Involvement of Cysteine Residues in Catalysis and Inhibition of Human Aldose Reductase

SITE-DIRECTED MUTAGENESIS OF CYS-80, -298, AND -303*

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In order to study the potential role of cysteiny] residues in catalysis and inhibition of human aldose reductase, mutants containing cysteine to serine substitution at positions 80 (ALR2:C80S), 298 (ALR2:C298S), and 303 (ALR2:C303S) were constructed. Mutation of Cys-298 resulted in the most profound changes, as ALR2:C298S displayed 4- to 6-fold elevation in $K_m^{\text{NADPH}}$, $K_m^{\text{glyceraldehyde}}$, and $K_m^{\text{glucose}}$. Relative to wild type aldose reductase as well as a 10-fold higher $K_i$, for the aldose reductase inhibitor sorbinil. Wild type and mutant reductases were equally sensitive to tolrestat, a structurally different reductase inhibitor. Carboxymethylation of the wild type enzyme or the C80S and C303S mutants led to a modest decrease in $K_m$ as well as an increase in $K_m$ and $K_i$. These parameters were not significantly changed when ALR2:C298S was subjected to carboxymethylation. Lithium sulfate caused activation of ALR2:WT, C80S, and C303S but did not significantly affect the activity of ALR2:C298S. The differential sensitivity of wild type and mutant reductases to inhibition by sorbinil and tolrestat, before and after carboxymethylation, indicates that these inhibitors bind at different sites. These results suggest that Cys-298 is present near the active site and constitutes a regulatory group which controls the catalytic activity and inhibitor sensitivity of the enzyme.

Aldose reductase (ALR2; alditol:NADPH oxidoreductase; EC 1.1.1.21), the first and rate-limiting enzyme of the polyol pathway, catalyzes the NADPH-linked reduction of glucose to fructose and other aldo-sugars to their corresponding sugar alcohols or polyols (1, 2). Sorbitol dehydrogenase completes the pathway by catalyzing the NAD-dependent oxidation of sorbitol to fructose. Although under hyperglycemia the polyol pathway activity may account for more than 30% of glucose utilized (3), the physiological relevance of this pathway remains unclear. Under hyperglycemic conditions, activation of the polyol pathway has been suggested to lead to extensive tissue pathogenesis particularly in tissues independent of insulin for glucose uptake (4, 5). The most convincing evidence linking aldose reductase and pathogenesis comes from the study of aldose reductase inhibitors which have been shown to prevent or significantly delay the onset and development of diabetic complications such as cataract, retinopathy, and neuropathy (5–7). However, most aldose reductase inhibitors are nonspecific and inhibit structurally related enzymes (8, 9) which precludes their long term clinical use. Design of effective and specific aldose reductase inhibitors will depend on an understanding of the mechanism of catalysis of the enzyme and synthesis of mechanism-based inhibitors or transition state analogs.

Little is known about the mechanism of catalysis by aldose reductase or the identity of amino acid residues involved in substrate binding and/or catalysis. One of the significant properties of the enzyme is its sensitivity to oxidation (10–12). Oxidative modification of the enzyme has been shown to result in its conversion to an "activated" form (12–16). Oxidation of the enzyme has also been shown to result in generation of multiple isoforms, presumably due to intramolecular disulfide formation, which display marked changes in kinetic behavior and altered susceptibility to inhibition by hydantoin derivatives such as sorbinil (15, 16).

Human placental aldose reductase contains 7 cysteine residues (17, 18), 3 of which are accessible to solvent and react readily with 5,5'-dithiobis(2-nitrobenzoic acid) (18). It has been proposed that oxidation-induced activation of the enzyme and alterations in the kinetic and inhibitor binding properties of the enzyme may be due to modification of cysteine residues (14–16, 19). Although, the specific role of the 3 exposed cysteine residues in enzyme catalysis and inhibitor binding has not been identified, chemical modification studies suggest that Cys-298 may be responsible for oxidation-induced activation of the enzyme (19).

In this study we have utilized an efficient bacterial plasmid for overexpression of human aldose reductase in Escherichia coli. The recombinant and native enzymes appear to have similar structural and kinetic properties. Kinetic characterization of mutant enzyme forms in which Cys-80, -298, or

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‡‡ The abbreviations used are: ALR2, aldose reductase; DTT, dithiothreitol; gL0-L, gene 10 leader sequence; IPTG, isopropylthio-β-D-galactopyranoside; SDSPAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sorbinil, (S)-6-fluoro-spiro-[chroman-4,4'-imidazolidine]-2'-N'-dione; tolrestat, N-[5-(trifluoromethyl)-6-methoxy-1-naphthalenyl][thioxomethyl]-N'-methyl]glycine.
TABLE I
Oligonucleotides used for site-directed mutagenesis
| Oligonucleotide | Sequence |
|-----------------|----------|
| C80S | 5'-GGTACGTCAGCTCACAGCTTG-3' |
| C298S | 5'-CAACGGGACGAGGCTCTCC-3' |
| C303S | 5'-TGGGAGGTAGAAGCTCAAAC-3' |

-303 was substituted with serine suggests that none of these residues is essential for catalytic activity of the enzyme. However, replacement of Cys-298 converts the enzyme from a low $V_{max}$/low $K_m$ ("unactivated") form to a high $V_{max}$/high $K_m$ (activated) form which displays lowered sensitivity to sorbinil but not to tolrestat. On the basis of these results it is suggested that Cys-298 is present near the active site and regulates the conversion of the enzyme from the unactivated to activated state.

MATERIALS AND METHODS

Isolation and Sequencing of Aldose Reductase cDNA Clones—Complementary DNA (cDNA) clones encoding aldose reductase were isolated from a human placenta library constructed in ZAPII vector (Stratagene, LaJolla, CA). Bacteriophage plaques were screened by hybridization with a bovine aldose reductase cDNA (20) radiolabeled by the Klenow primer method (21). Oligonucleotides used for site-directed mutagenesis

expression construct was as follows: FeCl$_3$·6H$_2$O, 5.4 mg/ml/liter; MnSO$_4$·H$_2$O, 0.25 mg/ml/liter; CuSO$_4$·5H$_2$O, 0.4 mg/ml/liter; H$_2$BO$_3$, 0.1 mg/ml/liter; Na$_2$MoO$_4$, 0.25 mg/ml/liter. When the cultures had grown to a density of approximately 150 Klett units, IPTG was added to a final concentration of 1 mm and cultures were grown for an additional 2 h. Aliquots (1 ml) of cells for electrophoretic analysis were harvested by centrifugation (7,500 × g, 1 min, 4 °C) immediately before and 2 h following IPTG induction and were processed separately through the following purification steps. The enzyme preparation was split into three aliquots, and each was concentrated. Glucose was added to maintain a final concentration of 1 mM and cultures were grown for an additional 6 h after the addition of IPTG.

Large Scale Osmotic Shock—Cells from 1 liter of cell suspension from a fermentation run (A$_{600}$ = 70) were collected by centrifugation (7,500 × g, 7 min, 4 °C) and were suspended in 3.6 liters of 20% sucrose, 30 mM Tris-HCl, pH 7.5, 1 mM EDTA. After suspension, cells were kept at 23 °C for 15 min. Cells were then collected by centrifugation (8,670 × g, 10 min, 4 °C) and were suspended in 1.8 liters of ice-cold water containing protease inhibitors (1 µg/ml each of chymostatin, leupeptin, and pepstatin A) and kept on ice for 10 min. After centrifugation (8,670 × g, 15 min, 4 °C), the supernatant (designated water wash) containing aldose reductase was collected and supplemented with DTT and Tris-HCl, pH 7.5, to 0.1 and 5 mm, respectively.

Purification of Recombinant Human Aldose Reductase—All purification steps were carried out at 4 °C in buffers containing 1.0 mm DTT. The following method was used to purify human recombinant aldose reductase from the osmotic shock extract (water wash) obtained from 1 liter of JM101 (pMON5997) fermentation culture. To concentrate the proteins present in the initial water wash, an ammonium sulfate fractionation step was carried out. Material precipitating in the presence of 50–50% ammonium sulfate was collected by centrifugation at 10,000 × g for 20 min, and resuspended in 100–200 ml of 25 mm imidazole-HCl, pH 7.4. From this point, the crude enzyme preparation was split into three aliquots, and each was processed separately through the following purification steps. The enzyme was applied to a 1.5-l Polyybuffer (Stratagene) packed with PBE 94 chromatofocusing resin (Pharmacia LKB Biotechnology Inc.) which had been previously equilibrated with 25 mm imidazole buffer, pH 7.4. Proteins were eluted from the column with a 1.8-luted Polybuffer 74, pH 5.0. The column eluate was continuously monitored by measuring A$_{600}$ mm. Fractions of 5 ml were collected and were measured for pH, aldose reductase activity, and protein content by the dye-binding method of Bradford (35). Fractions containing aldose reductase activity were pooled and di-
alyzed against 120 volumes of 10 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA. Fractions containing aldose reductase activity were pooled, concentrated to approximately 1-2 mg/ml, and stored at 4 °C.

**Amino Acid Sequence Analysis of Recombinant Aldose Reductase**—Aliquots of recombinant aldose reductase taken from selected chromatofocusing fractions (greater than 90% pure) were subjected to automated Edman degradation using an Applied Biosystems, Inc. Model 470A gas-phase sequenator (36). The respective phenylthiohydantoin derivatives were identified by reverse phase high performance liquid chromatography analysis in an on-line fashion using an Applied Biosystems, Inc. Model 120A phenylthiohydantoin analyzer fitted with a Brownlee 2.1-mm inner diameter phenylthiohydantoin-Cr column.

**Purification of Aldose Reductase from Human Placenta**—Aldose reductase was purified to apparent homogeneity from human placenta as described previously (37), except that ammonium sulfate fractionation was used as the first step. During purification, the enzyme solution was maintained in 10 mM imidazole-HCl, pH 7.0, containing 5 mM β-mercaptoethanol. Homogeneity of the purified enzyme was established by the movement of a single protein band on reducing SDS-PAGE at pH 8.6 and by the appearance of a single protein peak coincident with aldose reductase following Sephadex G-100 gel filtration. The purified enzyme was stored at 4 °C in 50 mM imidazole-HCl, pH 7.0, containing 5 mM β-mercaptoethanol.

**Enzyme Assays**—Aldose reductase activity in purification fractions was measured at 23°C in 10 mM sodium phosphate, pH 6.2, containing 5 mM β-mercaptoethanol, 10 mM DL-glyceraldehyde, and 0.15 mM NADPH in a total volume of 1 ml as described previously (38). The reaction was initiated by addition of NADPH and enzyme activity determined by measuring the rate of disappearance of NADPH by monitoring absorbance of the reaction mixture at 340 nm using a Beckman DU64 spectrophotometer. One unit of enzyme oxidized 1 μmol of NADPH/min. Reaction mixtures without enzyme and/or alddehyde substrate were used as controls. For kinetic studies, enzyme activity was determined in 50 mM potassium phosphate, pH 7.0, 0.1 mM NADPH, alddehyde substrate, 0.4 mM ammonium sulfate or lithium sulfate if indicated, and appropriate amounts of enzyme in a total reaction volume of 1 ml.

**Data Analysis**—Individual saturation curves used to obtain V_{max} and K_{m} were fitted to a general Michaelis-Menten equation using a nonlinear iterative fitting program (14)

\[
v = \frac{V_{max} A}{K_m + A}
\]

where v is the enzyme velocity and A is the substrate concentration. Data conforming to linear competitive, and noncompetitive or competitive inhibition were fitted to Equations 2-4, respectively.

\[
v = \frac{V_{max} A}{K_m + A(1 + 1/K_i)}
\]

\[
v = \frac{V_{max} A}{K_m(1 + I/K_i) + (A(1 + 1/K_i))}
\]

\[
v = \frac{V_{max} A}{(1 + I/K_i) + A}
\]

where I is the inhibitor concentration and K_{i} and K_{m} are the intercept and slope inhibition constants. In all cases, best fit to the data was chosen on the basis of the standard error of the fitted parameter and the lowest value of σ, which is defined as the sum of the squares of the residuals divided by the degrees of freedom (number of observations minus the number of parameters calculated). The concentration of the substrate/inhibitor used to estimate kinetic parameters was in most cases varied from 4 × K_{m} or K_{i} to one-fourth the value of K_{m} and K_{i}. Data are expressed as means ± S.E. Statistical significance was determined using unpaired Student’s t test. Data were considered statistically significant when the p value was <0.001.

**RESULTS**

**Expression of Wild Type and Mutant Human Aldose Reductase in E. coli**—A total of 36 aldose reductase cDNA clones were isolated by screening approximately 2.5 × 10^{6} plaques from a XZAPII human placental cDNA library. The cDNA insert of one of these clones, designated HuPAR53, contained a single open reading frame encoding the expected 316-amino acid aldose reductase translation product. Introduction of this cDNA into the expression plasmid pMON5842 yielded plasmid construct pMON5997. When introduced into E. coli strain JM101, plasmid pMON5997 directed the synthesis of a M_{r}~36,000 polypeptide which co-migrated with bovine lens aldose reductase and cross-reacted with antibodies to bovine lens aldose reductase (Fig. 1, lanes 2–6). The abundance of this polypeptide increased significantly in cells cultured for 2 h following the addition of 1 mM IPTG (compare Fig. 1, lanes 2 and 3). No corresponding immunoreactive material was observed in extracts of pMON5842, a control plasmid directing the expression of chloramphenical acetyltransferase (Fig. 1, lanes 7–11). Aldose reductase activity was abundant in the water wash extract fraction from cells containing pMON5997 but was undetectable in similarly treated extracts from a culture containing pMON5842 (data not shown). Comparison of whole cell extracts and material from osmotic shock fractions from cultures containing pMON5997 revealed that most of the aldose reductase expressed in E. coli can be released by osmotic shock. This confirms a previous report that recombinant human aldose reductase expressed in E. coli can be released by osmotic shock despite the absence of a recognizable secretion signal in the protein structure (39). Recovery of recombinant aldose reductase in the water wash fraction provides obvious advantages as it affords substantial partitioning of the enzyme from intracellular proteins and hence easier purification.

Table II summarizes the results of a typical purification of aldose reductase extracted from E. coli JM101 fermentation cultures containing the pMON5997 expression construct. As a final step, aldose reductase was purified by hydroxylapatite chromatography where it eluted as a single coincident peak of enzyme activity and protein (Fig. 2). The final enzyme preparation (specific activity, 3.34 units/mg protein) was homogeneous as evidenced by a single polypeptide band on SDS-PAGE (Fig. 2, inset). Purified recombinant and native human aldose reductase were examined by Western blot analysis as described in the text. Lane 1, 50 ng of bovine lens ALR2 purified as described (34); lanes 2 and 7, preinduction whole cell extract; lanes 3 and 8, 2-h postinduction whole cell extract; lanes 4 and 9, sucrose wash derived from 2-h postinduction cells; lanes 5 and 10, water wash derived from 2-h postinduction cells; lanes 6 and 11, cell pellet remaining after water wash, 2-h postinduction cells. Lane 12, 50 ng of purified human recombinant ALR2. Samples in lanes 2–11 each contained materials derived from 10 Klett unit equivalents. Mass of molecular weight markers are indicated. ALR2-immunoreactive material is indicated by an arrow. A faint background band at M_{r} ~17,000 was observed in some lanes containing aldose reductase and control expression lysates and is thought to represent an epitope unrelated to human aldose reductase.

![Fig. 1. Western blot analysis demonstrating expression of human aldose reductase in E. coli strain JM101. Extracts from E. coli JM101 cells containing pMON5997 (ALR2 expression construct; lanes 2–6) and pMON5842 (chloramphenical acetyltransferase expression construct; lanes 7–11) were subjected to electrophoresis and Western blotting as described in the text. Lane 1, 50 ng of bovine lens ALR2 purified as described (34); lanes 2 and 7, preinduction whole cell extract; lanes 3 and 8, 2-h postinduction whole cell extract; lanes 4 and 9, sucrose wash derived from 2-h postinduction cells; lanes 5 and 10, water wash derived from 2-h postinduction cells; lanes 6 and 11, cell pellet remaining after water wash, 2-h postinduction cells. Lane 12, 50 ng of purified human recombinant ALR2. Samples in lanes 2–11 each contained materials derived from 10 Klett unit equivalents. Mass of molecular weight markers are indicated. ALR2-immunoreactive material is indicated by an arrow. A faint background band at M_{r} ~17,000 was observed in some lanes containing aldose reductase and control expression lysates and is thought to represent an epitope unrelated to human aldose reductase.](image-url)
aldo reductase comigrated on SDS-PAGE and reacted with antibodies against human placental aldose reductase (Fig. 2, inset). This purification method resulted in a 40% recovery of starting enzyme units and greater than 15-fold purification with a total yield of 56 mg of recombinant aldose reductase derived from 1 liter of fermentation culture (Table II). Sequential Edman degradation of the purified human recombinant aldose reductase revealed the following sequence: NH2-A-S-R-L-L-N-N-G-A-K-M-P-, thus confirming the identity of the recombinant material as aldose reductase. Purified recombinant aldose reductase was stable for at least 6 months when stored at 4 °C. Approximately 50% activity was lost when the enzyme solution was stored at -20 or -70 °C in the presence of 3% polyethylene glycol (molecular weight 7000-9000). The enzyme was completely inactivated when stored at -20 or -70 °C in the absence of polyethylene glycol.

Expression of mutant aldose reductase cDNA sequences was accomplished by replacing the wild type aldose reductase cDNA sequence in pMON5997 with restriction fragments containing cDNA sequences encoding cysteine to serine substitutions at positions 80 (ALR2:C80S), 298 (ALR2:C298S), and 303 (ALR2:C303S). Conditions identical to those described above were used to express, extract, and purify wild type and mutant reductases from shaker flask cultures. Purified wild type and mutant reductases comigrated on SDS-PAGE and cross-reacted with antibodies to native human aldose reductase (Fig. 3).

Structural and Kinetic Comparison of Native and Recombinant Human Aldose Reductases—Analytical chromatofocusing of native and recombinant reductases revealed a slight difference in their apparent isoelectric pH values. When applied separately to different chromatofocusing columns, recombinant and native aldose reductases eluted at positions corresponding to pH 5.83 and 5.67, respectively (Fig. 4A). This minor difference in the apparent isoelectric pH was confirmed when the enzymes were mixed and subjected to chromatofocusing simultaneously (Fig. 4B). It is probable that the difference in acetylation of the amino-terminal alanine residue is responsible for this apparent difference in isoelectric pH (40). Apart from potential differences at the amino terminus, it is unlikely that the primary structure of the recombinant aldose reductase differs from the native human aldose reductase because the coding sequence in pMON5997 was identical to all aldose reductase cDNA clones isolated from the human placental cDNA library (data not shown).

Kinetic Constants for Wild Type and Mutant Aldose Reductases—Kinetic constants of the wild type and mutant reductases determined with a series of hydrophilic and hydrophobic substrates are shown in Table III. All of the enzyme forms preferentially utilized hydrophilic aldehyde substrates as the catalytic efficiencies (kcat/Km) measured with benzaldehyde and p-nitrobenzaldehyde were 1 to 4 orders of magnitude greater than that measured with the hexose and pentose substrates glucose and xylose, respectively. Among this series of substrates, the turnover number (kcat) measured with the C80S and C303S mutants deviated <2-fold from wild type, with the exception of glucose. In this case, the kcat values were either increased (>4.1-fold for C80S) or decreased (>2.3-fold for C303S) relative to wild type. No consistent trend was evident to associate mutation of Cys-80 and Cys-303 and pattern of substrate selectivity. For example, divergent changes in catalytic efficiency for the reduction of xylose and glucose were measured with the C80S mutant. This mutant also displayed enhanced utilization of benzaldehyde (kcat/Km) increased >4-fold relative to wild type although its utilization of para-nitro-substituted benzaldehyde (p-nitrobenzaldehyde) was decreased almost 3-fold. The catalytic efficiency of the C303S mutant was also decreased with both hydrophilic and hydrophobic substrates. In contrast, the kcat values measured with the C298S mutant were consistently elevated (≥4-fold) with all substrates tested even though the catalytic efficiency for these substrates was lower than wild type enzyme. Such behavior seems characteristic of ALR2:C298S because reduction in catalytic efficiency of the C80S and C303S mutants was usually not accompanied by an increase in the turnover number. Increased turnover number for the
reduction of DL-glyceraldehyde was also evident with ALR2:C298S but not with the other mutants (Table IV). Importantly, there was no statistically significant difference in $K_{m(NADPH)}$ observed among the wild type and mutants (Table IV).

Kinetic differences between ALR2:C298S and other forms of the enzyme were even more marked after carboxymethylation. Carboxymethylation of the wild type, C80S and C303S mutants resulted in an increase in $K_{m(DL-glyceraldehyde)}$. There was no statistically significant difference between the kinetic constants of the carboxymethylated wild type or carboxymethylated C80S or C303S mutants. Moreover, carboxymethylation had no significant effect on the kinetic parameters of C298S mutants. These results suggest that the functionally significant site of carboxymethylation of aldose reductase is Cys-298.

To further investigate the role of these cysteine residues, the inhibitor sensitivity of wild type and mutant reductases was determined. Substitution of Cys-80 caused a small but statistically significant increase in $K_{i(tolrestat)}$ (Table V). However, the $K_{i(sorbitinil)}$ was not affected on substitution of Cys-303 with serine. While the sorbinil inhibition constants for the wild type and C80S and C303S mutants were similar, the C298S mutant was approximately 10-fold less sensitive to this inhibitor than the other enzyme forms tested (Table V). Sensitivity to tolrestat was largely unaffected by serine substitutions at positions 80, 303, or 298. Although $K_{i(tolrestat)}$ was 2–3-fold higher for the ALR2:C303S mutant, the difference in the estimated $K_i$ values were slight compared to a 10-fold increase in $K_{i(sorbitinil)}$ observed with ALR2:C298S. The $K_{i(tolrestat)}$ for ALR2:C298S was also similar to the $K_i$ value of the wild type enzyme, indicating that the substitution of Cys-298 does not affect tolrestat binding but significantly attenuates the sensitivity of the enzyme to sorbinil.

Carboxymethylation dramatically elevated the $K_{i(sorbitinil)}$ for ALR2:WT, C80S, and C303S. In contrast, this treatment had virtually no effect on the $K_{i(sorbitinil)}$ observed with ALR2:C298S (Table V). Carboxymethylation also had no significant effect on the $K_{i(tolrestat)}$ observed for the wild type and C298S and C303S reductase mutants. However, carboxymethylation dramatically elevated the $K_{i(tolrestat)}$ for ALR2:C80S mutant.

**Modulation of Enzyme Activity by Lithium Sulfate**—To investigate the effect of sulfate ions on the activity of the enzymes, the enzyme activities were determined with and without 0.4 M Li2SO4 in the standard assay buffer ($n = 5$). Addition of Li2SO4 to the assay buffer increased the activity of the wild type enzyme 2.36 ± 0.22-fold. The activities of the C80S and C303S mutants were similarly affected (1.89 ± 0.04 and 2.27 ± 0.15-fold increases, respectively). However, the activity of the C298S mutant assayed in the presence of Li2SO4 was increased only 1.16 ± 0.04-fold. Compared to the wild type enzyme, the difference in fold activation was statistically significant only for ALR2:C298S ($p < 0.001$).

**DISCUSSION**

In spite of its proposed role in the etiology of secondary complications of diabetes (1–5), little is known about the active site and the reaction mechanism of aldose reductase. Difficulties in purifying adequate quantities of human aldose reductase and its marked sensitivity to oxidation have hampered efforts toward elucidation of the kinetic mechanism (14–16). In this article we describe methods to overexpress wild type and mutant forms of human aldose reductase using *E. coli* host cells. Use of an efficient bacterial plasmid in conjunction with a simple three-step purification method has provided yields similar or favorable to other bacterial and eucaryotic systems used for overexpression of human aldose reductase (39–44). The similarity in kinetic behavior observed

![Fig. 3: SDS-polyacrylamide gel electrophoresis and Western blotting of purified wild type and mutant reductases.](image)

![Fig. 4: Analytical chromatofocusing of native and recombinant human aldose reductases.](image)
TABLE III

Kinetic constants for wild type and mutant aldose reductases

|                | Wild type | C50S | C298S | C303S |
|----------------|-----------|------|-------|-------|
| D-Xylose       |           |      |       |       |
| $K'_m$ (mM)    | 10.2 ± 1.49| 15.6 ± 1.55| 543 ± 102$^a$| 290 ± 38$^a$|
| $k_{cat}$ (s$^{-1}$) | 1.24 ± 0.06 | 1.52 ± 0.04 | 8.48 ± 0.85$^a$ | 0.81 ± 0.4 |
| $k_{cat}/K'_m$ (s$^{-1}$ M$^{-1}$) | 122 ± 13.5 | 97.6 ± 8.07 | 15.7 ± 1.52$^a$ | 2.77 ± 0.23$^a$ |
| D-Glucose      |           |      |       |       |
| $K'_m$ (mM)    | 212 ± 26.7 | 196 ± 24 | >1000$^a$ | 881 ± 277$^a$ |
| $k_{cat}$ (s$^{-1}$) | 0.086 ± 0.004 | 0.350 ± 0.026$^a$ | >1.95$^a$ | 0.037 ± 0.004$^a$ |
| $k_{cat}/K'_m$ (s$^{-1}$ M$^{-1}$) | 0.407 ± 0.033 | 1.87 ± 0.269$^a$ | ND$^b$ | 0.085 ± 0.010$^a$ |
| p-Nitrobenzaldehyde |          |      |       |       |
| $K'_m$ (µM)    | 15.7 ± 1.70 | 34.6 ± 6.79$^a$ | 202 ± 14.1$^a$ | 254 ± 20.2$^a$ |
| $k_{cat}$ (s$^{-1}$) | 0.469 ± 0.011 | 0.377 ± 0.014 | 3.33 ± 0.092$^a$ | 0.486 ± 0.016 |
| $k_{cat}/K'_m$ (s$^{-1}$ M$^{-1}$) | 297.38 ± 2.659 | 10.065 ± 1.868$^a$ | 18.432 ± 74.1$^a$ | 1.912 ± 94$^a$ |
| Benzaldehyde   |           |      |       |       |
| $K'_m$ (µM)    | 72.8 ± 11.3 | 27.6 ± 3.49$^a$ | 1.061 ± 208$^a$ | 67.7 ± 9.39 |
| $k_{cat}$ (s$^{-1}$) | 0.443 ± 0.044 | 0.758 ± 0.018$^a$ | 4.62 ± 0.121$^a$ | 0.299 ± 0.011$^a$ |
| $k_{cat}/K'_m$ (s$^{-1}$ M$^{-1}$) | 6.983 ± 742 | 27.473 ± 3.033$^a$ | 4.404 ± 377$^a$ | 4.420 ± 501 |

*p < 0.001 compared to ALR2: WT.

*ND, value could not be accurately computed due to uncertainty in $K'_m$ and $k_{cat}$ components.

TABLE IV

Kinetic parameters of reduced and carboxymethylated wild type and mutant aldose reductases

The enzymes were reduced by incubating with 0.1 M DTT in 1 h at 37 °C in 0.1 M Tris-HCl, pH 8.0. Excess DTT was removed by gel filtration. The reduced enzymes were carboxamethylated by incubation with 1 mM iodoacetic acid at 23 °C for 1 h in 0.1 M Tris-HCl, pH 8.0. The concentration of Dl-glyceraldehyde was varied from 0.1 to 10 mM and NADPH from 10 to 100 µM and best fit parameters calculated using Equation 1. Values of the parameters are mean ± S.E. (n = 5). $K'_m$ are apparent values expressed in mM and $k_{cat}$ values in s$^{-1}$.

|                | ALR2:WT | ALR2:C50S | ALR2:C298S | ALR2:C303S |
|----------------|---------|-----------|------------|------------|
| Reduced enzyme |         |           |            |            |
| $k_{cat}$      | 4.49 ± 0.420 | 3.34 ± 0.155 | 15.70 ± 2.108$^a$ | 3.38 ± 0.207 |
| $K'_m$(Dl-glyceraldehyde) | 0.05 ± 0.003 | 0.07 ± 0.005 | 2.71 ± 0.146$^a$ | 0.07 ± 0.002$^a$ |
| $K'_m$(NADPH)  | 0.05 ± 0.009 | 0.06 ± 0.005 | 0.15 ± 0.029 | 0.05 ± 0.006 |
| Carboxymethylated enzyme |       |           |            |            |
| $k_{cat}$      | 2.73 ± 0.205 | 2.46 ± 0.134 | 18.24 ± 2.153$^a$ | 1.83 ± 0.139 |
| $K'_m$(Dl-glyceraldehyde) | 0.36 ± 0.089 | 0.38 ± 0.009 | 2.48 ± 0.257$^a$ | 0.30 ± 0.005 |
| $K'_m$(NADPH)  | 0.05 ± 0.010 | 0.09 ± 0.009 | 0.19 ± 0.031$^a$ | 0.05 ± 0.008 |

*p < 0.001 compared to ALR2: WT.

TABLE V

Inhibitor sensitivity of the reduced and carboxymethylated aldose reductases

Reduced and carboxymethylated forms of the enzymes were prepared as described for Table IV. $K_i$ values are expressed in micromolar. For these studies, the concentration of Dl-glyceraldehyde was varied from 0.1 to 10 mM. The concentration of sorbinil was varied from 0.01 to 10 µM for the reduced enzyme and from 1 to 150 µM for the carboxymethylated enzyme. The concentration of tolrestat was varied from 0.0025 to 0.1 µM. In all cases, Equation 2 gave a better fit to the data than Equations 3 or 4. Therefore, the $K_i$ values reported are intercept values ($K_i$). Values of the parameters are mean ± S.E. (n = 5).

|                | ALR2:WT | ALR2:C50S | ALR2:C298S | ALR2:C303S |
|----------------|---------|-----------|------------|------------|
| Reduced enzyme |         |           |            |            |
| $K_i$(sorbinil)| 0.37 ± 0.023 | 0.84 ± 0.06$^a$ | 3.81 ± 0.18$^a$ | 0.55 ± 0.057 |
| $K_i$(tolrestat) | 0.015 ± 0.002 | 0.022 ± 0.002 | 0.015 ± 0.002 | 0.042 ± 0.011 |
| Carboxymethylated enzyme |       |           |            |            |
| $K_i$(sorbinil)| >50 | >150 | 4.10 ± 0.26 | >100 |
| $K_i$(tolrestat) | 0.038 ± 0.007 | 0.428 ± 0.086$^a$ | 0.015 ± 0.002 | 0.053 ± 0.017 |

*p < 0.001 compared to ALR2: WT.

with native and recombinant human aldose reductases in this and other studies (44, 45) appears to validate the use of aldose reductase produced in procaryotic cells as a reliable source of enzyme for structural and functional studies. We and others (44, 45) have detected a minor difference in isoelectric pH between native and recombinant reductases produced in procaryotic expression systems, most likely a reflection of differences in amino-terminal acetylation (40). Although the potential contribution of the acetylated amino terminus to the gross conformation of the enzyme or its mechanism of catalysis was not directly studied, we consider it unlikely that acetylated and nonacetylated enzymes are functionally different as their kinetic properties appear to be virtually identical.

Crystallographic studies of porcine lens aldose reductase complexed with 2'-monophosphoadenosine-5'-diphosphoribose revealed that the enzyme is a single domain 8-stranded parallel $\beta$/$\alpha$-barrel similar to triose phosphate isomerase (46). From this structure, it may be inferred that the nucleotide cofactor binding domain, and presumably the active site, should be located at the COOH-terminal portion of the barrel. The structural model for porcine aldose reductase predicts three solvent-exposed cysteine residues, namely those corresponding to human Cys-80, Cys-298, and Cys-303, all of which are located in the carboxyl-terminal region of the $\beta$/$\alpha$-barrel.
The possibility that Cys-298 could be the proton donor in the catalytic reaction has been rejected on the argument that it appears that substitution of the thiol group with an alcohol has only a nominal effect on catalytic activity, unlike that expected if the mutation replaced a proton donating group.

The differences between ALR2:C298S and the carboxymethylated wild type enzyme may be due to different perturbations introduced into the active site environment by carboxymethylation and cysteine to serine substitution, respectively. Based on the close proximity of the Cys-298 thiol to the nicotinamide ring, it seems plausible that substitution of the thiol with an alcohol could alter the interaction of this side chain with the nicotinamide ring either directly or indirectly by changing the solvation properties of the active site pocket. A structural model of ALR2:C298S complexed to NADPH solved to 2.75Å resolution revealed that the interactions between the enzyme and NADPH appeared to be similar to those observed in the wild type holoenzyme structural model (see accompanying paper, Ref. 48). However, a higher resolution structure of the C298S mutant, as well as construction and analysis of additional Cys-298 mutants containing side chains with different physical properties, will be required to more fully evaluate the potential interactions of Cys-298 with the nicotinamide ring. It seems likely that introduction of a large charged group at Cys-298 by carboxymethylation would also be expected to alter the active site milieu. By virtue of its proximity to the active site, it is plausible that the increase in $K_m$ (DL-glyceraldehyde) observed after carboxymethylation of Cys-298 in the wild type and C80S and C303S mutants may reflect altered access of the substrate to the active site due to steric hindrance provided by the carboxymethyl group.

Recent studies have suggested that isomerization of the ALR2-NAD$^+$ binary complex is the rate-limiting step in the reaction catalyzed by unactivated aldose reductase (15, 49). Upon activation, the isomerization rate of the enzyme-coenzyme binary complex is increased, which facilitates NADP$^+$ release and increases the turnover rate of the catalytic cycle (14, 15). Therefore, if activation is due to oxidation of one or more critical cysteine residues, it seems plausible that modification of these residues should lead to an increase in $k_{cat}$. The evidence presented above demonstrates that substitution of Cys-298 with serine results in an increase in $k_{cat}$. Therefore, we suggest that modification of Cys-298 may be responsible for oxidation-induced activation of aldose reductase.

Stimulation by sulfate is a well known property of aldose reductase (38). Although the mechanism is not known, the inability of sulfate to stimulate ALR2:C298S suggests that this process involves the participation of Cys-298. However, unlike oxidation-induced activation, stimulation by sulfate does not alter the $K_m$ (sorbinil), although sulfate increases $K_m$ (NADPH), and $K_m$ (DL-glyceraldehyde), and $k_{cat}$ (40). Thus, stimulation by sulfate and activation by oxidation are not identical processes, although both seem to involve Cys-298. Substitution of Lys-262, a residue thought to participate in NADP$^+$ binding (50), is reported to attenuate the ability of sulfate to stimulate aldose reductase activity (44). Further investigations are necessary to delineate the role of Cys-298 and Lys-262 in the catalytic reaction, but it appears likely that alterations to residues involved in binding (and release) of NADP(H) may give rise to kinetic behaviors consistent with the activated form of the enzyme.

The observation that carboxymethylation or substitution of Cys-298 alters $K_m$ (sorbinil) but not $K_m$ (NADPH) suggests that the two inhibitors do not bind to the same site. These results are
consistent with our earlier observations that double inhibition plots of sorbinil and tolrestat are nonparallel (9). It has been suggested that all aldose reductase inhibitors bind at a unique site distinct from the substrate-binding site (51). However, mutations or structural modifications giving rise to concomitant changes in substrate binding and sorbinil binding suggest that the sorbinil-binding site may be at or close to the active site of the enzyme. Cys-298 may form a part of the sorbinil binding site, or the "S" site. The tolrestat-binding site, or "T" site, which is insensitive to carboxymethylation or substitution of Cys-298, is likely to be located at a different site possibly farther removed from the active site of the enzyme.

These results indicate that among the 3 solvent-exposed cysteine residues located in the COOH-terminal portion of the aldose reductase beta/a-barrel, cysteine 298 may play a key role in the conversion of aldose reductase from an unactivated to an activated state (19). The chemical and structural details of this conversion have practical importance in the design of aldose reductase inhibitors, as the activated form of aldose reductase displays markedly reduced sensitivity to some enzyme inhibitors. In view of these findings, it is possible that the in vivo oxidation state of the cell may regulate the proportion of activated and unactivated forms of aldose reductase.

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