Pig Muscle Aldehyde Reductase

IDENTITY OF PIG MUSCLE ALDEHYDE REDUCTASE WITH PIG LENS ALDOSE REDUCTASE AND WITH THE LOW $K_{m}$ ALDEHYDE REDUCTASE OF PIG BRAIN AND PIG KIDNEY

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A monomeric ($M_r = 43,000$) NADPH-dependent oxidoreductase with a broad substrate specificity for aliphatic and aromatic aldehydes and aldo sugars has been purified from the skeletal muscle of female and castrated pigs. The properties of this enzyme are consistent with those of aldose reductase (EC 1.1.1.21), and the enzyme is immunologically identical with aldose reductase from pig lens. However, antiserum to pig muscle aldehyde reductase also cross-reacts identically with the low $K_{m}$ aldehyde reductase from pig brain and kidney and the pattern of inhibition of the muscle reductase by anticonvulsant drugs is the same as that obtained for the low $K_{m}$ reductase. These intraspecies results yield the first definitive evidence that pig muscle aldehyde reductase is the same enzyme as aldose reductase and that the latter is identical with the low $K_{m}$ aldehyde reductase, one of two major aldehyde reductases found in mammalian tissues.

The aldehyde reductases (alcohol:NADP$^+$ oxidoreductases, EC 1.1.1.2) are a group of enzymes with a broad substrate specificity for aliphatic and aromatic aldehydes and aldo sugars. These enzymes are widely distributed throughout the animal and plant kingdoms (1), but their precise physiological role(s) is unknown. In mammals, the enzymes have been found in brain (2, 3), kidney (4, 5), liver (6-8), and muscle (9). There are two major aldehyde reductases in tissues and these are commonly referred to as the high $K_{m}$ and low $K_{m}$ aldehyde reductases, respectively (2). Also in tissues, there are a ketone or carbonyl reductase (3, 9) and a dimeric succinic semialdehyde reductase (3, 10). The aldehyde and ketone reductases have been shown to be monomeric NADPH-dependent enzymes with molecular weights in the 35,000-45,000 range (5, 11).

The broad, overlapping, substrate specificity and the lack of definitive physiological substrates for these enzymes have resulted in a confusing and often conflicting nomenclature. For example, the predominant aldehyde reductase has been called the high $K_{m}$ enzyme (2) or simply a tissue-specific aldehyde reductase, e.g. pig kidney aldehyde reductase (5), and has been abbreviated as AR1 (2) and AR3 (5). The other major enzyme has been called the low $K_{m}$ form (2) and abbreviated as AR2 (3). As discussed recently, none of these designations or abbreviations are satisfactory in the absence of a defined physiological substrate. 1 The problem of identity and nomenclature is exacerbated by the fact that in certain tissues, such as lens (12), there occurs a monomeric NADPH-dependent oxidoreductase of broad aldehyde substrate specificity which, because it has some affinity for D-glucose and other aldo sugars, has been classified separately as aldose reductase (EC 1.1.1.21). This enzyme is the first enzyme of the polyol pathway (13) and has been implicated in the etiology of the complications of diabetes. The general properties of aldose reductase are very similar to those of the aldehyde reductases, and there has been speculation as to whether aldose reductase is indeed a separate and unique monomeric oxidoreductase (14, 15). It is clear from immunological criteria, that aldose reductase and the high $K_{m}$ aldehyde reductase are not the same enzyme (16). Whittle and Turner (17) and others (3, 18) have suggested that aldose reductase is identical with the low $K_{m}$ aldehyde reductase, but no experimental data have been presented to substantiate this.

In the present paper, we demonstrate for the first time the intraspecies' identity of aldose reductase. Our study shows that an aldehyde reductase present in pig muscle is identical with pig lens aldose reductase and with the low $K_{m}$ aldehyde reductase of pig brain and pig kidney.

EXPERIMENTAL PROCEDURES

Materials—p-Nitrobenzaldehyde and p-nitroacetophenone were purchased from British Drug House (Canada) Ltd. D3-Glyceraldehyde, D-glucose, D-glucose, D-xylene, succinic semialdehyde, dihydroxyacetone, chlorpromazine, quercetin, and pyrazole were obtained from Sigma. NADPH was supplied by Boehringer-Mannheim, and chromatographic materials were purchased from Pharmacia. Materials for polyacrylamide gel electrophoresis were obtained from Bio-Rad. The drugs used in this study were gifts from various drug companies as indicated: sodium valproate (Abbott), Alrestatin (Ayerst), and Sorbinil (Pfizer). Ethacrynic acid and indomethacin were purchased from Sigma.

Preparation and Characterization of Enzyme—Aldehyde reductase activity was purified from 500 g of pig skeletal muscle (female or castrated pigs) by using, with minor modifications, a procedure for the purification of glycerol dehydrogenase from rabbit muscle (19). This method consists of 1), homogenization of tissue in 10 mM phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol, 2) fractionation with ammonium sulfate: an initial precipitation of protein at 0.5 saturation with the precipitate being discarded followed by addition of ammonium sulfate to a final saturation of 0.8 and the precipitate being retained, 3) gel filtration on Sephadex G-100, 4) chromatography on Blue Sepharose CL-6B, 5) chromatography on DEAE-Sephalac, and 6) gel filtration on Sephadex G-75. By this procedure, a single peak of aldehyde reductase activity was purified. When the procedure was attempted with boar muscle, two peaks of aldehyde dehydrogenase activity were evident, which is similar to the results obtained with rabbit muscle (19). Difficulty in obtaining boar muscle precluded preparation of both isoenzymes. The procedure used in this study resulted in the preparation of approximately 3 mg of aldehyde reductase from 500 mg of muscle. This enzyme migrated as a single band on polyacrylamide gel electrophoresis at pH 8.5 and on polyacrylamide gel electrophoresis in the presence of sodium...
dodecyl sulfate. By the latter technique, and using appropriate molecular weight standards (Bio-Rad), a molecular weight of 43,000 was obtained for pig muscle aldehyde reductase. A similar, but slightly lower (34,000 ± 4,000) molecular weight was obtained by gel filtration on Sephadex G-100. The enzyme is therefore a monomeric reductase.

Pig kidney aldehyde reductase was purified by the method of Flynn et al. (20). The low $K_\text{m}$ aldehyde reductase of pig kidney was used during the purification of pig kidney aldehyde reductase. Details of the purification will be published elsewhere. The low $K_\text{m}$ and high $K_\text{m}$ aldehyde reductase of pig brain were purified by major modifications of the method of Turner and Tipton (2), details of which will be published separately.

**Assay Methods**—For determining enzyme activity reaction mixtures contained 100 mM sodium phosphate buffer, pH 6.5, 0.4 M ammonium sulfate, 200 μM NADPH, 5 mM DL-glyceraldehyde, and 0.1 ml of enzyme solution in a total volume of 2.5 ml. Reactions were initiated by the addition of enzyme and the decrease in absorbance at 340 nm was measured in a Beckman model 25 spectrophotometer at 25 °C. Control cuvettes contained all reagents except DL-glyceraldehyde. One unit of enzyme activity is defined as the amount of enzyme which reduces 1 μmol of substrate/min under assay conditions. Protein concentrations were measured using the Bradford (21) method as described in Bio-Rad Technical Bulletin 1051 (1977).

**Immunological Techniques**—Purified pig muscle aldehyde reductase (1 mg in 1 ml of 10 mM phosphate buffer containing 0.9% NaCl) was emulsified in an equal volume of Freund's complete adjuvant and injected subcutaneously into rabbits at four sites (2 subscapular and 2 pelvic). Subsequent injections of the same amount of enzyme in Freund's incomplete adjuvant were administered intramuscularly at 2-week intervals. Antibodies were detected 6 weeks after the first injection. Double immunodiffusion was carried out essentially by the technique of Ouchterlony (22) using agarose (0.85%) in 0.1 M Tris-saline buffer, pH 7.2, as the diffusion medium. Immunodiffusion was carried out at room temperature for 24 h. All precipitin lines were visible without staining, but for photographic purposes, the lines were stained with Coomassie blue G-250.

**RESULTS AND DISCUSSION**

**Amino Acid Analysis**—The amino acid composition of pig muscle aldehyde reductase (Table I) was quite different from that of pig kidney aldehyde reductase (values in parentheses in Table I). Application of the method of Cornish-Bowden (23) for estimating the number of sequence differences between a pair of related proteins based on compositional data alone yielded a S&N value of 265 for a comparison of pig muscle and pig kidney aldehyde reductase. A value for S&N of 146 or less would be required to show significant homology between these enzymes (23). Thus, little or no sequence homology is indicated for pig muscle and pig kidney aldehyde reductase. This is consistent with the immunological results described below. Pig muscle and pig kidney aldehyde reductase were similar, however, in that both enzymes had blocked NH$_2$ termini.

**Immunological Cross-reactivity**—Antiserum to pig muscle aldehyde reductase gave a single precipitin line on double immunodiffusion with its homologous antigen (Fig. 1a). The antiserum also cross-reacted identically with the low $K_\text{m}$ aldehyde reductase from pig brain. Antiserum to pig muscle aldehyde reductase did not cross-react at all with pig kidney aldehyde reductase or with the high $K_\text{m}$ aldehyde reductase of pig brain (Fig. 1a). In separate experiments, it was shown that antiserum to pig kidney aldehyde reductase gave reactions of immunological identity with the high $K_\text{m}$ aldehyde reductase of pig brain.

### Table I

**Amino acid composition of pig muscle aldehyde (aldose) reductase**

| Amino acid | Residues/mol |
|-----------|--------------|
| Asp/Asn   | 39 (33)*     |
| Thr       | 17 (17)      |
| Ser       | 22 (17)      |
| Glu/Glu   | 47 (41)      |
| Pro       | 24 (29)      |
| Gly       | 32 (20)      |
| Ala       | 26 (36)      |
| Half-Cys* | 4 (4)        |
| Val       | 25 (25)      |
| Met*      | 3 (4)        |
| Ile       | 14 (17)      |
| Leu       | 37 (35)      |
| Tyr       | 13 (13)      |
| Phe       | 13 (10)      |
| Lys       | 31 (19)      |
| His       | 11 (8)       |
| Arg       | 12 (21)      |

* Values in parentheses are for pig kidney aldehyde reductase based on a molecular weight of 40,200 (20).

* Estimated as cysteic acid and methionine sulfone, respectively, after treatment of the enzyme with performic acid.

![Fig. 1. Double immunodiffusion analysis of pig aldehyde reductases.](image)

J. Cromlish, unpublished work.
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reductase of pig brain. Antiserum to pig muscle aldehyde reductase also gave a reaction of immunological identity with a preparation of pig lens aldose reductase (Fig. 1B) which was partially purified by the initial steps used by Shaff and Dougherty (12) for the purification of bovine lens aldose reductase. Pig lens did not contain the high $K_m$ aldose reductase since antiserum to pig kidney aldose reductase yielded no cross-reaction on double immunodiffusion with crude homogenates or with partially purified preparations. Antiserum to pig muscle aldose reductase also cross-reacted identically with the low $K_m$ reductase from pig kidney (Fig. 1C).

These results show that the aldehyde reductase present in pig muscle is immunologically identical with the low $K_m$ aldose reductase of pig brain and kidney and with pig lens aldose reductase. Muscle NADPH-dependent aldehyde reductase has been previously named glycerol dehydrogenase (9), but in recent work, we have shown that rabbit muscle contains two isoenzymes of glycerol dehydrogenase which have the characteristics of aldose reductase (19). It appears, therefore, that mammalian muscle characteristically contains a reductase which is identical with lens aldose reductase and the low $K_m$ aldose reductase of brain and kidney.

For example, with DL-glyceraldehyde as substrate, $K_m$ values of 0.093 mM, 0.08 mM, and 0.062 mM have been determined for aldose reductase of brain and kidney. A low $K_m$ aldehyde reductase of brain and kidney for which, using $p$-nitrobenzaldehyde as substrate, $K_m$ values of 0.013 mM and 0.018 mM, respectively, have been calculated (2, 16). Pig muscle aldose reductase also had lower $K_m$ values for aldo sugars than does pig kidney aldose reductase (Table II). In this respect, pig muscle aldose reductase resembles the low $K_m$ aldose reductases from rat brain and pig brain for which, using $p$-nitrobenzaldehyde as substrate, $K_m$ values of 0.015 mM and 0.018 mM, respectively, have been determined (2, 16). Pig muscle aldose reductase also had lower $K_m$ values for aldo sugars than does pig kidney aldose reductase (Table II; Refs. 4 and 5), a fact that has been previously observed in comparing the high $K_m$ aldose reductase and aldose reductase from the same species (16, 27, 28). The high $K_m$ aldose reductase from brain and kidney may also be distinguished from pig muscle aldose reductase and from aldose reductases in general by having a lower $K_a$ for $p$-glucuronate (16) (Table II).

In terms of catalytic efficiency, i.e. $k_{cat}/K_m$ (30), $p$-acetacetaldehyde was a much better substrate ($k_{cat}/K_m = 63,000$) for pig muscle aldose reductase than for any of the aldo sugars, especially $p$-glucose ($k_{cat}/K_m = 23$), even though the latter is purportedly a physiological substrate for aldose reductase (14). The high $K_m$ of pig muscle aldose reductase for other aldose reductases for glucose, however, may be somewhat misleading in that it is unlikely that the enzyme interacts with the pyranose form of the sugar. It is more likely that aldose reductase reduces the free aldehyde, and support for this has been obtained recently by Inagaki et al. (32), whose kinetic analysis of bovine lens aldose reductase suggested that the enzyme acts on the aldehyde form of glucose with a $K_m$ of 0.66 mM. With all the aldohydrate substrates tested, pig muscle aldose reductase exhibited linear kinetics which was also the case for pig brain aldose reductase (18). In contrast, aldose reductase from bovine lens (24), human brain (3), and sheep placenta and seminal vesicles (33) was found to exhibit curvilinear Lineweaver-Burk plots. Since it has been shown that aldose reductase often occurs as two isoenzymes (16, 19), it is possible that the nonlinear kinetics may have been due to the use of heterogeneous enzyme preparations.

Both pig kidney aldose reductase (4) and the same enzyme from pig brain (2) use NADPH exclusively as coenzyme. In contrast, pig muscle aldose reductase can also use NADH as cofactor, albeit with a 500-fold lower affinity than it has for NADPH (Table II). This dual coenzyme specificity is very similar to that found for aldose reductase from several sources, e.g. lens (34), and for the low $K_m$ aldose reductase (2). The ability to use NADPH and NADH is yet another way of distinguishing the low $K_m$ aldose reductase/aldose reductase/pig muscle aldose reductase from the other major aldose reductase.

Effect of Drugs—The high $K_m$ aldose reductase from brain (35–37) and from kidney (8, 38) is inhibited markedly (i.e. in excess of 75%) by barbiturates and other anticonvulsant drugs at concentrations of the order of 1 mM. In contrast, the low $K_m$ enzyme is less sensitive to barbiturate inhibition (10–30%) (16, 37). In the present study, the degree of inhibition of pig muscle aldose reductase by barbiturates (Table III) was similar to that found for the low $K_m$ reductase (37) and was quite different from that obtained for pig kidney aldose reductase (Table III, values in parentheses). Also, sodium valproate (1 mM), which selectively inhibits the high $K_m$ aldose reductase of rat brain (17), was a much better inhibitor (72% compared to 27%) of pig kidney aldose reductase than of pig muscle aldose reductase.

Alrestatin, an aldose reductase inhibitor commercially developed for the treatment of diabetic complications (39),

### Table II

**Substrate specificity and kinetic constants for pig muscle aldose (aldose) reductase**

| Substrate          | $K_m$ (mM) | $k_{cat}$ (s^{-1}) | $k_{cat}/K_m$ (s^{-1} M^{-1}) |
|--------------------|------------|-------------------|------------------------------|
| p-Nitrobenzaldehyde | 0.013 ± 0.002 | 1.7               | 72,000                      |
| Indole-3-acetaldehyde | 0.035 ± 0.005 | 2.2               | 62,000                      |
| D-Glucose         | 0.078 ± 0.005 | 1.8               | 23,000                      |
| Sucinic semialdehyde | 0.024 ± 0.007 | 0.8               | 198                         |
| NADPH             | 0.19 ± 0.003 | 0.7               | 136,000                     |
| NADH              | 1200 ± 200 µM | 0.5               | (N.R.)                      |

$^a$ Assayed in the presence of 2% (v/v) methanol.
$^b$ Values in parentheses are those obtained for pig kidney (high $K_m$) aldose reductase.
$^c$ Prepared from the bisulfite adduct by the method of Brown and Purves (29).

$^d$ No reaction at any substrate concentration.
The effect of drugs on the activity of pig muscle aldehyde (aldose) reductase

Solutions of the various drugs in 100 mM sodium phosphate buffer, pH 6.5, were added to a reaction mixture consisting of 160 μM NADPH, 30 mM DL-glyceraldehyde, and 100 mM sodium phosphate buffer, pH 6.5, immediately before starting the reaction by the addition of enzyme. The final reaction volume was 2.5 ml, and assays were performed at 30 °C. The values of per cent of inhibition are the average of duplicates.

| Drug            | Concentration (mM) | Inhibition (%) |
|-----------------|--------------------|---------------|
| Phenobarbital   | 1.0                | 19 (57)*      |
|                 | 0.1                | 8 (11)        |
| Diphenylhydantoin | 0.1                | 48 (56)       |
|                 | 0.01               | 12 (10)       |
| Sodium valproate | 1.0                | 27 (72)       |
|                 | 0.1                | 8 (38)        |
| Quercetin       | 0.01               | 70 (56)       |
|                 | 0.001              | 41 (21)       |
| Pyrazole        | 1.0                | 4 (5)         |
|                 | 0.01               | 73 (40)       |
| Chlorpromazine  | 0.1                | 54 (8)        |
|                 | 0.01               | 74 (83)       |
| Allestatin 4     | 0.01               | 55 (54)       |
| Indomethacin    | 0.1                | 67 (44)       |
|Ethacrynic acid  | 0.1                | 78 (33)       |

* Values in parentheses are per cent of inhibition of pig kidney aldehyde reductase.

1,3-Dixo-1H-benzene-(de)-isoquinoline(3H)-acetic acid.

The results of this drug inhibition study show that pig muscle and pig kidney aldehyde reductases, respectively; whereas, Sorbinil, a similarly developed drug (40), was about equally effective in inhibiting pig muscle and pig kidney aldehyde reductase (74 and 83%, respectively). It appears, therefore, that aldose reductase inhibitors do not discriminate effectively between aldose (i.e., low K_m) aldehyde reductase and aldehyde (high K_m) reductase. Also, of interest in the present study was the extent to which indomethacin and ethacrynic acid, known inhibitors of carbonyl reductase (10), inhibited both pig muscle and pig kidney aldehyde reductase. A 0.1 mM concentration of these drugs which caused a 50% inhibition of carbonyl reductase (10) yielded a 67 and 78% inhibition of pig muscle aldehyde reductase and 44 and 33% inhibition pig kidney aldehyde reductase, respectively (Table III).

The results of this drug inhibition study show that pig muscle aldehyde reductase exhibits the same pattern of inhibition as the low K_m reductase and aldose reductase. It was also apparent that the only effective discriminators between the three major aldehyde reductases in tissues are sodium valproate and barbitalates.

We conclude that the aldehyde reductase found in pig muscle (and very likely also in other mammalian muscle) is identical with the low K_m reductase of brain and kidney and with aldose reductase of lens. A separate designation for aldose reductase is, therefore, unnecessary. It now remains for a suitable nomenclature to be developed for the three predominant aldehyde reductases found in tissues, i.e., high K_m, low K_m, and carbonyl reductase. This topic was discussed recently and a proposed nomenclature has been published (41).

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