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Does sorbinil bind to the substrate binding site of aldose reductase?

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Abstract—With benzyl alcohol as the varied substrate, sorbinil was found to be a competitive inhibitor of aldose reductase, an enzyme implicated in the etiology of secondary diabetic complications. The $K_{is\,sorbinil}$ and the V_{max}/K_m (V/K) benzyl alcohol decreased at low pH with a pK of 7.5 and 7.7, respectively. These observations suggest that both sorbinil and benzyl alcohol bind to the same site on the enzyme. Active site inhibition by sorbinil is consistent with non-competitive inhibition patterns of sorbinil with nucleotide coenzyme or aldehyde as the varied substrate in the direction of aldehyde reduction.

Aldose reductase-catalyzed reduction of glucose to sorbitol constitutes the first step of the polyol pathway [1, 2]. Activation of this pathway has been implicated in the manifestation of tissue damage associated with secondary complications of diabetes mellitus [1, 2]. Inhibition of aldose reductase has been shown to attenuate, prevent or reverse hyperglycemic injury and, therefore, clinical use of such inhibitors has been suggested for the therapeutic management of secondary diabetic complications [1, 3].

A number of structurally diverse compounds inhibit aldose reductase. However, these compounds also inhibit related enzymes [4, 5], and their clinical efficacy has not been clearly established [6]. Most aldose reductase inhibitors display non- or uncompetitive inhibition profiles in the direction of aldehyde reduction [5, 7], which has led some investigators to suggest that the substrate and inhibitor binding site of the enzyme are different [7]. Structural analysis of the available inhibitors has been used to postulate a minimal model of the inhibitor binding site of the enzyme [7, 8]. However, the location of this inhibitor binding site and its relationship to the active site of the enzyme remains undetermined. Present studies indicate that sorbinil and alcoholic substrate bind to the same site on the enzyme.

Materials and Methods

Aldose reductase was purified from human placenta by the method of Das and Srivastava [9], except that ammonium sulfate was used as the first step in the purification protocol. During purification, enzyme activity was monitored on a Gilford Response Spectrophotometer at 340 nm using 10 mM DL-glyceraldehyde and 100 μM NADPH in 0.05 M phosphate, pH 6.0, containing 0.4 M lithium sulfate. For kinetic studies enzyme activity was determined using benzyl alcohol and 3-acetylpyridine-ADP (3-APADP$^+$) at 363 nm. Sorbinil was used for all inhibition kinetics because its K_i is much higher than the enzyme concentration used for kinetic assays to permit interpretation without correction for tight binding (see below).

Since storage of aldose reductase, even in thiol-containing solutions, leads to the generation of several partially oxidized forms of the enzyme with varying sensitivity to sorbinil [10], the stored enzyme was reduced immediately before use by incubation with 0.1 M dithiothreitol (DTT) at 37° for 1 hr. DTT was removed by filtration through a Sephadex G-25 solumn. The reduced enzyme migrates as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and, in 0.05 M potassium phosphate, 0.4 M Li_2SO_4, pH 6.0, the enzyme has a $K_{i\,sorbinil}$ of 1.5 μM, $K_{m\,NADPH}$ of 33.7 μM, and $K_{m\,DL\text{-}glyceraldehyde}$ of 103 μM [10].

To avoid changes in the composition of the buffer and ionic strength of the reaction mixture upon varying the pH, a three-buffer system was chosen which consisted of 2-[*N*-morpholino]ethanesulfonic acid (MES), Tris and HEPES. The pK values of these buffers at the ionic strength used (0.125 M) and the amount of the salt needed to keep the ionic strength constant throughout the experimental pH range were computed as described by Ellis and Morrison [11]. To evaluate the effects of the various components of the buffer system the enzyme was incubated with the three-buffer system at varying pH (5–11) for 10 min and the enzyme activity was determined at pH 6.0. No irreversible change in the enzyme activity was observed upon incubation in the buffer system in this pH range. Even so, between determinations, the enzyme was suspended in 0.1 M Tris–HCl, pH 8.0, containing 1 mM EDTA and added last to the reaction mixture (pH jump technique). No significant change in the pH was observed at the beginning or after 5 min of the reaction. There was no evidence of either 3-APADP$^+$ or benzyl alcohol reacting with any component of the buffer system. For all kinetic studies no thiol reagents were added in the assay mixture.

Data analysis. Inhibition patterns were fitted to equations for linear competitive, non-competitive or uncompetitive inhibition profiles [5]. Data for pH profiles showing an increase in $\log V_{max}/K_m(V/K)(Y)$ with a slope of 1 as the pH increased were fitted to Equation (1) and data in which the values of V, V/K and K_i (Y') decreased at both high and low pH were fitted to Equation (2) [12].

$$\log Y = \log[c/(1 + K_a/H^+)] \quad (1)$$

$$\log Y' = \log[c/(1 + H^+/K_a + K_b/H^+)] \quad (2)$$

where c is the pH-independent value of the parameter and K_a and K_b are the acid and base ionization constants. The 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 the residual sum of squares divided by the degrees of freedom. Sorbinil was a gift from the Pfizer Chemical Co. The sources of the other chemicals have been listed before [5].

Results and Discussion

In the direction of aldehyde reduction catalyzed by aldose reductase, sorbinil displays non-competitive inhibition profiles versus either NADPH or glyceraldehyde [4]. However, in the direction of alcohol oxidation, sorbinil displayed linear competitive inhibition with benzyl alcohol ($K_m = 1.67 \pm 0.11$ mM) as the varied substrate, and 3-APADP$^+$ as the coenzyme ($K_m = 0.01 \pm 0.001$ mM), with a K_{is} of 0.15 ± 0.01 μM (Fig. 1). 3-APADP$^+$ was used as the coenzyme in the direction of alcohol oxidation as in this direction NADP$^+$ is a very poor substrate which precludes detailed kinetic studies. However, NADPH is a competitive inhibitor versus 3-APADP$^+$ in the reaction direction ($K_{is} = 0.42 \pm 0.005$ μM), and E:NADP$^+$–glycol-

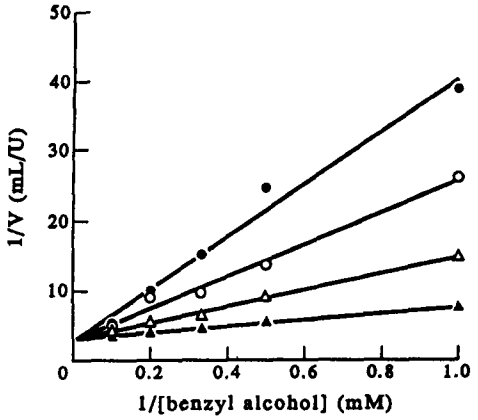
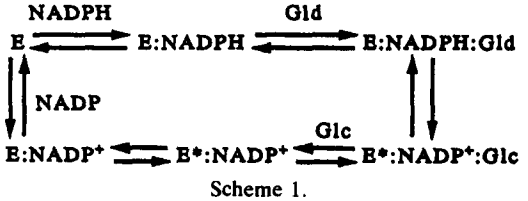


Fig. 1. Double-reciprocal plot of the rate of substrate consumption vs substrate concentration at different concentrations of inhibitor. The enzyme activity was assayed in 0.125 M MES–Tris–HEPES, pH 8.5, with the indicated concentrations of benzyl alcohol and 100 μM 3-APADP$^+$. The concentrations of sorbinil used were: 0 (▲), 0.2 (△), 0.5 (○) and 1 (●) μM.

aldehyde and E : APADP$^+$–glycolaldehyde adduct complex have similar fluorescence spectra [13], suggesting that both these nucleotides bind to the same site on the enzyme. A replot of the inhibition profile was also consistent with competitive inhibition (data not shown). Analyzed on the basis of the reaction scheme of the enzyme (Scheme 1), the data suggest that sorbinil binds selectively to enzyme–nucleotide complexes, at the substrate binding site.

On kinetic grounds alone, lack of competitive inhibition in one direction of the reaction does not exclude the possibility that the inhibitor binds to the substrate binding site. Alcoholic products of the enzyme do not show competitive inhibition profiles versus aldehyde [14], due to the conformation change in the enzyme–substrate ternary complex, but nevertheless bind to the substrate binding site of the enzyme. Thus, a competitive inhibition pattern of sorbinil with alcohol and a non-competitive pattern with aldehyde as the varied substrate indicate kinetically significant isomerization of the enzyme before alcohol release and after aldehyde binding and may be a reflection of selective binding of sorbinil to enzyme-oxidized nucleotide (3-APADP$^+$ or NADP$^+$) binary complex.

Based on the competitive inhibition pattern observed between benzyl alcohol and the aldose reductase inhibitor AL-1567 [15], high affinity binding of the inhibitor to the free enzyme has been suggested. However, inhibitor binding to the free enzyme is unlikely since product release steps of aldose reductase are ordered, with NADP$^+$ being released last [14, 16]. Since benzyl alcohol does not bind to the free enzyme, $K_{is\,AL\text{-}1567}$ may represent the dissociation constant of the inhibitor from E : APADP$^+$ and not the free enzyme. Moreover, unlike sorbinil, a $K_{is\,AL\text{-}1567}$ of 5.7×10^{-9} M [15] is close to the concentration of the enzyme used, which makes interpretation of the data difficult.



Scheme 1.

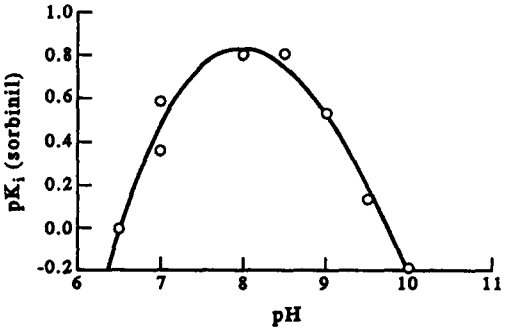


Fig. 2. Effect of pH on $K_{is\,sorbinil}$ of aldose reductase. $K_{is\,sorbinil}$ was determined at 100 μM 3-APADP$^+$, by varying the concentration of benzyl alcohol (1 to 10 mM) in 0.125 M MES–Tris–HEPES at the indicated pH values. The inhibition pattern of sorbinil was competitive at all the pH values used. Data are shown as open circles and the curve is the best fit of Equation (2) to the data. The pH-independent value of the parameter and the estimated pK values are given in the text.

Figure 2 shows that $K_{is\,sorbinil}$ with benzyl alcohol as the varied substrate displays a bell-shaped dependence on the pH. Two pK_i values, estimated on fitting Equation (2) to the data were: 7.5 ± 0.04 and 8.75 ± 0.65 and the pH-independent value of $K_{is\,sorbinil}$ was 8.53 ± 0.52 μM. If sorbinil is a true competitive inhibitor of the enzyme versus the alcohol, p$K_{i\,sorbinil}$ should be similar to the pK for alcohol binding. The pH dependence of V/K benzyl alcohol displayed only one pK value of 7.70 ± 0.10 (Fig. 3). The pH-independent value of V/K benzyl alcohol was 0.25 ± 0.016 sec^{-1}. These data suggest that binding of both benzyl alcohol and sorbinil decreases at low pH with similar pK values. The decrease in $K_{is\,sorbinil}$ at high pH with a pK_i of 8.7 may be due either to sorbinil binding to another group, the deprotonation of which prevents sorbinil (but not alcohol) binding, or to deprotonation of sorbinil itself, which would prevent binding.

Competitive inhibition profiles of sorbinil with benzyl alcohol as the varied substrate and similar pK values

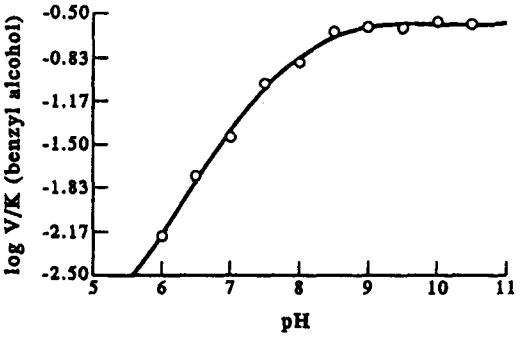


Fig. 3. Effect of pH on V/K benzyl alcohol of aldose reductase. The V/K values of the benzyl alcohol were determined from the complete initial velocity profile of the enzyme generated by varying 3-APADP$^+$ from 0.01 to 0.1 mM and benzyl alcohol from 1 to 10 mM in 0.125 M MES–Tris–HEPES at the indicated pH values. Data are shown as open circles and the curve is the best fit of Equation (1) to the data. The pH-independent value of the parameter and the estimated pK values are given in the text.

obtained for $K_{is\,sorbinil}$ and V/K benzyl alcohol suggest that the two compounds probably bind to the same site on the enzyme. Further experiments will be necessary to establish the identity of the residue with a pK of 7.5 to 7.7 involved in sorbinil and alcohol binding, which is consistent with the residue being either cysteine or histidine. Oxidation of the enzyme or carboxymethylation of Cys-298 increase in $K_{m\,aldehyde}$ and $K_{i\,sorbinil}$ simultaneously [17, 18]. It is also likely that this residue could be a histidine. In aldehyde reductase-catalyzed reaction the V/K alcohol shows a pK of 8.0 which has been proposed to be due to the ionization of a catalytic histidine residue [19]. In view of 60% structural homology between aldose reductase and aldehyde reductase [20], it is likely that the two enzymes share a similar catalytic mechanism.

The suggestion that sorbinil binds selectively to the enzyme–nucleotide binary complex at the substrate binding site has important practical consequences in the design and synthesis of aldose reductase inhibitors. We have shown that most aldose reductase inhibitor, with the exception of tolrestat, bind to the same site on the enzyme [4]. A structural model based on X-ray analysis of the enzyme crystals suggests that the active site of the enzyme is located near the C-terminal region of the protein [21], which may also be the inhibitor binding site. Primary isotope effects on the aldose reductase-catalyzed reaction [17] and transient kinetic studies [14] suggest that isomerization of the enzyme–nucleotide complex may be the rate-limiting step in the catalytic cycle. Thus, under steady-state conditions, most of the enzyme will be present as enzyme–nucleotide binary complex and compounds that selectively bind to the enzyme–nucleotide complex rather than the free enzyme will be more effective inhibitors of the enzyme. Our observation that sorbinil (and presumably most such aldose reductase inhibitors) bound selectively to E:NADP$^+$ complex suggests that the currently available inhibitors are indeed targeted to the most abundant form of the enzyme. However, since binding of these inhibitors to enzyme–nucleotide is sensitive to the oxidation state of the enzyme [17, 18], which may change with hyperglycemia-induced changes in the cell, more selective inhibitors will be ones that bind to enzyme–nucleotide complex and are substrate analogs insensitive to the oxidation state of the enzyme.

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