Kinetic and Spectroscopic Evidence for Active Site Inhibition of Human Aldose Reductase[†,‡]

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ABSTRACT: Aldose reductase is an NADPH-dependent oxidoreductase that catalyzes the reduction of a variety of aldehydes and carbonyls, including monosaccharides. Intense interest in the discovery and characterization of inhibitors has developed since the action of this enzyme has been linked to the pathogenesis of some diabetic complications. Since past studies indicated that most inhibitors act noncompetitively or uncompetitively versus substrate in the direction of aldehyde reduction, it was assumed that they bind at one or more sites distinct from the active site. However, the crystal stucture of aldose reductase complexed with inhibitor [Wilson *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9847−9851] unambiguously revealed the inhibitor bound at the active site. The present study was undertaken to address this apparent discrepancy. Using a fluorometric assay, it was determined that zopolrestat, an acetic acid-type inhibitor, bound to aldose reductase complexed with either NADPH or $NADP^+$. In contrast, the spirohydantoin-type inhibitor sorbinil demonstrated preferential binding to the binary enzyme·NADPH complex. Prior incubation of the enzyme·NADPH complex with zopolrestat prevented subsequent sorbinil binding. These results, together with the published structure of the ternary enzyme·NADPH·zopolrestat complex, are consistent with the conclusion that both sorbinil and zopolrestat bind at the active site. We propose that mixed inhibition patterns previously observed with sorbinil are due to inhibitor binding to both structural isomers of the enzyme·NADPH complex. Similar patterns for inhibition by zopolrestat are due to tight binding of the inhibitor. Substrate inhibition in the direction of aldehyde reduction occurs as a result of substrate binding to the enzyme·$NADP^+$ complex.

Aldose reductase (alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.21; ALR2[1]) catalyzes the NADPH-dependent conversion of aldoses and various aromatic and aliphatic aldehydes to their corresponding alcohols. While a physiologic role for mammalian ALR2 has not been firmly established, recent studies suggest potential roles in osmotic homeostasis (Burg, 1988), steroid conversion (Warren *et al.*, 1993), and detoxification of reactive aldehydes (Vander Jagt *et al.*, 1992; Kolb *et al.*, 1994). As a catalyst of the first step in the polyol pathway, ALR2 converts glucose to sorbitol. Enhanced metabolism of glucose through the polyol pathway may lead to biochemical imbalances associated with diabetic complications such as retinopathy and neuropathy (Kinoshita & Nishimura, 1988). Inhibition of ALR2 therefore provides an attractive therapeutic strategy for prevention of some diabetic complications.

Models for the catalytic mechanism of ALR2 have recently been proposed on the basis of kinetic and crystallographic studies of wild-type and mutant forms of the enzyme. In the direction of aldehyde reduction, the enzyme follows a sequential ordered mechanism in which NADPH binds before the aldehyde substrate and $NADP^+$ is released after the alcohol product (Grimshaw *et al.*, 1990; Kubiseski *et al.*, 1992). A kinetically detectable conformational change involving a hingelike movement of a surface loop (residues 213−217) occurs upon binding of NADPH (E·NADPH → *E·NADPH)[2] and immediately prior to release of $NADP^+$ (Kubiseski *et al.*, 1992; Wilson *et al.*, 1992; Borhani *et al.*, 1992). Reorientation of this loop (*E·$NADP^+$ → E·$NADP^+$) to permit release of $NADP^+$ appears to represent the rate-limiting step in the catalytic mechanism (Grimshaw *et al.*, 1990, 1995a,b; Kubiseski *et al.*, 1992).

Site-directed mutagenesis and X-ray crystallography studies have been instrumental in identifying functional groups

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[1] Abbreviations: ALR2, aldose reductase; sorbinil, (S)-6-fluorospiro-[chroman-4,4′-imidazolidine]-2′,5′-dione; zopolrestat, 3,4-dihydro-4-oxo-3-[[5-(trifluoromethyl)-2-benzothiazolyl]methyl]-1-phthalazineacetic acid.

[2] E·NADPH and *E·NADPH refer to the binary enzyme·coenzyme complexes before and after the structural isomerization that occurs on coenzyme binding. In cases where no distinction between the structural isomers is intended, the binary complex is referred to as the enzyme·NADPH complex. Similar nomenclature applies to binary complexes involving $NADP^+$.

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at the active site of ALR2. Crystallography of human ALR2 complexed with NADP(H) revealed three residues that could potentially function as the proton donor in the catalytic mechanism: tryrosine-48, histidine-110, and cysteine-298 (Wilson *et al.*, 1992; Harrison *et al.*, 1994). Mutagenesis and structural-modeling studies, together with detailed kinetic analysis, provided compelling evidence that tyrosine-48 is most likely the proton donor (Tarle *et al.*, 1993; Bohren *et al.*, 1994; Grimshaw *et al.*, 1995a,b). This was further corroborated by crystallographic studies which showed that zopolrestat, an ALR2 inhibitor containing a carboxylate functional group, binds with high affinity directly at the active site (Wilson *et al.*, 1993). One of the inhibitor's carboxylate oxygen atoms, which would be analogous to an aldehyde substrate carbonyl oxygen, was within a favorable distance (2.65 Å) of the phenolic hydroxyl of tyrosine-48. Likewise, the inhibitor's carbonyl carbon atom, analogous to an aldehyde substrate carbonyl carbon, was appropriately separated (3.63 Å) from the nicotinamide C4 of NADPH.

Two major classes of ALR2 inhibitors, carboxylic acids and spirohydantoins, have been extensively studied and tested in experimental animal models over the past decade (Sarges & Oates, 1993). Lineweaver−Burk plots typically revealed noncompetitive and/or uncompetitive inhibition patterns when inhibitors were tested in the direction of aldehyde reduction with aldehyde as the variable substrate [reviewed by Larson *et al.* (1988)]. These results appeared to be consistent with the inhibitor binding site being distinct from either substrate or cofactor binding sites. This conclusion now appears to be contradicted by recent crystallographic evidence showing that the potent carboxylic acid-type inhibitor zopolrestat binds directly at the active site of human ALR2 (Wilson *et al.*, 1993). In the present study, we address this apparent discrepancy by examining the inhibition kinetics of recombinant human ALR2 using sorbinil and zopolrestat, ALR2 inhibitors of the spirohydantoin and carboxylic acid classes, respectively.

MATERIALS AND METHODS

Preparation of Enzyme. Aldose reductase was overexpressed in *Escherichia coli* strain JM101 host cells harboring the cDNA (pHuALR2-1) encoding wild-type aldose reductase (Tarle *et al.*, 1993). The recombinant enzyme was purified by chromatofocusing on PBE 94 (Pharmacia LKB Biotechnology Inc.) and hydroxylapatite chromatography (Bio-Rad) essentially as described previously (Petrash *et al.*, 1992). Enzyme isolated by these steps was further purified by chromatography on Affi-Gel Blue resin (Bio-Rad) in 10 mM potassium phosphate (pH 7.1) containing 1 mM EDTA and 1 mM DTT. Bound enzyme was eluted from the pseudoaffinity resin by a linear gradient of NaCl (0 to 1.4 M). Apparent homogeneity of the purified material was established by the appearance of a single $M_r \sim 36\,000$ polypeptide on SDS−PAGE and Coomassie Brilliant Blue R-250 staining. The enzyme concentration was determined from the quenching of protein intrinsic fluorescence that occurs upon binding of NADPH essentially as described earlier (Grimshaw *et al.*, 1989).

Enzyme Assay Reagents and Inhibitors. NADPH and L-glyceraldehyde were obtained from Sigma Chemical Co. Zopolrestat and sorbinil were obtained from Pfizer Central Research, Groton, CT.

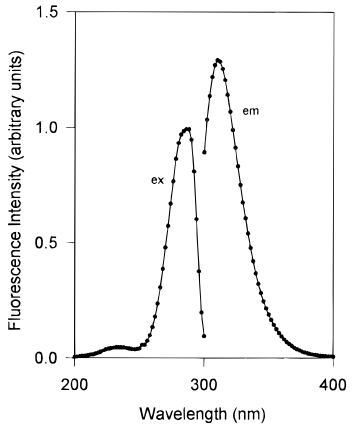


FIGURE 1: Fluorescence spectra of sorbinil. Spectra were obtained at pH 7.0 in 100 μM sorbinil contained in the triple buffer system described in Materials and Methods. Excitation (ex) and emission (em) spectra were collected with emission and excitation wavelengths set to 310 and 280 nm, respectively.

Determination of Inhibitor Binding by Fluorescence Titration. Binding of inhibitors was studied by fluorimetry by using a PTI Alphascan fluorimeter (Photon Technology Inc.). Binding assays were carried out at pH 7.0 in a triple buffer system consisting of 25 mM 2-(*N*-morpholino)-ethanesulfonic acid, 25 mM potassium phosphate, and 90 mM Tris. Solutions were maintained at 25 °C with the use of a thermostatted water circulator. Fluorescence scans of sorbinil revealed excitation and emission maxima at approximately 285 and 310 nm, respectively (Figure 1). To measure binding of sorbinil to the enzyme and its complexes, fluorescence scans from 300 to 345 nm with excitation at 285 nm were typically collected. In the measurement of sorbinil binding to the E·NADPH complex, protein intrinsic fluorescence was quenched by approximately 85% as measured near 310 nm. The fluorescence intensities at appropriate wavelengths were plotted as a function of the inhibitor concentration. Because we observed some photodegradation of the fluorophore as a result of repeated measurements, it was necessary to use fresh enzyme solution for each scan when measuring the binding of inhibitor to the apoenzyme or the enzyme·$NADP^+$ complex. Control mixtures (blank reactions) containing no enzyme were measured at each inhibitor concentration.

For binding of zopolrestat to the enzyme·$NADP^+$ complex, approximately 0.1 μM enzyme preincubated with 300 μM $NADP^+$ was titrated with 10 μM zopolrestat solution. Titration of the enzyme·NADPH complex with zopolrestat was carried out by adding aliquots of 100 μM zopolrestat solution to 0.92 μM enzyme preincubated with 10 μM NADPH. Fluorescence quenching at 320−325 nm upon zopolrestat binding was measured with the excitation wavelength set to 282 nm.

The data for sorbinil binding to the enzyme·NADPH complex, after correcting for background from enzyme blank titrations, was fitted to the following equation:

$$F_{obs} = F_o - (F_o - F_\infty)[[L]_t + K_d + [E]_t - \sqrt{([L]_t + K_d + [L]_t)^2 - 4[L]_t[E]_t}/2[E]_t] \quad (1)$$

where K_d is the thermodynamic dissociation constant, F_{obs} is the observed fluorescence intensity, F_o is the fluorescence intensity measured in the absence of NADPH, F_∞ is the

fluorescence intensity extrapolated to a saturating concentration of sorbinil, $[L]_t$ is the total concentration of sorbinil, and $[E]_t$ is the total concentration of the enzyme.

The data for zopolrestat binding were fitted to the same equation with the exception that F_∞ in the equation was substituted with $F_\infty + C[L]_t$, where C is the constant to describe the fluorescence of the ligand.

In addition, evidence for binding of sorbinil to the ternary enzyme·NADPH·zopolrestat complex was sought in order to determine if sorbinil could utilize a binding site distinct from the active site. To test this possibility, enzyme solution (0.57 μM) was preincubated with 300 μM NADPH and 1.0 μM zopolrestat and was titrated with 1 mM sorbinil. Fluorescence spectra were collected at sorbinil concentrations ranging from 0.1 to 10 μM.

Enzyme Assay. Aldose reductase activity was determined spectrophotometrically from the decrease in absorbance at 340 nm upon oxidation of NADPH. Approximately 0.1−0.5 μM enzyme was preincubated in 150 μM NADPH and varied concentrations of the inhibitors in a 1 mL triple buffer system consisting of 25 mM 2-(*N*-morpholino)ethanesulfonic acid, 25 mM potassium phosphate, and 90 mM Tris at pH 7.0. Reactions were initiated by the addition of L-glyceraldehyde. All enzyme assays and determination of the inhibition constant K_i were carried out at 25 °C with a Cary 1E spectrophotometer interfaced with a peltier temperature-controlling device.

Initial velocity rate data as a function of inhibitor and substrate concentrations were fitted to the equations given below. For inhibition with sorbinil

$$\frac{v}{[E]} = [S]/[P_1 + P_2[I] + (P_3 + P_4[I])[S] + P_5[S]^2] + [S]/(P_6 + P_7[S]) \quad (2)$$

where [I] and [S] are the concentration of the inhibitor and substrate, respectively. Terms P_1-P_7 are parameters made up of a combination of various dissociation constants and microscopic rate constants.

For inhibition with zopolrestat, the data were fitted to the equation for tight-binding inhibitors (Cha, 1975) with the modification for the high-K_m enzyme component.

$$\frac{v}{[E]_t} = [2K_i/[K_i + [I]_t - [E]_t + \sqrt{(K_i + [I]_t + [E]_t)^2 - 4[I]_t[E]_t}]\,[[S]/(P_1 + P_3[S])] + [S]/(P_6 + P_7[S]) \quad (3)$$

Expression of parameters P_1-P_5 are given in the Appendix. Parameters P_6 and P_7 are discussed in the Results.

RESULTS

Binding of the Inhibitors. Sorbinil binding to the enzyme·NADPH complex (K_d of 1.7 ± 0.3 μM) leads to enhancement of fluorescence measured near 310 nm (Figure 2A). However, fluorescence enhancement does not occur when sorbinil is incubated either with the apoenzyme or with the enzyme·$NADP^+$ complex. In such cases, the fluorescence spectra corrected for the ligand fluorescence were independent of the ligand concentration (not shown). Binding of sorbinil to the enzyme·NADPH complex and not to the apoenzyme indicates that these ligands bind in an ordered fashion.

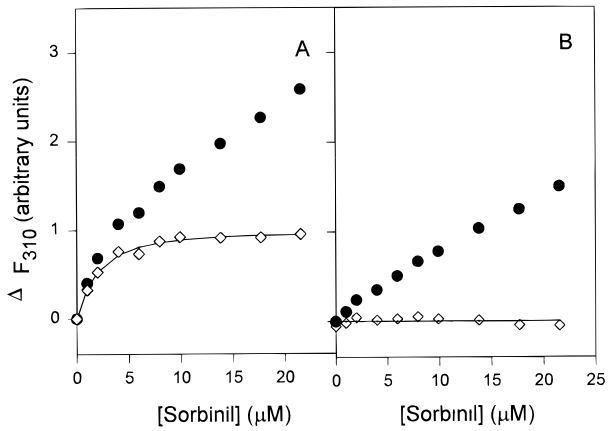


FIGURE 2: Measurement of the thermodynamic dissociation constant for sorbinil for the aldose reductase·NADPH complex and for the aldose reductase·NADPH·zopolrestat complex. Filled circles and open diamonds represent raw data and the data after the correction of the fluorescence from the ligand, respectively. The excitation wavelength was 285 nm. (A) Aldose reductase (0.57 μM) was titrated with sorbinil solution (1 mM) in the presence of 300 μM NADPH. (B) Aldose reductase (0.57 μM) was titrated with sorbinil solution (1 mM) in the presence of 300 μM NADPH and 1 μM zopolrestat.

However, we observed that the apparent K_d for NADPH in the presence of sorbinil, as measured by protein intrinsic fluorescence, was not strictly dependent on the inhibitor concentration (data not shown). The latter observation would seem to be inconsistent with an ordered binding pattern. It is possible that the transient existence of an enzyme·sorbinil complex (i.e. equilibrium is much less favored for the enzyme·sorbinil complex than either for the apoenzyme or for the enzyme·NADPH complex) provides the pathway between the apoenzyme and the enzyme·NADPH·sorbinil complex.

Binding of zopolrestat to the enzyme·NADPH complex quenches both protein intrinsic fluorescence near 330 nm and the energy-transfer fluorescence to the dihydronicotinamide moiety of NADPH observed near 450 nm (Figure 3). We expected that fluorescence quenching upon binding of the zopolrestat to the enzyme·NADPH complex would permit determination of the K_d value. However, binding was so tight that accurate estimation of the K_d was not possible (Figure 3). Unlike sorbinil, zopolrestat does not bind exclusively with the enzyme·NADPH complex. Instead, it binds very tightly to both the enzyme·NADPH and enzyme·$NADP^+$ complexes as indicated by quenching of fluorescence near 330 nm. As with sorbinil, zopolrestat does not bind the apoenzyme. No ligand-dependent fluorescence change was observed when the inhibitor was incubated with the apoenzyme (data not shown).

To examine whether sorbinil and zopolrestat bind at overlapping sites, we compared the fluorescence enhancement in enzyme·NADPH complexes preincubated with zopolrestat and buffer control. No sorbinil-dependent fluorescence enhancement is seen if the tight-binding inhibitor zopolrestat is previously incubated with the enzyme·NADPH complex (Figure 2B).

Inhibition with Sorbinil. At low substrate concentrations, the profile of v/[E] versus [S] in the absence of inhibitor was found to fit very well to the Michaelis−Menten equation (Figure 4A). However, at high substrate concentrations (>∼5 mM), substantial substrate inhibition was observed

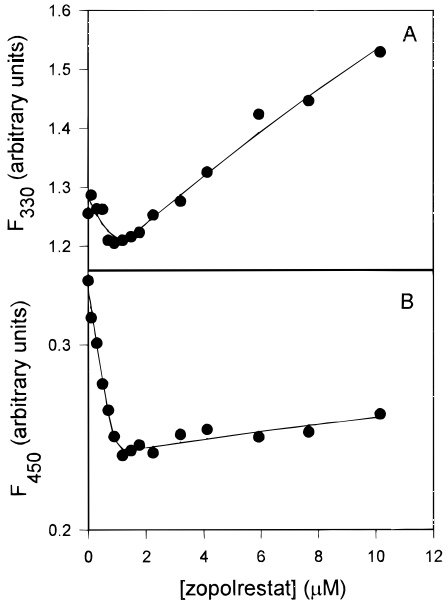


FIGURE 3: Estimation of the thermodynamic dissociation constant for zopolrestat for the aldose reductase·NADPH complex. A solution containing 0.92 μM aldose reductase and 10 μM NADPH was titrated with 100 μM zopolrestat. The excitation wavelength was 282 nm. Fluorescence emission as a function of zopolrestat was collected at (A) 330 nm and (B) 450 nm.

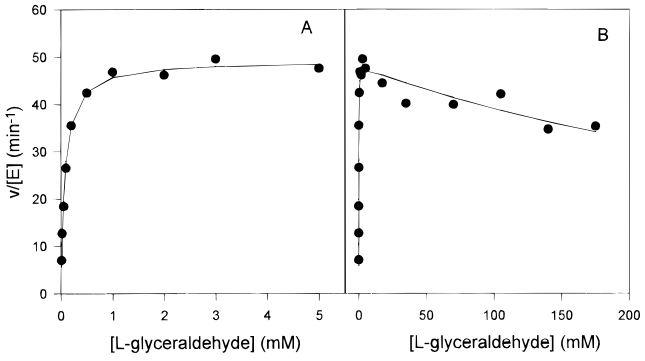


FIGURE 4: Initial catalytic rate of aldose reductase. (A) The rate data from assays conducted with L-glyceraldehyde up to the concentration of 5 mM were fitted to the Michaelis−Menten equation. (B) The data including the points at the high substrate concentration were fitted to eq 2 with $P_6 = P_7 = 0$.

(Figure 4B). Rate data were found to fit well to eq 2 with $[I]_t = 0$ and $P_6 = P_7 = 0$. The obtained parameter values are summarized in Table 1. Deviation from Michaelis−Menten kinetics was observed as apparent substrate activation even in the absence of sorbinil. This deviation became more evident at progressively higher concentrations of sorbinil (Figure 5A). In the double-reciprocal plot, the deviation is seen as a curvature of the plots near the ordinate (Figure 5B). This observation is consistent with the existence of two forms of enzyme: a high-K_m form and a low-K_m form. The existence of high- and low-K_m forms has been previously reported for aldose reductase isolated from various sources (Ward *et al.*, 1991; Vander Jagt *et al.*, 1990; Del Corso *et al.*, 1987). Although the nature of the high-K_m form is not clear, the rate data were found to fit very well to the equation after inclusion of an additional term to accommodate the existence of the high-K_m form.

Inhibition with Zopolrestat. Unlike that of sorbinil, binding of zopolrestat to the enzyme·coenzyme complex is sufficiently tight to invalidate the assumption that the concentration of unbound inhibitor is equal to the total inhibitor concentration. Zopolrestat inhibition data were found to fit well to eq 3 (Figure 6). Stoichiometry of the enzyme and the inhibitor obtained from the fit was close to equimolar ([E]/[zopolrestat] = 1/1.16). Deviation from the kinetic profile expected for a Michaelis−Menten mechanism was observed again (Figure 6). The values of the parameters P_6 and P_7, which represent the high-K_m component of the enzyme, agreed reasonably well with the values obtained from the data of sorbinil inhibition (Table 1). Because of the good agreement in P_6 and P_7, despite the large difference in the K_d values for each of these two inhibitors, it may be concluded that the high-K_m component is almost totally insensitive to both sorbinil and zopolrestat.

DISCUSSION

Most well-studied aldose reductase inhibitors may be grouped into one of two major structural classes: carboxylic acids and acidic cyclic amides [reviewed in Larson *et al.* (1988)]. Zopolrestat and sorbinil, respectively, are representative of each class of inhibitor. In Lineweaver−Burk plots of aldose reductase assayed in the direction of aldehyde reduction in the presence of various inhibitors of either class, profiles characteristic of uncompetitive or noncompetitive inhibition have been reported. This led to the conclusion that aldose reductase inhibitors of both structural classes bind at sites other than the substrate binding site [reviewed in Kador (1988)]. However, it was recently demonstrated by X-ray crystallography that zopolrestat, a carboxylic acid-type inhibitor, binds directly at the active site of human aldose reductase (Wilson *et al.*, 1993). Thus, the previous deduction that aldose reductase inhibitors bind at one or more allosteric sites appears to be discordant with more recent structural evidence. The present studies were undertaken in an attempt to reconcile this apparent discrepancy.

A refined 1.8 Å X-ray structure of the human aldose reductase holoenzyme complexed with zopolrestat revealed the drug to be stabilized by numerous contacts with residues lining the large, predominantly hydrophobic active site pocket (Wilson *et al.*, 1993). The X-ray structure of a ternary complex of zopolrestat and FR-1, a murine aldo-keto reductase with 70% identity with human aldose reductase, revealed that the inhibitor bound at the active site in a manner strikingly similar to that observed for human aldose reductase (Wilson *et al.*, 1995). The extensive contacts with hydrophobic residues in the active site cavity of aldose reductase are consistent with the tight-binding affinity we observed in the present kinetics studies, which revealed an approximately stoichiometric inhibition by zopolrestat. Since no allosteric zopolrestat binding sites appear to be present on either aldose reductase or FR-1 (Wilson *et al.*, 1993, 1995), it seems reasonable to conclude that zopolrestat binds human aldose reductase exclusively at the active site.

In the absence of an X-ray structure of aldose reductase complexed with sorbinil, efforts to determine a binding site for this inhibitor depend on kinetic analysis and indirect binding assays of protein intrinsic fluorescence changes occurring upon inhibitor binding and/or quantification of inhibitor binding by ultrafiltration assays (Ehrig *et al.*, 1994). Several recent observations made with these procedures support the hypothesis that sorbinil binds at or near the active site. Sorbinil competitively inhibits benzyl alcohol oxidation

Table 1: Kinetic Parameters Obtained by Fitting the Steady-State Initial Velocity for Human Aldose Reductase as a Function of the Substrate and Inhibitor Concentrations to the Equations Described in the Text

parameter	inhibitor		
	none	sorbinil	zopolrestat
P_1 (min mM)	0.00149 ± 0.00016	0.00141 ± 0.0007	0.0161 ± 0.0008
P_2 (min)	−	0.384 ± 0.154	−
P_3 (min)	0.0207 ± 0.0004	0.0207 ± 0.0004	0.0205 ± 0.0005
P_4 (min mM^{-1})	−	62.1 ± 20.1	−
P_5 (min mM^{-1})	$4.91 \times 10^{-5} \pm 7.7 \times 10^{-6}$	−	−
P_6 (min mM)	−	2.80 ± 0.90	4.26 ± 2.51
P_7 (min)	−	0.131 ± 0.045	0.142 ± 0.167
k_{cat}[a] (min^{-1})	48 ± 1	48 ± 1	48 ± 1
K_m (mM)	0.07 ± 0.01	0.07 ± 0.04	0.08 ± 0.04
k_{cat}/Km (min^{-1} mM^{-1})	700 ± 100	700 ± 100	600 ± 300
k_{cat}/K_m for high-*K*m component (min^{-1} mM^{-1})	−	0.36 ± 0.012	0.23 ± 0.14

[a] The values of k_{cat} shown here are the turnover number for the low-K_m component enzyme relative to the total of the low-K_m and high-K_m component enzymes.

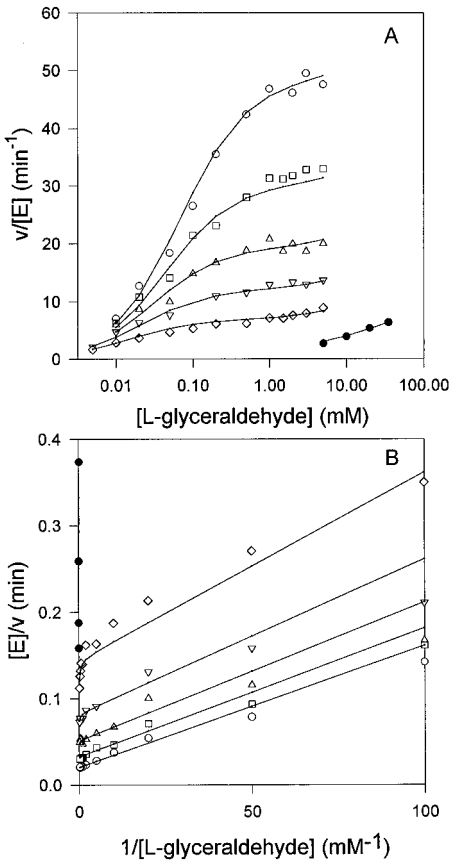


FIGURE 5: Initial catalytic rate of aldose reductase at varied concentrations of sorbinil. Inhibitor concentrations were (○) 0 μM, (□) 0.2 μM, (△) 0.5 μM, (▽), 1.0 μM, (◇), 2.0 μM, and (●) 10 μM. All the data points were analyzed by simultaneous nonlinear least-squares fitting to eq 2. The value of the parameter in the present fit was assumed to be 0 since the magnitude of parameter P_5 for the present data should be insignificantly small: (A) semilog plot and (B) Lineweaver−Burk plot.

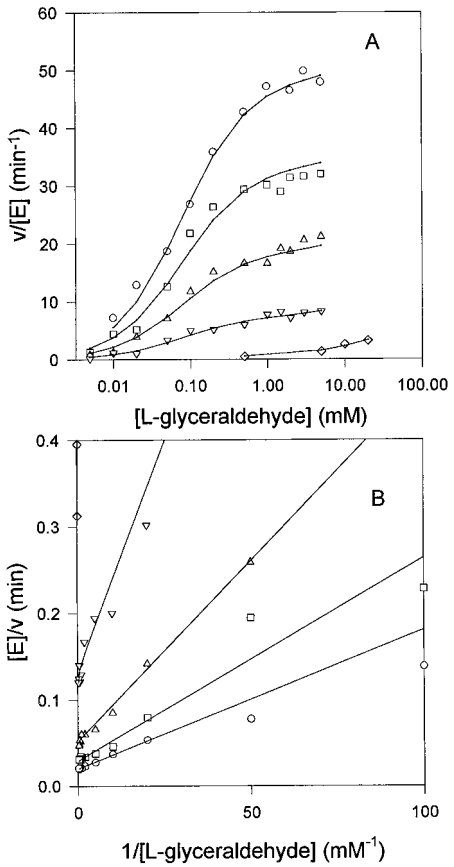


FIGURE 6: Initial catalytic rate of aldose reductase at varied concentrations of zopolrestat. Inhibitor concentrations were (○) 0 nM, (□) 72 nM, (△), 144 nM, (▽), 216 nM, and (◇) 719 nM zopolrestat: (A) semilog plot and (B) Lineweaver−Burk plot.

when assayed in the presence of the NADP$^+$ analog 3-acetylpyridine adenine dinucleotide phosphate (Liu *et al.*, 1992). Our present studies show that prior binding of zopolrestat to the binary enzyme·NADPH complex prevents subsequent binding of sorbinil. This indicates either that zopolrestat and sorbinil utilize overlapping binding sites or that prior binding of zopolrestat induces a conformational change that abolishes a crucial binding element in the sorbinil site. The latter possibility is strengthened by the observation that zopolrestat binding in the active site cavity of human aldose reductase induces a conformational change involving movement of a loop (residues 121−135) and a short peptide segment (residues 298−303) near the C-terminal end of the protein (Wilson *et al.*, 1993). Prior mutagenesis and chemical modification studies demonstrated that Cys298, which is contained within the mobile peptide segment, marks a domain for sorbinil binding. In the binary enzyme·NADP(H) complex, the thiol group of Cys298 is approximately 4 Å from the C4 of the nicotinamide group of the coenzyme (Wilson *et al.*, 1992). Modification of Cys298 by substitution with serine, as well as by carboxymethylation, resulted in enzyme forms demonstrating marked alterations in sensitivity to sorbinil (Petrash *et al.*, 1992; Bhatnagar *et al.*, 1994). We interpret the ability of sorbinil to bind the enzyme·NADPH complex but not the apoenzyme or the

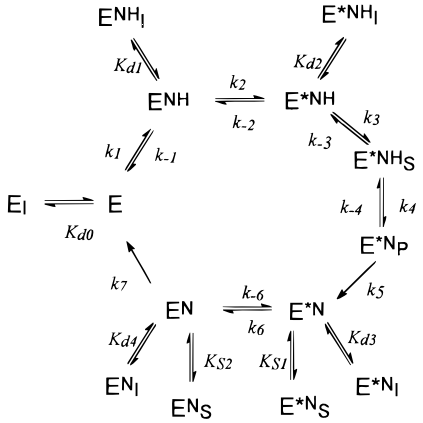
binary enzyme·NADP$^+$ binary complex as evidence that the sorbinil site is associated with a domain near the nicotinamide ring, which in turn marks the active site domain. Unfortunately, the lack of an X-ray structure for aldose reductase complexed with sorbinil makes it virtually impossible to propose a structural basis for selective binding of sorbinil to the E·NADPH complex. We hypothesize that differences in the charge state and structures of the binary E·NADPH and E·NADP$^+$ complexes, as yet unsolved at atomic resolution, underlie the selective binding of sorbinil.

If sorbinil and zopolrestat bind at the active site, why do they fail to produce Lineweaver−Burk profiles typical of competitive inhibition when tested with aldehyde as the variable substrate (Bhatnagar *et al.*, 1990; Mylari *et al.*, 1991)? With tight-binding inhibitors such as zopolrestat, Lineweaver−Burk plots acutely curve near the ordinate. In addition, extrapolation from the asymptotic lines at lower substrate concentrations reveals a convergence at the abscissa as in plots for noncompetitive inhibition. To obtain classical linear Lineweaver−Burk plots, it must be assumed that the total inhibitor concentration is equal to the concentration of free inhibitor. In the case of zopolrestat, this assumption is not appropriate. The fraction of bound zopolrestat relative to total inhibitor is very large at low zopolrestat concentrations because of tight binding. Therefore, linear Lineweaver−Burk plots typical of competitive inhibition will not be observed with zopolrestat in the direction of aldehyde reduction even though its binding site overlaps that of the aldehyde substrate.

An additional consideration relates to the distribution of enzyme forms in the reaction sequence of aldehyde reduction. Previous studies demonstrated that reduction of aldehyde substrates by aldose reductase follows an ordered Bi−Bi mechanism in which the substrate binds only after the enzyme has formed the binary complex with NADPH and release of the alcohol product precedes release of NADP$^+$ (Grimshaw *et al.*, 1990; Kubiseski *et al.*, 1992). In addition, isomerization of the enzyme to an alternate conformational isomer after binding of NADPH (E·NADPH → *E·NADPH) and after the release of the alcohol product (*E·NADP$^+$ → E·NADP$^+$) has been reported. The latter, kinetically significant structural isomerization is thought to represent the rate-limiting step in the direction of aldehyde reduction (Grimshaw *et al.*, 1990; Kubiseski *et al.*, 1992). Since the predominant enzyme form at steady state is *E·NADP$^+$, inhibitors which bind this binary complex will give rise to uncompetitive inhibition patterns when tested in the direction of aldehyde reduction (Liu *et al.*, 1992; Ward *et al.*, 1993; Grimshaw *et al.*, 1995c).

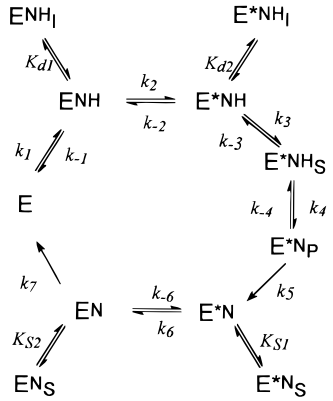
To account for all known steps in the reaction scheme and to evaluate points of inhibition by sorbinil, we considered the catalytic mechanism shown in Scheme I (adapted from Kubiseski *et al.* (1992)]. Dependence of the catalytic rate on the substrate concentration is described by eq 2. According to this mechanism, it may be seen that the ordinate intercept of the Lineweaver−Burk plot is independent of the inhibitor concentration only if the parameter P_4 is equal to zero. This condition is satisfied if the inhibitor binds only to the binary enzyme·NADPH complex, namely *E·NADPH. Since our fluorescence quenching measurements indicate that sorbinil does not bind the apoenzyme or the enzyme·NADP$^+$ complex, a noncompetitive or uncompetitive pattern is most likely brought about by the inhibitor binding to E·NADPH and *E·NADPH.

Scheme 1: Catalysis and Inhibition Scheme for Aldose Reductase



[a] E, aldose reductase; E*, aldose reductase of an alternative conformation; NH, NADPH; N, NADP; I, inhibitior; S, substrate; and P, product.

Scheme 2: Catalysis and Inhibition Scheme for Aldose Reductase and Sorbinil



A characteristic feature of aldose reductase is its susceptibility to inhibition by high concentrations of substrate. We propose that substrate inhibition is manifest by binding of the substrate to either or both of the binary E·NADP$^+$ or *E·NADP$^+$ complexes, which gives rise to a non-zero value of the parameter P_5.

These studies provide evidence that both sorbinil and zopolrestat bind at the active site of human aldose reductase. Mixed inhibition patterns previously observed in Lineweaver−Burk plots for inhibition by zopolrestat are due to the tight binding of this inhibitor. Similar patterns for sorbinil inhibition are due to binding of the inhibitor to the binary enzyme·NADPH complex. Substrate inhibition occurs from binding of aldehyde to the enzyme·NADP$^+$ complex. While many structurally diverse aldose reductase inhibitors may share binding domains at the active site of aldose reductase, it seems likely that they will possess different affinities for various binary enzyme·coenzyme complexes. Further study will be required to more fully understand their mechanisms of action.

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APPENDIX

Parameters P_1-P_5 in eq 2 for Scheme 1 derived with the King−Altman method (1956) are expressed as follows:

$$P_1 = (k_4k_5k_{-1}k_{-2} + k_5k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_{-3}k_{-4} + k_4k_5k_1k_{-2} + k_5k_1k_{-2}k_{-3} + k_1k_{-2}k_{-3}k_{-4} + k_4k_5k_1k_2 + k_5k_1k_2k_{-3} + k_1k_2k_{-3}k_{-4})/k_1k_2k_3k_4k_5$$

$$P_2 = (k_4k_5k_{-2} + k_5k_{-2}k_{-3} + k_{-2}k_{-3}k_{-4})/K_{d1}k_5k_2k_3k_4 + (k_4k_5k_2 + k_5k_2k_{-3} + k_2k_{-3}k_{-4})/K_{d2}k_5k_2k_3k_4 + (k_4k_5k_{-1}k_{-2} + k_5k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_{-3}k_{-4})/K_{d0}k_1k_2k_3k_4k_5$$

$$P_3 = (k_2k_4k_5k_6k_7 + k_4k_5k_6k_7k_{-1} + k_6k_7k_1k_2k_4 + k_7k_1k_2k_4k_5 + k_1k_2k_4k_5k_{-6} + k_1k_2k_4k_5k_6 + k_4k_5k_6k_7k_1 + k_5k_6k_7k_1k_2 + k_6k_7k_1k_2k_{-4})/k_5k_6k_7k_1k_2k_4$$

$$P_4 = (k_7 + k_{-6})/K_{d3}k_6k_7 + 1/K_{d4}k_7 + (k_2 + k_{-1})/K_{d0}k_1k_2 + 1/K_{d1}k_2$$

$$P_5 = (k_7 + k_{-6})/K_{S1}k_6k_7 + 1/K_{S2}k_7$$

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