canada. Boehringer Mannheim (Canada) supplied carboxypeptidase A and NADPH, and *p*-nitrobenzaldehyde, anhydrous hydrazine and *p*-nitroacetophenone were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Sodium valproate was a gift from Abbott Laboratories, Montreal, Canada, and Sorbinil (CP-45,534) was a gift from Pfizer, Groton, CT, U.S.A. Alrestatin (AY-22,284) was a gift from Ayerst, Montreal, Canada. All other chemicals were of the highest purity available.

Free indolylacetaldehyde was prepared from the bisulphite adduct as described by Brown & Purves (1976). The concentration of free indolylacetaldehyde was determined by using an absorption coefficient of $5400\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 280 nm. NADPH and NADH concentrations were determined by using an absorption coefficient of $6.22 \times 10^3\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 340 nm.

Purification of enzymes

The initial steps of the purification procedure, i.e. homogenization of kidneys, precipitation with $(\text{NH}_4)_2\text{SO}_4$ and column chromatography on Sephadex G-100, were identical with those described previously for the purification of pig kidney aldehyde reductase (Flynn *et al.*, 1982).

Chromatography on Blue Sepharose CL-6B. Fractions from the Sephadex G-100 column containing enzyme activity were pooled and added to a suspension [18 g of Blue Sepharose CL-6B in sufficient 10 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol (Buffer A) to make a thick slurry] of Blue Sepharose CL-6B, previously equilibrated in Buffer A, and the mixture was stirred for 12 h. After that the mixture was allowed to settle and all but 100 ml of the supernatant was aspirated. The slurry remaining was poured into a column (2.5 cm $\times$ 12.5 cm) and washed with 1 litre of Buffer A. The column was then eluted with a linear salt gradient [750 ml of Buffer A + 750 ml of Buffer A containing 1 M-NaCl (Buffer B)]. Fractions containing enzyme activity were pooled and concentrated to 100 ml by ultrafiltration. The concentrated solution was then dialysed against 9 litres of 5 mM-sodium phosphate buffer, pH 7.4, containing 5 mM-2-mercaptoethanol (Buffer C). The specific conductivity of the enzyme solution was less than 1 mS/cm after dialysis.

Chromatography on DEAE-Sephacel. The dialysed enzyme solution was applied to a DEAE-Sephacel column (2.5 cm $\times$ 30 cm) which had been previously equilibrated with Buffer C. The column was washed with 500 ml of Buffer C and then eluted with a linear salt gradient [1 litre of Buffer C + 1 litre of 200 mM-sodium phosphate buffer, pH 7.4, containing 5 mM-mercaptoethanol (Buffer D)]. Two peaks of aldehyde reductase activity were eluted from this column (Fig. 1). The fractions comprising each peak were pooled and concentrated to approx. 10 ml by ultrafiltration.

Chromatography on Sephadex G-75. The solutions of each peak of aldehyde reductase obtained in the previous step were applied separately to a column of Sephadex G-75 (2.5 cm $\times$ 80 cm) previously equilibrated with Buffer A. Fractions containing enzyme activity were pooled. Enzyme solutions intended for kinetic analysis were dialysed twice against 3 litres of distilled water containing 30 mg of polyethylene glycol/ml and 5 mM-mercaptoethanol and then frozen in 10 ml batches at -20°C . Enzyme solutions for chemical and physical characterization studies were dialysed against 4×3 litres of distilled water and then freeze-dried.

Assay methods

Protein concentrations were measured by the method of Bradford (1976) with bovine γ -globulin as standard as described in Bio-Rad Technical Bulletin

1051 (1977). Aldehyde reductase activity was determined spectrophotometrically in a Beckman 25 recording spectrophotometer by measuring the decrease in A_{340} of NADPH. A standard assay mixture consisted of 200 μ M-NADPH, 6 mM-pyridine-3-aldehyde, 100 mM-sodium phosphate buffer, pH 7.0, and 0.1 ml of enzyme solution in a total volume of 2.5 ml. Reactions were carried out at 22°C and were initiated by addition of enzyme. One unit of enzyme activity is the amount of enzyme that catalyses the oxidation of 1 μ mol of NADPH/min at 22°C.

For the determination of kinetic constants slightly different conditions were used. Assays were performed at 30°C and assay mixtures contained 100 mM-sodium phosphate buffer, pH 6.5, 160 μ M-NADPH, enzyme solution and various concentrations of aldehyde substrates in a total volume of 2.5 ml. Kinetic constants for NADPH were determined with 25 mM-pyridine-3-aldehyde as substrate. Kinetic constants were calculated by using a linear-regression computer program for double-reciprocal plots, which yielded values for K_m , V_{max} , and their standard deviations.

In studying the effects of drugs on the activity of aldehyde reductase, the various inhibitors were added to a reaction mixture consisting of 160 μ M-NADPH, 15 mM-pyridine-3-aldehyde and 100 mM-sodium phosphate buffer, pH 6.5, immediately before the reaction was started by the addition of enzyme. Assays, in a total volume of 2.5 ml, were performed at 30°C. Values of percentage inhibition are expressed as averages of duplicates.

Electrophoresis

Anionic polyacrylamide-gel electrophoresis was performed by the method of Gabriel (1971). The separating gel consisted of 7.5% acrylamide and 0.18% bisacrylamide at pH 8.9 and the stacking gel (riboflavin-polymerized) consisted of 2.0% acrylamide and 0.625% bisacrylamide at pH 6.7. The slab gels were run in a Bio-Rad electrophoresis apparatus at a constant current of 20 mA. The upper electrode buffer contained 5 mM-2-mercaptoethanol to prevent oxidation of protein during electrophoresis. Gels were stained for enzyme activity by incubating them at room temperature in the dark in a mixture consisting of 30 mg of NADP⁺, 17.5 mg of Tetrazolium Violet, 1 mg of phenazine methosulphate, 4.5 g of glycerol and 45 ml of 60 mM-sodium phosphate buffer, pH 8.0. After staining, the gels were fixed and stored in a mixture of water/glycerol/acetic acid/ethanol (2:1:1:1, by vol.). Gels were stained for protein with Coomassie Brilliant Blue R-250.

Polyacrylamide-gel electrophoresis in SDS was performed with a 10%-acrylamide separating gel as described by Laemmli (1970). The gels were stacked at a constant current of 20 mA and run at a constant

current of 40 mA. Gels were stained for protein as described above.

Immunological techniques

Antiserum to purified pig kidney aldehyde reductase was obtained after injection of the antigen (1 mg of enzyme in 1.0 ml of 10 mM-sodium phosphate buffer containing 0.9% NaCl emulsified in 1 ml of Freund's complete adjuvant) into young New Zealand White rabbits. The antigen was injected subcutaneously at four sites (two subscapular and two pelvic). Subsequent doses of 1 mg of aldehyde reductase in Freund's incomplete adjuvant were injected intramuscularly at 2-weekly intervals. Antibodies were detected by double immunodiffusion 6 weeks after the first injection. Animals were bled out by cardiac puncture and the pooled serum was stored frozen at -20°C. Double immunodiffusion (Ouchterlony, 1968) was performed in 0.83% agarose in 0.1 M-Tris/saline buffer, pH 7.2, for 24 h at room temperature. Rocket immuno-electrophoresis was performed in 1.0% agarose in 0.2 M-Tris/citrate buffer, pH 8.2, containing 2% (v/v) of partially purified antiserum. Electrophoresis was for 12 h at 2 V/cm. Fused rocket immunoelectrophoresis was performed under identical conditions, except that the applied enzyme was allowed to diffuse into the gel for 30 min before the current was applied. All precipitin lines were visible without staining. However, for photographic purposes the gels were washed with 0.3 M-NaCl and then stained with Coomassie Blue G-250 as described by Blakesley & Boezi (1979).

Peptide-map analysis

Pig kidney aldehyde reductases were reduced and carboxymethylated before tryptic digestion. Enzyme (2.0 mg) was added to 1.0 ml of 0.1 M-Tris/HCl buffer, pH 8.0, containing 5 M-guanidinium chloride and 2 mM-dithiothreitol and, after flushing with N₂, the mixture was heated at 37°C for 40 min. Iodoacetic acid (a 50 mM solution previously neutralized with NaOH) was then added to give a 1:1:1 molar ratio of thiol reagent to thiol groups. The mixture was again flushed with N₂ and incubated in the dark at 37°C for 1 h. Then further additions of dithiothreitol and iodoacetic acid were made as described above, except that this time only half the amounts used previously were added. The reaction was terminated by the addition of 10 μ l of 2-mercaptoethanol followed by dialysis against 4 litres of 2.5% (v/v) acetic acid. The non-diffusible material was then freeze-dried.

The freeze-dried enzyme was dissolved in 0.8 ml of 0.1 M-(NH₄)₂CO₃, and diphenylcarbamoyl chloride-treated trypsin (0.2 mg of trypsin/ml of 1 M-HCl) was added in three batches 8 h apart to give a final enzyme/substrate ratio of 1:100 (w/w).

Before each addition of trypsin, the mixture was sonicated for 1 min with a Biosonik III (Bronwill Scientific Co., Rochester, NY, U.S.A.). 300 W-power-source sonicator with a 5 mm probe operating at one-third maximum intensity. The reaction was terminated by freeze-drying the mixture.

Peptide mapping was performed by the method of Yaguchi & Wittman (1975). The digested protein (0.3–0.5 mg) was applied to microcrystalline cellulose sheets. Electrophoresis was run for 2.5 h and chromatography required 7 h. For detection of peptides the dried sheets were dipped in ninhydrin/cadmium reagent (Heilman *et al.*, 1957) and heated in an oven for 10–15 min at 60°C.

Amino acid analysis

Enzyme samples (approx. 0.5 mg) were hydrolysed *in vacuo* in 6 M-HCl at 110°C for 48 h. Each sample was analysed in duplicate on a Beckman model 120C analyser modified for single-column analysis. Analysis was also performed on performic acid-oxidized (Hirs, 1967) samples in order to determine cysteine as cysteic acid and methionine as methionine sulphone.

C-Terminal sequence analysis

The sequence of amino acids at the C-terminal of both pig kidney aldehyde reductases was determined as follows by using carboxypeptidase A. Enzyme (1.5 mg) was dissolved in 0.4 ml of 0.2 M-*N*-ethylmorpholine/acetate buffer, pH 8.5, containing 1% SDS and 20 nmol of norleucine as internal standard. Carboxypeptidase A, previously washed with water to remove contaminating amounts of free amino

acids, was added to the mixture to give an enzyme/substrate ratio of 1:40 (w/w). The concentration of carboxypeptidase A was determined by using an absorption coefficient of $8.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 278 nm (Ambler, 1967). A control containing buffer and carboxypeptidase alone was used to correct for amino acids not removed by the washing procedure. The reaction mixtures were incubated at 37°C. Samples were removed at 0.5, 1.0, 2.0 and 5.0 h and mixed with an equal volume of 1 M-acetic acid. After centrifugation at 2°C for 20 min at 10000g, the supernatants were freeze-dried and subjected to amino acid analysis.

N-Terminal sequence analysis

N-Terminal analysis was performed on a Beckman 890C automatic sequencer by using a 0.33 M-Quadrol program [a modification of the Beckman dilute Quadrol (0.1 M) program no. 12.11.78]. Amino acids were converted into their phenylthiohydantoin derivatives and quantified by high-pressure liquid chromatography as described by Johnson *et al.* (1979). The method of Schmer & Keil (1969) was used for the determination of *N*-acetylated amino acids. Acetyldansylhydrazide was identified by the chromatographic system of Woods & Wang (1967) as described by Takagi & Doolittle (1974).

Results and discussion

Purification and isolation of aldehyde reductase forms

Aldehyde reductase activity was resolved into two peaks of activity by chromatography on DEAE-Sephacel (Fig. 1).

Fig. 1. Chromatography on DEAE-Sephacel of pig kidney aldehyde reductases. A, pig kidney aldehyde reductase; B, pig kidney aldehyde reductase (minor form). Fractions comprising each peak (horizontal bars) were collected. A_{280} represents protein.

The enzyme eluted first from the DEAE-Sephacel column was eluted in a position coincident with the pig kidney aldehyde reductase previously purified in this laboratory (Davidson & Flynn, 1979; Flynn *et al.*, 1982). The second aldehyde reductase activity eluted from the DEAE-Sephacel column has not been purified previously. Both aldehyde reductases

were homogeneous by the criteria of polyacrylamide-gel electrophoresis either alone (Fig. 2) or in the presence of SDS (Fig. 3). The results of a typical purification procedure are summarized in Table 1. However, when the purification was performed in the presence of 5mM-EDTA, only pig kidney aldehyde reductase was eluted from the DEAE-

Fig. 2. Polyacrylamide-gel electrophoresis of pig kidney aldehyde reductases
Lanes 1 and 3, pig kidney aldehyde reductase (minor form); lanes 2 and 4, pig kidney aldehyde reductase and pig kidney aldehyde reductase (minor form). Panel (a) was stained for protein with Coomassie Brilliant Blue; panel (b) was stained for enzyme activity as described in the Materials and methods section.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of pig kidney aldehyde reductases
Lane 1, standard proteins [phosphorylase b (*M_r* 92 500), bovine serum albumin (*M_r* 66 200), ovalbumin (*M_r* 45 000), carbonic anhydrase (*M_r* 31 000), soya-bean trypsin inhibitor (*M_r* 21 500) and lysozyme (*M_r* 14 300)]; lane 2, pig kidney aldehyde reductase and pig kidney aldehyde reductase (minor form); lane 3, pig kidney aldehyde reductase (minor form); lane 4, pig kidney aldehyde reductase.

Table 1. Purification of pig kidney aldehyde reductase and pig kidney aldehyde reductase (minor form)
The Table shows the purification of aldehyde reductase activity from 845 g of pig kidney in the absence of 0.5mM-phenylmethanesulphonyl fluoride and 5mM-EDTA. For details see the text.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Supernatant after first centrifugation	1830	30 500	1620	0.053	100
Pellet from second (NH ₄) ₂ SO ₄ fractionation	145	6840	1436	0.21	89
Sephadex G-100	1600	3111	1478	0.48	91
Blue Sepharose pool	156	124	523	4.22	32
DEAE-Sephacel					
Aldehyde reductase	152	30.1	351	11.7	22
Aldehyde reductase (minor form)	86	5.4	32	5.9	2
Sephadex G-75					
Aldehyde reductase	113	28.9	341	11.8	21
Aldehyde reductase (minor form)	69	4.3	28	6.6	2

Sephacel column and, by the criterion of polyacrylamide-gel electrophoresis followed by staining for enzyme activity as described in the Materials and methods section, only traces of the second aldehyde reductase were evident at other stages of the purification procedure. In other experiments, the serine-proteinase inhibitor phenylmethanesulphonyl fluoride (0.5 mM) was used with and without EDTA, but it was clear that only EDTA was able to prevent the formation of the additional peak of aldehyde reductase activity. Neither phenylmethanesulphonyl fluoride nor EDTA had any effect on the enzymic activity of either of the enzymes. It therefore appears that EDTA acted as a metallo-proteinase inhibitor (Barrett, 1977) in preventing formation of the minor form. The small amounts of this form that could be detected may be the result either of incomplete inhibition of proteolytic activity during homogenization, or of proteolysis before homogenization. In view of these results, and because of the relative peak heights of the enzymes eluted from the DEAE-Sephacel column, we have designated the enzyme present in the second peak as pig kidney aldehyde reductase (minor form).

Aldehyde reductase from pig kidney has been purified and characterized by several workers (Bosron & Prairie, 1972; Flynn *et al.*, 1975; Morpeth & Dickinson, 1980), but it is apparent that many of these preparations were not homogeneous by the criterion of polyacrylamide-gel electrophoresis and that additional enzymically active enzyme forms were present (Bosron & Prairie, 1972; Flynn *et al.*, 1975). The results described in the present paper reveal the dangers inherent in identifying, on the basis of enzyme activity and in the absence of suitable precautions, forms of aldehyde reductase which are not necessarily the expressed gene products.

Determination of molecular weight

Molecular weights were determined by the technique of SDS/polyacrylamide-gel electrophoresis by using appropriate molecular-weight standards (see Fig. 3). From the curve of the relative mobilities of the standards plotted against the logarithm of their molecular weights an estimate of M_r 40 200 was obtained for pig kidney aldehyde reductase and M_r 35 000 for pig kidney aldehyde reductase (minor form). By gel filtration on Sephadex G-75 and by reference to appropriate molecular-weight markers the M_r values of both enzymes were calculated to be $34\,000 \pm 3000$ (s.d.). Despite the discrepancy in the estimates of molecular weight by the different methods, which have been noted previously (Bosron & Prairie, 1972; Flynn *et al.*, 1975; Morpeth & Dickinson, 1980), it is apparent that both aldehyde reductases are monomeric.

Immunological studies

Antiserum to pig kidney aldehyde reductase cross-reacted with complete identity with its homologous antigen and pig kidney aldehyde reductase (minor form) when examined by double immunodiffusion (Fig. 4). Fused rocket immunoelectrophoresis of fractions from stages in the purification procedure, e.g. the Sephadex G-100 stage, showed a continuous single precipitin line, indicating that a single antigen, or immunologically identical antigens which behave identically on gel filtration, cross-reacted with the antiserum to purified pig kidney aldehyde reductase. In addition, equal amounts of pig kidney aldehyde reductase and the minor form gave identical responses on rocket immunoelectrophoresis (i.e. rockets of the same width and height), and when both antigens were combined in equal proportions an additive response was obtained on rocket immunoelectrophoresis (results not shown). Antiserum to pig kidney aldehyde reductase was also effective in inhibiting the enzymic activity of both aldehyde reductases (Fig. 5). The immunological identity of pig kidney aldehyde reductase and pig kidney aldehyde reductase (minor form) indicate that they have the same antigenic determinants and the same or closely similar tertiary structures despite the difference in their molecular weights. These observations, together with the fact

Fig. 4. Double immunodiffusion of pig kidney aldehyde reductases

The centre well contained antiserum to pig kidney aldehyde reductase. Wells 1 and 3, pig kidney aldehyde reductase (minor form); wells 2 and 4, pig kidney aldehyde reductase. No precipitin lines were obtained when antiserum to pig kidney aldehyde reductase (centre well) was replaced by pre-immune serum.

Fig. 5. Inhibition of enzyme activity of pig kidney aldehyde reductase (Δ) and pig kidney aldehyde reductase (minor form) ($\blacktriangle$) by antiserum to pig kidney aldehyde reductase

Each enzyme ($2.0\ \mu\text{g}$ in $60\ \mu\text{l}$ of $0.1\ \text{M}$ -phosphate buffer, pH 7.0) was incubated with portions (2.5 – $40\ \mu\text{l}$) of antiserum and buffer in a final volume of $100\ \mu\text{l}$ at 4°C for 30 min. Each sample was assayed for enzyme activity by the standard assay.

that the minor form is absent when the purification procedure is performed in the presence of the metallo-proteinase inhibitor EDTA, suggest that the minor form is produced by proteolysis of pig kidney aldehyde reductase.

Enzyme stability

Exhaustive dialysis of pig kidney aldehyde reductase against several changes of distilled water followed by freeze-drying of the enzyme resulted in the loss of approx. 5% of the total enzyme activity. In contrast, similar treatment of pig kidney aldehyde reductase (minor form) resulted in a progressive loss of enzyme activity and precipitation of the enzyme during dialysis. The dialysed freeze-dried preparation of the minor form had only 5% of the activity of the enzyme before dialysis. When this preparation was reconstituted in $10\ \text{mM}$ -sodium phosphate buffer, pH 7.0, containing $5\ \text{mM}$ -2-mercaptoethanol the enzyme exhibited a decreased antigenic response (as shown by rocket immunoelectrophoresis) by comparison with an equal amount of undialysed non-freeze-dried enzyme. It seems therefore that antiserum to pig kidney aldehyde reductase only interacts favourably with enzymically active pig kidney aldehyde reductase (minor form), indicating that immunoreactivity depends on the intact tertiary structure of the antigen.

Catalytic properties

Both aldehyde reductases exhibited broad

superimposable pH-activity profiles, with pH optima in the region of 6.5.

The equilibrium of the catalysed reaction was found to lie markedly in the direction of the reduction of aldehyde for both enzymes. Attempts at detecting the reverse reaction by using $10\ \text{mM}$ -pyridine-3-methanol and $200\ \mu\text{M}$ -NADP⁺ in $0.1\ \text{M}$ -sodium phosphate buffer, pH 10, were not successful. Both aldehyde reductases appear to use NADPH exclusively as coenzyme, since no activity could be detected in either case when $200\ \mu\text{M}$ -NADH was used in place of NADPH. In addition, no inhibition of enzyme activity was observed for either enzyme when NADH was included in assay mixtures.

Kinetic constants were obtained for both pig kidney aldehyde reductase and pig kidney aldehyde reductase (minor form) with a variety of aldehyde substrates (Table 2). The Michaelis constants obtained for all the aldehydes tested, and for NADPH, were very similar for both enzymes. In terms of catalytic efficiency (K_{cat}/K_m ; Fersht, 1977), *p*-nitrobenzaldehyde, succinic semialdehyde and indol-3-ylacetaldehyde were the best substrates, with the preferred specificity of both enzymes being for *p*-nitrobenzaldehyde. Table 2 also shows that, in terms of V_{max} and k_{cat}/K_m , pig kidney aldehyde reductase is the most efficient enzyme. Nevertheless, considerable enzymic activity is exhibited by the minor form, which is surprising for an enzyme that has lost approx. 50 amino acids from its *N*-terminal end. For all the substrates tested the values of V_{max} obtained for the minor form were 40–50% lower than the corresponding values obtained with pig kidney aldehyde reductase. The lower values for k_{cat} may indicate a slower rate-limiting step for the reduction of aldehydes by pig kidney aldehyde reductase (minor form) or that, compared with pig kidney aldehyde reductase, 40–50% of the enzyme molecules of the cleaved product are inactive.

Non-competitive inhibitors of aldehyde reductase, such as phenobarbital ($1\ \text{mM}$; Erwin & Deitrich, 1973), diphenylhydantoin ($0.1\ \text{mM}$) and sodium valproate (Turner & Whittle, 1981) caused considerable inhibition (70–100%) of both enzymes. Alrestatin (1,3-dioxo-1*H*-benzo[*de*]isoquinolin-3-yl-acetic acid) and Sorbinil (*d*-6-fluorospiro[chroman-4,4'-imidazolidine]-2',5'-dione), non-competitive inhibitors of aldose reductase (EC 1.1.1.21; Gabbay *et al.*, 1979; Peterson *et al.*, 1979), were also equally effective in inhibiting pig kidney aldehyde reductase and the minor form. Sorbinil proved to be a particularly effective inhibitor, giving 70% inhibition of the activity of both enzymes at a concentration ($0.01\ \text{mM}$) of the same order of magnitude as that which inhibited calf lens aldose reductase (Peterson *et al.*, 1979). Alrestatin pro-

Table 2. *Kinetic constants for pig kidney aldehyde reductase and pig kidney aldehyde reductase (minor form)*
For details see the text.

Substrate	Pig kidney aldehyde reductase*			Pig kidney aldehyde reductase† (minor form)		
	K_m (mM)	V_{max} ($\mu\text{mol/min}$)	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{M}^{-1}$)	K_m (mM)	V_{max} ($\mu\text{mol/min}$)	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{M}^{-1}$)
<i>p</i> -Nitrobenzaldehyde‡	0.43 ± 0.07	0.17 ± 0.02	130 000	0.47 ± 0.12	0.09 ± 0.01	54 500
Pyridine-3-aldehyde	2.4 ± 0.3	0.18 ± 0.04	16 800	2.2 ± 0.3	0.06 ± 0.002	8000
DL-Glyceraldehyde	2.8 ± 0.3	0.03 ± 0.00	3900	3.1 ± 0.4	0.02 ± 0.002	2100
D-Glucuronate	5.8 ± 1.8	0.06 ± 0.01	3600	4.5 ± 1.0	0.03 ± 0.002	2200
D-Xylose	2300 ± 1900	0.04 ± 0.002	6	2010 ± 1050	0.02 ± 0.007	3
Succinic semialdehyde	0.19 ± 0.03	0.04 ± 0.001	66 000	0.18 ± 0.3	0.02 ± 0.002	36 000
Indol-3-ylacetaldehyde	0.7 ± 0.6	0.05 ± 0.02	25 000	0.94 ± 0.7	0.03 ± 0.01	7800

NADPH 5.0 ± 1.8 (μM)

6.4 ± 2.1 (μM)

* Assay mixture contained 50 pmol of enzyme.

† Assay mixture contained 57 pmol of enzyme.

‡ Assayed in the presence of 2% (v/v) methanol.

duced a 65% inhibition of both reductases at a concentration of 0.1 mM. Quercetin (0.01 mM), also used previously as an aldose reductase inhibitor (Varma, 1980), inhibited pig kidney aldehyde reductase and the minor form by 48% and 63% respectively. Pyrazole (1 mM) and chlorpromazine (0.1 mM) caused only slight inhibition (2–10%) of both reductases.

It has also been shown previously that the high- K_m aldehyde reductase of ox brain is non-competitively inhibited ($K_i = 8.9 \times 10^{-3}$ M) by Alrestatin (Whittle & Turner, 1981). Both Alrestatin and Sorbinil have been used effectively in the treatment of the complications of diabetes in rats (Gabbay *et al.*, 1979; Peterson *et al.*, 1979), but the results of studies with human diabetic patients have not been successful. Little attention has been paid, however, to the effect of these drugs on other aldehyde reductases. The results of the present study show that these effects have to be taken into account in studies on the inhibition of aldose reductase in relation to the complications of diabetes.

Amino acid analysis

The amino acid compositions of both aldehyde reductases are shown in Table 3. Compositional differences between the two enzymes are quite marked and reflect the lower molecular weight of the minor form. The composition of the fragment or fragments removed by protolysis was Asp₂, Glu₂, Pro₈, Ala₉, Cys₁, Ile₁, Leu₈, Tyr₈, His₃, Arg₇, Met₁, which in itself is not unusual in any respect. However, the lower number of arginine residues present in the minor form resulted in the production of fewer tryptic peptides for peptide mapping (see below).

Table 3. *Amino acid composition of pig kidney aldehyde reductase and pig kidney aldehyde reductase (minor form)*

For details see the text. The values shown are the nearest integer values of the average of two sets of duplicate analyses.

Amino acid	Pig kidney aldehyde reductase (no. of residues/molecule)	Pig kidney aldehyde reductase (minor form) (no. of residues/molecule)
Aspartic acid/asparagine	33	29
Threonine	17	17
Serine	17	18
Glutamic acid/glutamine	41	39
Proline	28	20
Glycine	20	20
Alanine	36	27
Half-cystine*	4	3
Valine	25	26
Methionine*	4	3
Isoleucine	17	16
Leucine	35	27
Tyrosine	13	5
Phenylalanine	10	10
Lysine	19	19
Histidine	8	5
Arginine	21	14
Total no. of residues (excluding tryptophan)	348	300

* Estimated as cysteic acid and methionine sulphone respectively after treatment of the enzymes with performic acid.

Peptide maps

Tryptic peptide maps of pig kidney aldehyde reductase and pig kidney aldehyde reductase (minor form) are shown in Fig. 6. Equal amounts of enzymes were applied in each case and the fingerprints obtained were similar overall. However, more peptides were present in the map of pig kidney

aldehyde reductase than in that of the minor form, which is consistent with the additional cleavage points provided for by the greater number of arginine residues in pig kidney aldehyde reductase. In addition, two peptides (indicated by 'X' in Fig. 6b) are present in the tryptic map of pig kidney aldehyde reductase (minor form) that were not present in the map of pig kidney aldehyde reductase. This may be explained if the minor form was heterogeneous with respect to its *N*-terminal end, i.e. proteolysis of pig kidney aldehyde reductase has resulted in a 'ragged end' giving additional sites for tryptic cleavage. This suggestion is corroborated by the results of amino acid sequencing at both *N*- and *C*-termini.

N-Terminal analysis

Both aldehyde reductases were subjected to automatic sequencing analysis. Before sequencing, four pre-cycles of Polybrene and glycylglycine were run as described by Klapper *et al.* (1978). After five cycles of the sequencer no *N*-terminal amino acid was detected in pig kidney aldehyde reductase, indicating that the *N*-terminal of this enzyme is blocked. This is consistent with results previously obtained by us for pig kidney aldehyde reductase (T. G. Flynn, unpublished work) and by others for human liver aldehyde reductase (Omar *et al.*, 1980).

When pig kidney aldehyde reductase was treated with anhydrous hydrazine and dansyl chloride (Schmer & Keil, 1969) and analysed as described in the Materials and methods section, a fluorescent compound was obtained which, when subjected to two-dimensional chromatography, had an R_F value identical with that of acetyldansylhydrazide. No fluorescent material with the same chromatographic mobility as acetyldansylhydrazide was observed when pig kidney aldehyde reductase (minor form) was treated in the same way. In contrast, a distinct *N*-terminal sequence was obtained for pig kidney aldehyde reductase (minor form). However, at least two overlapping sequences were present, indicating that the proteolytic cleavage that had occurred during purification had not been at a specific site. The two major *N*-terminal sequences of pig kidney aldehyde reductase (minor form) are shown below:

C-Terminal analysis

In separate experiments both aldehyde reductases were incubated with carboxypeptidase A, and samples taken and analysed for released amino acids as described in the Material and methods section. The results obtained (see Fig. 7) show that both enzymes have identical *C*-terminal sequences:

Fig. 6. Tryptic peptide maps of pig kidney aldehyde reductase (a) and pig kidney aldehyde reductase (minor form) (b)

Electrophoresis in the first dimension (horizontal) was in pyridine/acetic acid/water buffer (1:10:89, by vol.), pH 3.6, and chromatography in the second dimension (vertical) was in pyridine/butan-1-ol/acetic acid/water (10:15:3:12, by vol.). For other details see the Materials and methods section.

Fig. 7. C-Terminal sequence analysis of pig kidney aldehyde reductase (a) and pig kidney aldehyde reductase (minor form) (b) by using carboxypeptidase A
Amino acids released; ▲, serine; ○, glycine; □, lysine; △, alanine; ●, valine. For details see the text.

The results of both C- and N-terminal analysis indicate that cleavage of pig kidney aldehyde reductase had occurred at two major sites, resulting in a cleavage product heterogeneous with respect to its N-terminal end.

The study described here yields the first report of an aldehyde reductase which is produced by proteolytic cleavage of a native form. In addition to the cleavage product, we have also recently detected aldose reductase and small amounts of another, as yet unidentified, aldehyde reductase in pig kidney (J. A. Cromlish & T. G. Flynn, unpublished work). Post-translational proteolytic modifications are often an obligatory step in the activation of many enzymes and proteins, e.g. digestive enzymes and proteins involved in blood coagulation. An example with perhaps the closest parallel to the present study is that of Horecker *et al.* (1975), who characterized an enzymically active cleavage product of fructose 1,6-bisphosphatase from rabbit liver and kidney. In this study proteolytic cleavage of a M_r -5000 fragment from the native enzyme was thought to be a physiologically significant enzyme activation. The proteolysis of pig kidney aldehyde reductase, however, is unlikely to represent activation, since kinetically the enzyme is largely unchanged and, moreover, formation of the cleavage product can largely be prevented by EDTA. It is more likely that the limited proteolysis of pig kidney aldehyde reductase is the first step in the complete degradative process of protein turnover, as suggested to be the case for any enzymes (Beynon, 1980, and references therein). Present studies on aldehyde reductase in

our and in other laboratories are directed towards determining the precise number of distinct aldehyde reductases present in a given tissue.

The research described in this paper was supported by a grant from the Medical Research Council of Canada. We are grateful to Mrs. Joyce Stacey and Mrs. Christine Gallerneault for invaluable technical assistance and to Ms. Janet Knox for typing the manuscript. We also thank Mr. Hans Metz for drawing the Figures.

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