Human aldose reductase and aldehyde reductase are members of the aldo-keto reductase superfamily that share three domains of homology and a nonhomologous COOH-terminal region. The two enzymes catalyze the NADPH-dependent reduction of a wide variety of carbonyl compounds. To probe the function of the domains and investigate the basis for substrate specificity, we interchanged cDNA fragments encoding the NH2-terminal domains of aldose and aldehyde reductase. A chimeric enzyme (CH1, 317 residues) was constructed in which the first 71 residues of aldose reductase were replaced with first 73 residues of aldehyde reductase. Catalytic effectiveness ($k_{cat}/K_m$) of CH1 for the reduction of various substrates remained virtually identical to wild-type aldose reductase, changing a maximal 4-fold. Deletion of the 13-residue COOH-terminal end of aldose reductase, yielded a mutant enzyme (ARA303-315) with markedly decreased catalytic effectiveness for uncharged substrates ranging from 80- to more than 600-fold (average 300-fold). The $K_{m}$NADPH of CH1 and ARA303-315 were nearly identical to that of the wildtype enzyme indicating that cofactor binding is unaffected. The truncated ARA303-315 displayed a NADPH/D isotope effect in $k_{cat}$ and an increased $\delta(k_{cat}/K_m)$ value for D-glyceraldehyde, suggesting that hydride transfer has become partially rate-limiting for the overall reaction. We conclude that the COOH-terminal domain of aldose reductase is crucial to the proper orientation of substrates in the active site.

Aldose reductase (EC 1.1.1.21) and aldehyde reductase (EC 1.1.1.12), are members of the aldo-keto reductase superfamily (1). They catalyze the NADPH-dependent reduction of a wide variety of carbonyl compounds such as sugars, aldehyde metabolites derived from biogenic amines, and corticosteroid hormones, as well as xenobiotic aldehydes (2). Aldose reductase has been linked to diabetic complications affecting the lens, retina, nerves, and kidneys (3-6) via its ability to reduce excess intracellular glucose resulting from the hyperglycemia of diabetes mellitus. Our goal is to understand the mechanism of enzyme-cofactor-substrate interactions in these two enzymes and develop a rational basis for the design of specific enzyme inhibitors.

The two enzymes share, with varying degrees of identity, three domains of homology beginning at the NH2-terminal domain. The COOH-terminal domains, on the other hand, are distinctly different and nonidentical (1). To probe the function and roles of the NH2-terminal and COOH-terminal domains, we constructed a chimera aldose reductase protein, CH1, in which the NH2-terminal domain is replaced with that of aldehyde reductase, as well as an aldose reductase with a deleted COOH-terminal (ARA303-315). In addition, an aldose reductase mutant, AR303, was created to determine the possible contribution of a deleted Cys303 in the COOH-terminal domain to the observed effects. We hereby describe the properties and kinetic characteristics of the modified enzymes, which show that the COOH-terminal domain of aldose reductase is a key determinant of catalytic effectiveness.

**MATERIALS AND METHODS**

Construction of Expression Plasmids—Construction of wild-type human aldose and aldehyde reductase expression plasmids in the pET system was described earlier (7). To replace the first homologous domain of aldose reductase with its counterpart of aldehyde reductase, a construct in pKK223-3 containing the complete coding sequence of aldose reductase as a EcoRI/HindIII insert was completely digested with EcoRI and SnaI to release a 241-bp piece which encodes the first 73 residues. The released piece was replaced by ligating into the remaining vector a EcoRI/Sacl digest of a PCR product that was obtained as follows: The NH2-terminal domain of aldose reductase was amplified by PCR using a T7 promoter primer and primer 1 (Table I), and the aldehyde reductase cDNA in pGEM-3 as template (1). Two Ndel sites, using primers 2 and 3 (Table I) were introduced by PCR at both 5'- and 3'-ends of the chimeric cDNA in pKK223-3, to introduce the cDNA into the NdeI site of the expression plasmid pET11a (Novagen, Madison, Wis), yielding pET11aCH1.

The COOH-terminal of aldose reductase was deleted by constructing an expression vector, pET11aARA303-315, in which the last 13 residues were deleted. The aldose reductase expression plasmid pET11aAR was digested with NcoI and NdeI to release a 545-bp piece encoding the COOH-terminal half of human aldose reductase. This fragment was replaced by a Ncol/NdeI digest of a PCR fragment with which the 13-residue COOH-terminal tail was deleted by PCR. The amplified was obtained by using primers 4 and 5 (Table I) and plasmid pET11aAR as a template.

The Cys303 to Ser374 mutation in aldose reductase, ARCOH, was created by oligo-directed mutagenesis. The aldose reductase cDNA was subcloned into M13 mp18 using the EcoRI restriction sites. Mutagenesis was done by using the Amersham Corp. In vitro mutagenesis system (version 2.1) and primer 6 (Table I). The cDNA bearing the mutation was introduced into pET11a as previously

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Oligonucleotide primers

<table>
<thead>
<tr>
<th>Primers used to construct pET11α(Ch1)</th>
<th>pET11αARala303 and pETARchOOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 5' CAAAGGCTTCCCGAGCCAAGC 3' (SacI)</td>
<td></td>
</tr>
<tr>
<td>2 5' CCATCGTGGGAGCGGACCA 3' (NdeI)</td>
<td></td>
</tr>
<tr>
<td>3 5' GTCTATGGAGGCAGCAG 3' (NdeI)</td>
<td></td>
</tr>
<tr>
<td>4 5' GTGCTATGTCAGTCAAAAGGACGAC 3' (NdeI)</td>
<td></td>
</tr>
<tr>
<td>5 5' CATTGGATGCTGGCGC 3'</td>
<td></td>
</tr>
<tr>
<td>6 5' GCTTGTGGAGCTTCACTCAGCAAG 3' (G-C)</td>
<td></td>
</tr>
</tbody>
</table>

Expression in E. coli and Enzyme Purification—The recombinant plasmids were overexpressed and purified as described in detail elsewhere (7). We used a more alkaline (pH 8.4) running buffer to purify ARKZezM and the DEAE-Sephacel since the IEP of this truncated enzyme is 6.9 (see below), and thus higher than the IEP of the wild-type enzyme (6.2).

SDS-Polyacrylamide Gel Electrophoresis, Western Blot Analysis, and Isoelectric Focusing—SDS-polyacrylamide gels were run on a precast 10–15% gradient gels on the Phastsystem (Pharmacia LKB Biotechnology Inc.) in the presence of 2% β-mercaptoethanol as described by the manufacturer. Isoelectric focusing was carried out using precast isoelectric focusing gels (pH 5–8), according to the manufacturer's (Pharmacia) instructions. Western blot analysis was performed with the ProteoBlot kit (Promega) containing goat anti-rabbit IgG alkaline phosphatase conjugate, and developed according to the manufacturer's procedures. Antibodies to human placental aldose reductase were previously described (7). Rabbit anti-human liver aldose reductase serum was a gift from Dr. Bendicht Wermuth (University of Bern, Switzerland). Sera were purified with the Econo-Pac Serum IgG purification kit (Bio-Rad). The protein concentration of the purified enzymes was determined with the Bio-Rad protein assay kit, using bovine γ-globulin as the standard.

Enzyme Assays and Kinetic Analysis—Enzymatic activities were assayed during purification by measuring the rate of enzyme-dependent decrease of NADPH absorption at 340 nm in a Gilford Response spectrophotometer at 25 °C. The standard reaction mixture contained 0.1 M sodium phosphate buffer, pH 7.0, 0.2 mM NADPH, and 5 mM DL-glyceraldehyde or 1 mM p-nitrobenzaldehyde in a total volume of 1 ml. Kinetic constants were determined in the same way except for varying the substrate or cofactor concentrations. Each data point (initial velocity) was determined in duplicate over at least six different substrate concentrations. Control assays, lacking either substrate or enzyme, were routinely included, and the rates, if any, were subtracted from the reaction rates. Kinetic constants were calculated by fitting the Michaelis-Menten function directly in the hyperbolic form to the data with an unweighted least-squares analysis using the Fortran programs of Cleland (13).

\[ v = \frac{v_{\text{max}}}{K_{m} + A} + A \]  

where \( v \) is the initial velocity, \( A \) is the substrate concentration, \( v \) is the apparent maximal velocity, \( K_{m} \) is the apparent Michaelis constant, and \( A \) is the apparent substrate inhibition constant. To measure the reverse reaction, 0.2 mM NADP⁺ was substituted for NADPH in the standard reaction buffer, and various concentrations of xylitol were used.

NADP⁺ and NADPH labeled with deuterium in the pro-R position were synthesized from unlabeled and 2-deuterio-3-malate using malic enzyme by the method of Viola et al. (9), and were purified by fast protein liquid chromatography on a Mono-Q column using the procedure of Orr and Blanchard (10). A value of \( K_{m} = 1.00 \) observed under conditions where NADPH release is the rate-limiting step for \( K_{m} \) (11) confirms the lack of inhibitory impurities in the NADPH and NADP⁺ samples (12). Primary deuterium isotope effects on \( k_{\text{nuc}} \) and \( k_{\text{cat}}/K_{m} \) for DL-glyceraldehyde were measured by direct comparison at saturating NADPH/D concentrations (500 μM for ARKZezM, 40 μM for all others) in 50 mM K-MES buffer at pH 6.5, 25 °C. Initial velocities, corrected for background, were fitted to Equation 2 as follows,

\[ v = \frac{v_{\text{max}}}{K_{m} + A} + A \left( 1 + F_{\text{iso-effect}} \right) \]

where \( v \) is the initial velocity, \( A \) is the DL-glyceraldehyde concentration, \( K_{m} \) is the Michaelis constant, \( F \) is the fraction of deuterium in the NADPH/D cofactor, and \( E_{\text{ iso-effect}} \), \( K_{m} \), and \( E_{\text{nuc}} \) are the isotope effects minus 1 on \( k_{\text{nuc}}/K_{m} \) and \( k_{\text{cat}}/K_{m} \), respectively, by the nonlinear least-squares method using the Fortran programs of Cleland (13).

RESULTS

Characterization of Constructs—The expression plasmid pET11αCH1 encodes 318 residues of the chimeric enzyme, CH1, including the initiation methionine. Complete sequence analysis of the construct revealed one A → G mutation just upstream of the 5’-NdeI cloning site, but downstream of the Shine-Dalgarno consensus sequence. The reason for this mutation is not clear, although we had previously observed other mutations leading to an incompletely reconstituted cloning site in this region in pET11aAR which, nevertheless, did not affect efficient expression (7). The expression plasmid pET11αARala303-315, encodes a truncated aldose reductase missing the COOH-terminal 13 residues. The encoded protein sequences of both the CH1 and the ARala303-315 constructs are shown aligned to the wild type aldose reductase sequence in Fig. 1. The chimeric enzyme (CH1) incorporates 22% (70 residues) of human aldohexose reductase on its NH2-terminal end and 78% (247 residues) of human aldose reductase on its COOH-terminal side. To ensure that the effects observed in the truncated enzyme are not due to the disruption of a potential disulfide bridge eliminated by the truncation, the expression plasmid pET11aARchOOG was constructed to express a mutant aldose reductase ARala303, where Cys300 was replaced by a serine residue.

Expression and Physical Characterization of Enzymes—Maximal expression of all constructs was obtained 6–8 h following induction with B-β-thiogalactoside, with a yield of 10–20 mg of purified protein per liter culture. The purified enzymes show single bands on SDS-polyacrylamide gel electrophoresis, and Western blot analysis (Fig. 2) shows that the chimeric enzyme, CH1, is recognized by specific antibodies to both human aldose and aldehyde reductase. The two antibodies show no cross-reactivity to wild type aldose and aldehyde reductase.
Aern blot analysis of purified recombinant aldose reductase (36.4 kDa, 324 residues), and seems to Western blot analysis of identical gel to aldose reductase. Molecular mass standards were run in lane M. B. Western blot analysis of identical gel to A probed with antibody to human aldose reductase. C, Western blot analysis of identical gel to A probed with antibody to human aldose reductase. Arrows indicate direction of electrophoresis.

reductase. Fig. 2 also shows that the chimeric enzyme (35.6 kDa, 317 residues) has comparable mobility to the larger wild type aldohexose reductase (36.4 kDa, 324 residues), and seems larger than the wild type aldose reductase (35.7 kDa, 315 residues). The truncated ARA303-315 enzyme (33.9 kDa, 302 residues) has appropriately greater mobility than the wild type aldose reductase and the ARG305S mutant.

The IEP of the chimeric enzyme of 5.9 is intermediate between the wild type aldose and aldohexose reductase isoelectric points of 6.2 and 5.6, respectively (7). The truncated ARA303-315 has a more basic IEP of 6.9, and no change in the IEP of the ARG305S was observed.

Steady State Kinetic Constants—Table II compares the apparent kinetic constants for the recombinant aldose and aldohexose reductase (hAR and hGR, respectively), the chimeric enzyme (CH1), the truncated aldose reductase (ARA303-315), and the ARG305S mutant aldose reductase. The chimeric enzyme, CH1, has Km and kcat values for all tested substrates that more closely resemble those of aldose than aldohexose reductase. For example, the largest increase in Km value of CH1 as compared to wild type aldose reductase was for Dl-glyceraldehyde as a substrate, and that was only 5-fold. Similarly, the kcat/Km values of the chimeric enzyme for the various substrates, which reflect the specificity constants (14), more closely resemble those of aldose reductase. The KmNADPH for CH1, 2 μM, is identical to that of both hAR and hGR. The kinetic parameters for the reverse reaction (xyitolol oxidation) of CH1 were virtually identical to those of the wild type aldose reductase. The similarity of CH1 and hAR is clearly demonstrated by a pairwise comparison of the ratios of kcat/Km values calculated for pairs of enzymes (Table III). The kcat/Km ratios for hAR/CH1 are all close to 1, while those for hGR/CH1 and hGR/hAR are similar but quite different from those of hAR/CH1. Thus, exchanging the first domains of the aldose and aldohexose reductases does not seem to seriously affect aldohexose substrate specificity or NADPH binding and kinetics.

In contrast to the results of NH2-terminal domain exchange in CH1, the COOH-terminally truncated aldose reductase, ARA303-315, showed striking increases in the Km values for all substrates tested with the exception of D-glucuronate. The kcat values of the truncated enzyme for the various substrates, on the other hand, showed only modest changes in comparison to hAR, ranging from no change for p-nitrobenzaldehyde and D-xylene, to 3- and 5-fold decreases for Dl-glyceraldehyde and D-glucose, respectively. These changes result in 80- to 650-fold decreases in kcat/Km, the substrate specificity/catalytic effectiveness constant, for the reduction of uncharged aldohexose substrates by the truncated ARA303-315 as compared to wild type hAR (Table III). In contrast, the Km for a negatively charged substrate, D-glucuronate, barely changed from 4.9 to 2.0 mM, while the corresponding kcat decreased a mere 4-fold, and the kcat/Km remained identical to that of hAR. These findings suggest that the COOH-terminal domain of aldose reductase is crucial to the substrate specificity and catalytic efficiency of the enzyme for uncharged substrates.

The interpretation of results of the COOH-terminal domain deletions is confounded by the presence among the deleted residues of a cysteine (Cys305) which potentially could participate in modifying the enzyme’s tertiary structure by forming a disulfide linkage. To rule out this possibility, we mutated the wild type hAR to yield an ARG305S mutant. Within experimental error, the measured kinetic constants of the ARG305S mutant are identical to those for wild type hAR, indicating that the entire 13-residue COOH-terminal tail of aldose reductase is participating in maintenance of the tertiary configuration required for optimal orientation of substrates in the active site and catalytic effectiveness.

Isotope Effects with NADPH/D Cofactor—Comparison of the primary deuterium isotope effects for the wild type and mutant hAR enzymes (Table IV), determined by using NADPH and NADPD with Dl-glyceraldehyde as the substrate, provides further insight into the mechanistic basis for the observed changes in kinetic parameters. The isotope effect on kcat was equal to 1.00 for all but the ARA303-315 mutant, consistent with the rate of NADP+ release (or the EnNADP+ isomerization step preceding NADP+ release) being the slowest step for the overall forward reaction of aldohexose reduction (11). A significant isotope effect on kcat/Km for Dl-glyceraldehyde was found for all the enzymes tested, indicating that hydride transfer is partially rate-limiting for the steps between aldohexose addition to the E-NADP complex and the release of the alcohol product. There is no significant difference in D(kcat/Km) between the wild type hAR and CH1 or ARA303-315; however, the substantial increase in D(kcat/Km) for ARA303-315 suggest that the large decreases in kcat/Km for the various aldohexose is at least partially due to the hydride transfer step becoming more rate-limiting. The ARA303-315 mutant is the only enzyme which displays a D(kcat) value (1.08 ± 0.05) significantly different from 1.00, suggesting that catalysis has, in fact, been slowed to the point where it partially limits the overall turnover rate as well. The substrate-dependent change in kcat seen for ARA303-315 is consistent with this idea, and suggests that the COOH-terminal tail is indeed important in the proper alignment of the various elements involved in catalysis.

The deuterium isotope effects (Table IV) clearly differentiate the large decreases in kcat/Km for aldohexose substrates previously described for the ARG305S mutant (7) from the similar effects seen with the truncated ARA303-315 mutant. The
Role of COOH-terminal Domain of Aldose Reductase

Table II
Apparent kinetic constants of hAR, hGR, CH1, AR303-315, and AR323

<table>
<thead>
<tr>
<th></th>
<th>hAR</th>
<th>hGR</th>
<th>CH1</th>
<th>AR303-315</th>
<th>AR323</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_m (mM)</td>
<td>K_m (mM)</td>
<td>K_m (mM)</td>
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<td>K_m (mM)</td>
</tr>
<tr>
<td>DL-Glyceraldehyde</td>
<td>0.02</td>
<td>1.60</td>
<td>0.10</td>
<td>4.30</td>
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<tr>
<td></td>
<td>k_cat (s^{-1})</td>
<td>k_cat (s^{-1})</td>
<td>k_cat (s^{-1})</td>
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<td>k_cat (s^{-1})</td>
</tr>
<tr>
<td></td>
<td>0.45</td>
<td>1.20</td>
<td>0.59</td>
<td>0.15</td>
<td>0.34</td>
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<td>P-Nitrobenzaldehyde</td>
<td>22.7 \times 10^3</td>
<td>0.7 \times 10^3</td>
<td>6.0 \times 10^3</td>
<td>35</td>
<td>12.8 \times 10^3</td>
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<tr>
<td>D-Xylose</td>
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<td>193</td>
<td>5</td>
<td>319</td>
<td>9</td>
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<td></td>
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<td>93.0 \times 10^4</td>
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<td>147 \times 10^4</td>
<td>1.2 \times 10^5</td>
<td>34.7 \times 10^5</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>6</td>
<td>711^a</td>
<td>19.6</td>
<td>2.5 \times 10^5 b</td>
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<tr>
<td></td>
<td>0.30</td>
<td>1.25</td>
<td>0.47</td>
<td>0.29^a</td>
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</tr>
<tr>
<td></td>
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<td>24.1</td>
<td>0.1</td>
<td>40.0</td>
</tr>
<tr>
<td>D-Glucuronate</td>
<td>0.1 \times 10^4</td>
<td>6.4 \times 10^4 a,b</td>
<td>0.2 \times 10^4</td>
<td>3.0 \times 10^5 b</td>
<td>0.1 \times 10^5</td>
</tr>
<tr>
<td></td>
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<td>NADPH</td>
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<tr>
<td></td>
<td>58</td>
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</tr>
<tr>
<td>Xylitol</td>
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<td>734^a</td>
<td>332</td>
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<td>0.1</td>
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</tr>
</tbody>
</table>

^a New data.

^b 30% > standard deviation > 15%.

^c DL-Glyceraldehyde as substrate.

AR323M mutation was shown to affect NADPH binding and thus the k_cat/K_m for DL-glyceraldehyde does not change significantly for the AR323M mutant enzyme as compared to wild type hAR, despite large decreases in k_cat/K_m for both NADPH and glyceraldehyde (7). The decrease in k_cat/K_m value for DL-glyceraldehyde seen with AR303-315 (Table II), however, leads to significant increases in both k_cat/K_m and k_cat. These findings indicate that the Lys^363 \rightarrow Met substitution, by weakening NADPH binding, alters the apparent interaction with the aldehyde substrate without affecting catalysis (hydride transfer) per se. In contrast, deletion of the COOH-terminal 13 residues of hAR leads to a direct effect on the catalytic step by altering the ability of the substrate to orient properly in the catalytic site, with little perturbation of the nucleotide’s interaction with the active site.

**DISCUSSION**

Aldose and aldehyde reductase have broad, overlapping substrate specificities which include aromatic and aliphatic aldehydes of wide structural diversity as well as certain ketones. Aldose reductase, however, exhibits K_m values for most substrates that are one or two orders of magnitude lower than those of aldehyde reductase (2). The exceptions are negatively charged substrates, e.g. D-glucuronate and succinic semialdehyde, which are more efficiently converted to alcohols by aldehyde reductase than by aldose reductase.

We previously proposed the partition of aldose and aldehyde reductases into structural/functional domains, based on the degree of homology seen in the two enzymes (1). There are three highly homologous domains with the NH2-terminal domain of aldose reductase spanning Ala^1-Glu^G0, the second domain spanning Val^67-Leu^319, and a third domain spanning Lys^319-Arg^326, and the highly dissimilar COOH-terminal domains of Val^327-Phe^318 in aldose reductase and Tyr^329-Tyr^334 in aldehyde reductase. The residues connecting domains 1 to 2, and 2 to 3, are also very dissimilar in the two enzymes.

In a continuing effort to determine the function of the various homologous domains of aldose and aldehyde reductase, we first created a chimeric enzyme where the NHZ-terminal domain of aldose reductase spanning Ala^1-Glu^G0, the second domain spanning Val^67-Leu^319, and a third domain spanning Lys^319-Arg^326, and the highly dissimilar COOH-terminal domains of Val^327-Phe^318 in aldose reductase and Tyr^329-Tyr^334 in aldehyde reductase. The residues connecting domains 1 to 2, and 2 to 3, are also very dissimilar in the two enzymes.
terminal 71 residues are changed in the chimeric enzyme in the course of replacement with the NH₂-terminal domain of aldehyde reductase. The findings suggest that all important residues necessary for activity in the NH₂-terminal domains of both enzymes are conserved, and further that substrate specificity is not determined in a major way by the NH₂-terminal domain. Nevertheless, the specific function of the NH₂-terminal domain in the enzyme is not immediately discernible.

On the other hand, the COOH-terminal domains of aldose and aldehyde reductase are very dissimilar. To determine the function of the COOH-terminal domain in aldose reductase, we created a truncated AR₃₀₃-₃₁₅ mutant in which the COOH-terminal residue is Ser³⁰², thus eliminating 13 residues including Cys³⁰³. In addition, since aldose reductase contains 2 cysteine residues at positions 288 and 303 that have been proposed to form a disulfide bridge (15), we also made an AR₃₀₃S mutant to eliminate the possibility of disulfide bond formation involving Cys³⁰³.

The truncated AR₃₀₃-₃₁₅ enzyme showed an alkaline shift in the IEP (6.9 vs. 6.2 for the wild type), due to the loss of 3 acidic residues (Asp³⁰⁶, Gly³¹¹, and Glu³¹⁴) and only 1 basic residue (Lys³⁰⁷). The AR₃₀₃S mutant, on the other hand, is indistinguishable from wild type hAR.

Kinetic analyses of the truncated AR₃₀₃-₃₁₅ revealed extensive decreases in kₗ/Kₘ values relative to those for the wild type hAR for all aldohexoses tested, except D-glucuronate, and little effect on kₗ/Kₘ for the nucleotide. Again, the AR₃₀₃S mutant is virtually indistinguishable from the wild type hAR, thus showing that the deletion of the Cys³⁰³ cannot be responsible for the observed kinetic changes in the truncated AR₃₀₃-₃₁₅. A recently published low resolution crystal structure of the pig lens aldose reductase, and our own crystallographic data, indeed confirm that this cysteine residue exists in the reduced state (16, 17).

Aldose reductase follows a sequential ordered mechanism whereby NADPH forms an E-NADPH complex prior to substrate binding (11, 18, 19). The rate-limiting step in the overall mechanism has been shown to be the rate of isomerization of the E-NADP⁺ complex following hydride transfer and release of the alcohol product (11). The absence of any significant primary deuterium isotope effect on kₗ (kₗ/Kₘ) for the wild type enzyme and all of the mutant enzymes, with the exception of AR₃₀₃-₃₁₅, is consistent with the idea that the rate-limiting step for overall turnover occurs after the first irreversible step. The observation of a small, but significant D/kₗ effect (1.08 ± 0.05 combined with a large increase in D/kₗ/Kₘ for DL-glyceraldehyde from an average value of 1.87 ± 0.16 to 2.64 ± 0.04 for the AR₃₀₃-₃₁₅ mutant, however, suggests that deletion of COOH-terminal 13 residues has led to a slowing of the hydride transfer step to the point where it now partially limits the overall reaction. The lack of a significant effect on Kₐ for the AR₃₀₃-₃₁₅ mutant relative to the wild type hAR confirms that these changes have occurred without affecting the interaction between the enzyme and the nucleotide substrate.

Additional support for this view is provided by our studies of the AR₃₀₃S mutation which was shown to interfere with the overall catalytic mechanism primarily by affecting NADPH binding (7). The absence of a significant primary deuterium isotope effect on kₗ indicates that the hydride transfer step was not affected in the AR₃₀₃S mutant enzyme. Thus, the large decreases in kₗ/Kₘ for aldohexose substrates seen with the AR₃₀₃S mutant (7) must be due to the relatively lesser ability of the Lysine³⁰⁷-lacking mutant to form a productive E-NADPH complex. The contrasting results for the AR₃₀₃S and the truncated AR₃₀₃-₃₁₅ mutants clearly show that we have been able to differentiate the effects on nucleotide binding from those on substrate binding and catalysis of hydride transfer to the aldohexose substrate.

The kinetic findings in the truncated AR₃₀₃-₃₁₅ mutant provide an important clue to the mechanism of action of aldose reductase. The recently reported crystal structure of the porcine enzyme, and our refined 1.65 Å structure of the human aldose reductase (16, 17), show that the enzyme is structurally composed of a parallel α/β barrel motif with eight central β strands and eight peripheral α helices that connect the strands. The NH₂-terminal domain exchanged in CH1 includes the NH₂-terminal loop β₁-α₁-β₂-α₂ (17), and as stated above, is functionally homologous to that of aldohexose reductase. On the other hand, it is clear from the data herein reported that the COOH-terminal region is crucial to substrate specificity and catalytic efficiency as demonstrated by remarkable decreases in kₗ/Kₘ for aldohexose substrates upon its deletion. Although the crystal structure of lens pig aldose reductase is not sufficiently refined to allow a determination of the locus of the active site, or to describe the interaction of the full NADPH molecule (16), it is well known that the active site of α/β barrel enzymes is usually located at one end of the α/β barrel motif, where the carboxyl ends of the β strands connect with the amino ends of the α helices (20).

Our refined high resolution atomic structure of human aldose reductase (17) indeed shows that the active site of aldohexose reductase is located in a deep elliptical, highly hydrophobic pocket at the COOH-terminal end of the barrel. The nicotinamide ring of the tightly bound NADPH is at the bottom of the pocket with the C4 of the nicotinamide and the pro-R hydrogen of NADPH facing outwards. The COOH-terminal end, including residues Cys³⁰³-Pho³¹⁵, forms a loop around a portion of the barrel in the vicinity of the NADPH binding site and the elliptical active site pocket. Although knowledge of the atomic structure of the COOH-terminal loop has hitherto been insufficient to reveal its function, the deletion studies reported here clearly point to its being very important in determining the enzyme's tertiary structure and proper alignment of substrates in the active site pocket. Preliminary observations indicate that several residues of the COOH-terminal loop are involved in stabilizing some of the key residues forming the active site.²

² K. H. Gabbay and K. M. Bohren, manuscript in preparation.
contains 2 positively charged arginine residues that can interact with the negatively charged glucuronate. Since such positively charged residues are not present in the COOH-terminal loop of aldose reductase, its deletion apparently has a minor or no effect on D-glucuronate. Alternatively, D-glucuronate may take advantage of a different mode of binding to aldose reductase that is not available to uncharged substrates, and which is not affected by removal of the COOH-terminal domain. Further experiments are needed to distinguish these two possibilities.

The structure/function studies reported here identify the COOH-terminal loop of aldose reductase as an important determinant of catalytic effectiveness, a term which combines both substrate binding and catalytic rate (14). The function of the COOH-terminal loop is apparently distinct from that of nucleotide binding and enzyme isomerization, and may thus be very significant to the understanding of the catalytic mechanism and to the rational design of specific inhibitors of this enzyme that may be useful in the prevention of diabetic complications.

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