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Reaction and Inhibition Mechanisms of Aldose Reductase from Rabbit Lens

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The reaction and inhibition kinetics of rabbit lens aldose reductase were investigated. The kinetic reaction mechanism of the enzyme is compatible with an ordered Bi Bi reaction mechanism in which reduced nicotinamide adenine dinucleotide phosphate (NADPH) binds first to the enzyme, before glyceraldehyde, and nicotinamide adenine dinucleotide phosphate (NADP⁺) is released last. The Michaelis constants for NADPH and glyceraldehyde were 1.7×10^{-5} and 8.9×10^{-5} M, respectively, and the dissociation constant of NADPH was 7.5×10^{-6} M. The inhibition constant for NADP⁺ as a product was 1.5×10^{-5} M. Benzyl 4-isopropyl-5-phenyl-2-oxazolocarbamate (4-isopropyl-BPOC), a potent aldose reductase inhibitor, exhibited a linear un-competitive inhibition with respect to NADPH, and a linear non-competitive inhibition with respect to glyceraldehyde. It was suggested that 4-isopropyl-BPOC binds to the enzyme-NADPH binary complex but not to the free enzyme. The inhibition constants of the inhibitor for NADPH and glyceraldehyde were 4.3×10^{-7} and 3.6×10^{-7} M, respectively. The inhibitory effect of 4-isopropyl-BPOC on aldose reductase was reduced when the enzyme was treated with pyridoxal 5-phosphate. From this fact, it may be speculated that 4-isopropyl-BPOC interacts with a lysine residue in the enzyme molecule.

Keywords—aldose reductase; rabbit lens; kinetics; kinetic mechanism; initial velocity study; product inhibition study; aldose reductase inhibitor

Aldose reductase (EC 1.1.1.21) is an enzyme which catalyzes the reduction of aldo-sugars to the corresponding sugar-alcohols, and possesses broad substrate specificity for aldoses and aldehydes. Aldose reductase has been found widely in a variety of mammalian and microbial sources, and the enzyme catalyzes the first step of the sorbitol pathway. Aldose reductase in mammalian tissues such as lens, peripheral nerves, renal papillae and retina plays a central role in the pathogenesis of diabetic complications.¹⁾ Therefore, the development of aldose reductase inhibitors has been attempted in order to prevent or treat chronic complications caused by diabetes.²⁾ Some aldose reductase inhibitors, *e.g.*, sorbinil, tolrestat and ONO-2235, are now undergoing clinical trials.

It has been reported that some benzyl oxazolocarbamate derivatives strongly inhibit aldose reductase,³⁾ and these compounds are candidate drugs for the prevention and treatment of diabetic complications.⁴⁾ However, the mode of action of the derivatives has been little investigated. In this paper, we describe the reaction mechanism of an aldose reductase from rabbit lens and the inhibition mechanism of an inhibitor of the enzyme, benzyl 4-isopropyl-5-phenyl-2-oxazolocarbamate.

Experimental

Materials—Aldose reductase was prepared from rabbit lens by the method described in the previous paper,⁵⁾ and aldose reductase 1b was used in this study. The enzyme was purified about 1100-fold, and was homogeneous on

polyacrylamide gel electrophoresis. Nicotinamide adenine dinucleotide phosphate (NADP⁺) and its reduced form (NADPH) were purchased from Oriental Yeast Co. Diethyl pyrocarbonate, phenylglyoxal and pyridoxal 5-phosphate were obtained from Nakarai Chemicals Ltd., Aldrich Chemical Co. and Sigma Chemical Co., respectively. Benzyl 4-isopropyl-5-phenyl-2-oxazolecarbamate (4-isopropyl-BPOC) was prepared by the method reported previously.⁶⁾

Standard Assay Method for Aldose Reductase—The enzyme activity was determined at 25 °C by measuring the decrease in absorption of NADPH at 340 nm on a Hitachi 557 dual-wavelength double-beam spectrophotometer equipped with a temperature-controlled cuvette chamber. The assay mixture consists of 100 mM sodium phosphate buffer (pH 6.2) containing 0.3 M ammonium sulfate, 10 mM DL-glyceraldehyde, 0.15 mM NADPH and an appropriate amount of enzyme in a total volume of 3.0 ml. The inhibition study was carried out by adding 5 μl of 4-isopropyl-BPOC dissolved in dimethyl sulfoxide to the reaction mixture. The appropriate blanks to correct for nonspecific oxidation of NADPH were prepared. The reaction was initiated by the addition of enzyme. One unit of the enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH per min under the conditions described here.

Kinetic Determinations and Data Processing—The kinetic measurements of initial velocity and product inhibition were carried out in duplicate by the standard assay method except that the concentration of the variable substrate was changed. As regards data processing, the nomenclature used herein is that of Cleland.⁷⁾ The reciprocal of velocity was plotted graphically against the reciprocal of substrate concentration. The slopes and intercepts of the double-reciprocal plots were plotted graphically against the reciprocal of the non-varied substrate concentration for initial velocity experiments or that of the inhibitor concentration for inhibition experiments, to determine the linearity of these replots. Data conforming to a sequential initial velocity pattern, a linear competitive inhibition pattern, a linear un-competitive inhibition pattern, and a linear non-competitive inhibition pattern were fitted to Eqs. 1–4, respectively.

$$v = \frac{VAB}{K_{ia}K_m^b + K_m^aB + K_m^bA + AB} \quad (1)$$

$$v = \frac{VS}{K_m(1 + I/K_{is}) + S} \quad (2)$$

$$v = \frac{VS}{K_m + S(1 + I/K_{ii})} \quad (3)$$

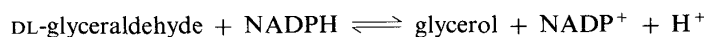
$$v = \frac{VS}{K_m(1 + I/K_{is}) + S(1 + I/K_{ii})} \quad (4)$$

In all equations, v is the experimentally observed rate, and V is the maximum velocity. In Eq. 1, K_m^a and K_m^b are Michaelis constants for substrates A and B, respectively, and K_{ia} is the dissociation constant for substrate A. In Eqs. 2–4, S is the substrate concentration, K_m is the Michaelis constant for the substrate, K_{is} and K_{ii} are apparent inhibition constants for slope and intercept, and I is the inhibitor concentration.

Results and Discussion

Initial Velocity and Product Inhibition Studies

In the present paper, the reaction mechanism of aldose reductase was investigated on the basis of the following reaction.



Double-reciprocal plots of initial velocity for the forward reaction are shown in Fig. 1. When NADPH was the variable substrate, with different concentrations of DL-glyceraldehyde as the changing fixed substrate (32–158 μM), the plots gave a family of straight lines intersecting in the third quadrant (Fig. 1 A). With DL-glyceraldehyde as the variable substrate and NADPH kept constant at a concentration ranging from 5.2 to 75 μM, double reciprocal plots of initial velocity *versus* DL-glyceraldehyde concentration again yielded straight lines converging in the third quadrant (Fig. 1 B). These results indicate that no irreversible step (such as release of a product not present initially) occurs before attachment of the second substrate to the enzyme and that the mechanism of the enzyme reaction is a sequential mechanism, *i.e.*, the substrates must both bind to the enzyme before release of the products. In

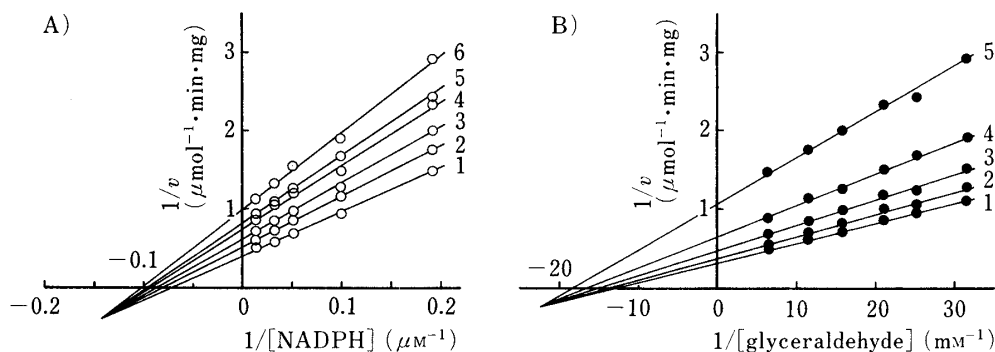


Fig. 1. Double-Reciprocal Plots of Initial Velocity

A) The concentration of NADPH as the variable substrate was varied between 5.2 and 75 μM . The concentrations of DL-glyceraldehyde as the fixed substrate were: 1, 158 μM ; 2, 87 μM ; 3, 63 μM ; 4, 47 μM ; 5, 40 μM and 6, 32 μM . B) The concentration of DL-glyceraldehyde as the variable substrate was varied between 32 and 158 μM . The concentrations of NADPH as the fixed substrate were: 1, 75 μM ; 2, 39 μM ; 3, 20 μM ; 4, 10 μM and 5, 5.2 μM .

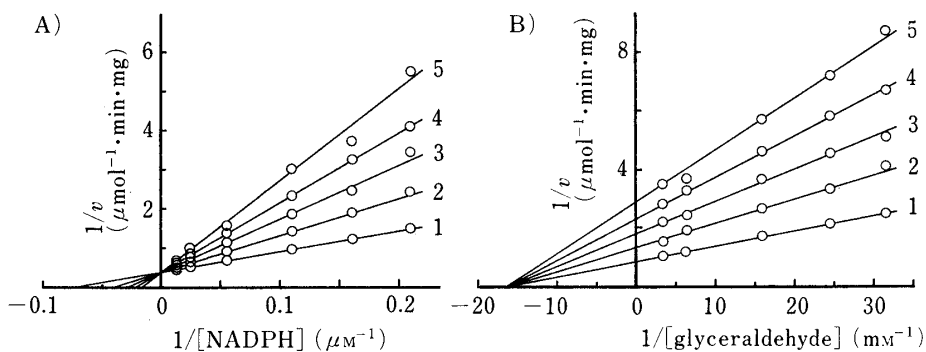


Fig. 2. Product Inhibition of Aldose Reductase by NADP⁺

A) The concentration of NADPH as the variable substrate was varied between 4.7 and 68 μM , and that of DL-glyceraldehyde as the fixed substrate was 158 μM . B) The concentration of DL-glyceraldehyde as the variable substrate was varied between 32 and 316 μM , and that of NADPH as the fixed substrate was 6.1 μM . The concentrations of NADP⁺ were: 1, 0 μM ; 2, 13 μM ; 3, 27 μM ; 4, 40 μM and 5, 54 μM .

addition, the double-reciprocal plots shown in Fig. 1 strongly suggested that the enzyme followed the ordered Bi Bi mechanism rather than the random bi-reactant mechanism. However, if the binding of one ligand decreases the affinity for the second ligand, the family of reciprocal plots would also intersect below the abscissa in a rapid equilibrium random Bi Bi mechanism. Therefore, from these initial velocity studies alone, it is not possible to distinguish between an ordered mechanism in which one substrate of a bi-substrate reaction binds initially to the enzyme and a rapid random equilibrium mechanism in which two substrates may bind to the enzyme in any order.

Thus, product inhibition studies were undertaken to clarify the mechanism. One of the products, NADP⁺, was found to be an inhibitor of the forward reaction. The product inhibition pattern of NADP⁺ is of the following type: competitive when NADPH is varied (Fig. 2.A), non-competitive when glyceraldehyde is varied (Fig. 2.B) and no inhibition when glyceraldehyde is varied with a saturated amount of NADPH. Such an inhibition pattern indicates an ordered or rapid equilibrium random mechanism, but it still does not clearly distinguish between the two mechanisms because another product, glycerol exhibited no inhibition in any case. According to Cleland,⁸⁾ these two mechanisms may be distinguished by determining the K_i slope of a competitive inhibitor at various fixed levels of one substrate in a bi-substrate reaction; a constant K_i slope indicates an ordered mechanism, and a changing K_i

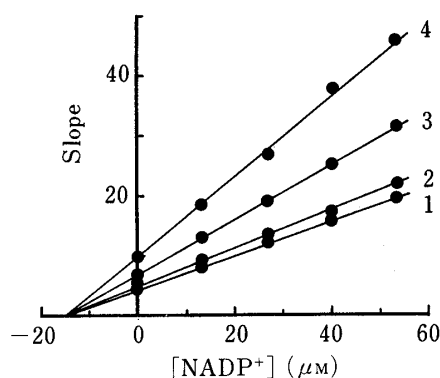
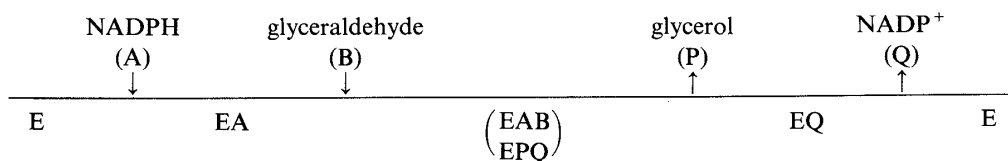


Fig. 3. Determination of K_i Slope for NADP^+

Plots of $1/v$ versus $1/[\text{NADPH}]$ as shown in Fig. 2A were made at four different concentrations of DL-glyceraldehyde with NADP^+ concentrations ranging from 0 to $54 \mu\text{M}$. The slopes of the resulting lines were plotted versus NADP^+ concentration, and the value of K_i slope was determined by linear regression for each concentration of DL-glyceraldehyde. The concentrations of DL-glyceraldehyde were: 1, $316 \mu\text{M}$; 2, $158 \mu\text{M}$; 3, $64 \mu\text{M}$ and 4, $32 \mu\text{M}$.

slope indicates a random mechanism. This kinetic diagnostic method has been used to determine the character of the mechanism for aldose reductase from rat lens⁹⁾ and *Rhodotorula*.¹⁰⁾ To determine this kinetic parameter, initial velocities were studied at 32, 64, 158 and $316 \mu\text{M}$ glyceraldehyde at various NADP^+ levels with NADPH as the variable substrate. Double reciprocal plots of initial velocity versus NADPH concentration were formed at each NADP^+ level, and then the slopes obtained from these double-reciprocal plots were replotted versus NADP^+ concentration. Similar replots were done at each concentration of glyceraldehyde, and the values of K_i slope were determined from the intersecting point of the regression line on the abscissa. The results of this analysis showed the K_i slope to be constant under the conditions investigated (Fig. 3) and indicated that addition of the substrate is ordered.

From the results of the initial velocity and product inhibition studies described herein, it was indicated that the rabbit lens aldose reductase follows an ordered Bi Bi reaction mechanism as depicted below in which NADPH is bound first, before glyceraldehyde, and glycerol is released, followed by NADP^+ . The reaction mechanism proposed here for aldose reductase from rabbit lens is different from that for rat lens aldose reductase reported by Doughty and Conrad,⁹⁾ who found that the forward reaction follows a random mechanism, and is identical with that for the enzyme from *Rhodotorula*.¹⁰⁾



When secondary plots of the slopes and intercepts of data obtained from Fig. 1 were made according to Cleland's method, these plots were linear and gave Michaelis constants for NADPH and glyceraldehyde of 1.7×10^{-5} and $8.9 \times 10^{-5} \text{M}$, respectively. The Michaelis constants of rabbit lens aldose reductase for NADPH and glyceraldehyde were the same as those (in the high substrate concentration range) of rat, human and bovine lens enzymes.¹¹⁾ Cooperativity for both NADPH and glyceraldehyde was demonstrated for aldose reductase obtained from rat, human and bovine lenses,^{11,12)} and the Michaelis constants of all three enzymes increased above a certain level of substrate concentration. However, cooperativity was not observed with the enzyme from rabbit lens. The distinction between these enzymes as regards cooperative behavior may be explained on the basis of conformational change, such as tertiary structural change, owing to binding of the ligand.

The dissociation constant for NADPH was $7.5 \times 10^{-6} \text{M}$. The fact that the intersecting point in double-reciprocal plots of initial velocity versus NADPH concentration is below the horizontal axis also supports the idea that the dissociation constant is lower than the Michaelis constant for NADPH .

Kinetics of Inhibition by 4-Isopropyl-BPOC

When the DL-glyceraldehyde concentration was maintained constant at 10 mM and the concentration of NADPH was varied from 4.5 to 72 μM at different concentrations of 4-isopropyl-BPOC, double-reciprocal plots of initial velocity against NADPH concentration showed that 4-isopropyl-BPOC is an un-competitive inhibitor of NADPH (Fig. 4 A). A replot of the intercepts of these graphs *versus* 4-isopropyl-BPOC concentration was linear (Fig. 4 B). The data obtained from this experiment were fitted by Eq. 3, and the value of K_{ii} was found to be $4.3 \times 10^{-7}\text{M}$. When the NADPH concentration was maintained constant at 120 μM and the DL-glyceraldehyde concentration was varied from 47.5 to 316 μM , double-reciprocal plots of initial velocity against glyceraldehyde concentration at different fixed 4-isopropyl-BPOC concentrations showed that 4-isopropyl-BPOC is a non-competitive inhibitor of glyceraldehyde (Fig. 5 A). A replot of the intercepts of these graphs *versus* 4-isopropyl-BPOC concentration was linear (Fig. 5 B). The data obtained from this experiment were fitted by Eq. 4. The values of K_{ii} and K_{is} were the same, because the double-reciprocal plots converged on the abscissa, and the inhibition constant was $3.6 \times 10^{-7}\text{M}$. These results indicate that the inhibitor combines with an enzyme-NADPH binary complex and an enzyme-NADPH-glyceraldehyde ternary complex, but not with the free enzyme.

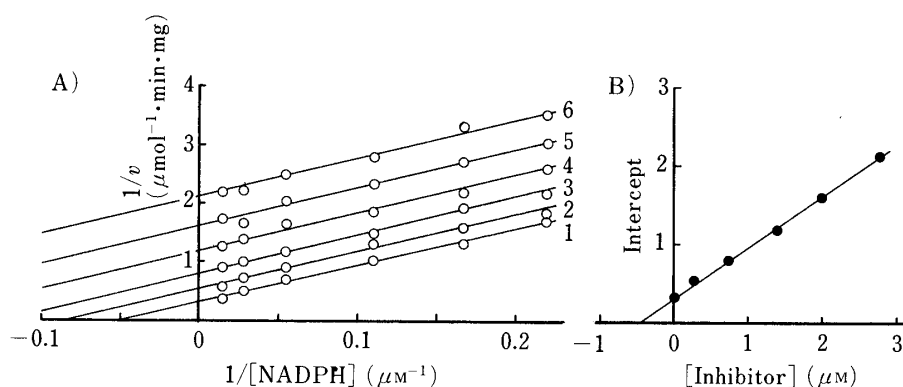


Fig. 4. Inhibition of Aldose Reductase by 4-Isopropyl-BPOC with NADPH as the Variable Substrate

A) The concentration of NADPH as the variable substrate was varied between 4.5 and 72 μM , and DL-glyceraldehyde was held constant at 10 mM. The concentrations of 4-isopropyl-BPOC were: 1, 0 μM ; 2, 0.28 μM ; 3, 0.79 μM ; 4, 1.4 μM ; 5, 2.0 μM and 6, 2.8 μM . B) Intercepts from A were replotted *versus* the concentration of 4-isopropyl-BPOC.

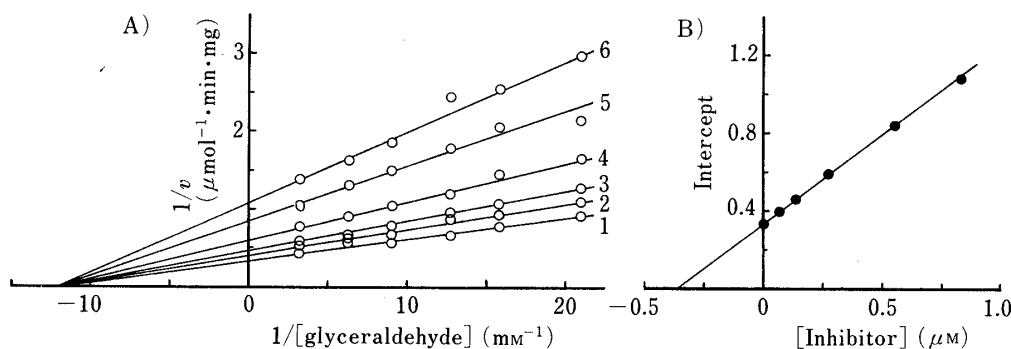


Fig. 5. Inhibition of Aldose Reductase by 4-Isopropyl-BPOC with DL-Glyceraldehyde as the Variable Substrate

A) The concentration of DL-glyceraldehyde as the variable substrate was varied between 47.5 and 316 μM , and NADPH was held constant at 120 μM . The concentrations of 4-isopropyl-BPOC were: 1, 0 μM ; 2, 0.07 μM ; 3, 0.14 μM ; 4, 0.28 μM ; 5, 0.56 μM and 6, 0.83 μM . B) Intercepts from A were replotted *versus* the concentration of 4-isopropyl-BPOC.

Inhibitory Effect of 4-Isopropyl-BPOC on Aldose Reductase Treated with Chemical Modifier

In order to examine the interaction between the enzyme molecule and 4-isopropyl-BPOC, inhibition of the enzyme treated with diethyl pyrocarbonate, phenylglyoxal or pyridoxal 5-phosphate by 4-isopropyl-BPOC was investigated. As the inactivation of the enzyme with these modifiers reached a plateau after 20 to 30 min, the enzyme treated for 30 min was used in the inhibition studies. The results are shown in Table I. Aldose reductase treated with diethyl pyrocarbonate, a modifier of histidine residues, was inhibited to 21% activity by 2×10^{-6} M 4-isopropyl-BPOC, and the extent of this inhibition was the same as that of non-treated enzyme. The enzyme treated with phenylglyoxal, a modifier of arginine residues, was also inhibited similarly to non-treated enzyme to about 25% activity by 2×10^{-6} M inhibitor. Aldose reductase incubated with 0, 3 and 6 mM pyridoxal 5-phosphate exhibited activities of 2.94, 2.14 and 1.93 units/mg, respectively, and, at 2×10^{-6} M 4-isopropyl-BPOC they were inhibited to 0.75, 1.06 and 1.38 units/mg (these activities correspond to 25, 50 and 72% of the non-inhibited enzyme activities), respectively. At 4×10^{-7} M 4-isopropyl-BPOC, although non-pyridoxal 5-phosphate-treated aldose reductase activity was decreased to about 55% compared to the control and was 1.63 units/mg, the activity of the enzyme treated with 6 mM pyridoxal 5-phosphate was 1.73 units/mg, corresponding to 90% of the control non-inhibited enzyme activity. Thus, the inhibitory effect of 4-isopropyl-BPOC was reduced in the enzyme treated with pyridoxal 5-phosphate. As pyridoxal 5-phosphate is a modifier of lysine residues, it could be speculated from this result that a lysine residue, but not a histidine or arginine residue, may be involved in the region of the aldose reductase molecule where 4-isopropyl-BPOC interacts as an aldose reductase inhibitor.

TABLE I. Inhibition of Aldose Reductase Treated with Diethyl Pyrocarbonate, Phenylglyoxal and Pyridoxal 5-Phosphate by 4-Isopropyl-BPOC

Modifier	Conc. (mM)	Enzyme activities (units/mg)		
		4-Isopropyl-BPOC (μ M)		
		0	0.4	2.0
Diethyl pyrocarbonate	0	2.95 (100)	1.68 (56.9)	0.70 (23.7)
	10	2.10 (100)	1.25 (59.5)	0.44 (21.0)
Phenylglyoxal	0	2.98 (100)	1.71 (57.4)	0.72 (24.2)
	1.4	2.72 (100)	1.38 (50.9)	0.65 (24.0)
Pyridoxal 5-phosphate	0	2.94 (100)	1.63 (55.4)	0.75 (25.5)
	3	2.14 (100)	1.58 (73.8)	1.06 (49.5)
	6	1.93 (100)	1.73 (89.6)	1.38 (71.5)

The enzyme (2×10^{-6} M) was incubated at the indicated concentrations of diethyl pyrocarbonate, phenylglyoxal and pyridoxal 5-phosphate for 30 min at 25°C, the enzyme activities were assayed in the presence of the indicated concentration of 4-isopropyl-BPOC by the method described in Experimental. The enzyme activities were expressed as units per mg of enzyme. The numbers in parentheses are percentages with respect to the activities with no inhibitor at each concentration of chemical modifier.

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