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Change in Stereospecificity of Bovine Lens Aldose Reductase Modified by Oxidative Stress*

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Bovine lens aldose reductase (alditol:NADP$^+$ oxidoreductase, EC 1.1.1.21) undergoes an oxidative modification, greatly stimulated by high ionic strength, upon incubation in the presence of oxygen radical generating systems (Del Corso, A., Camici, M., and Mura, U. (1987) *Biochem. Biophys. Res. Commun.* 148, 369–375). The enzyme modification is accompanied by a change in stereospecificity toward the two enantiomers of glyceraldehyde. In particular, the K_m for L-glyceraldehyde of the native form increased over 150 times after the enzyme modification, with a decrease in the catalytic efficiency of over 200 times. By contrast, for the D-enantiomer the K_m increased only 7 times with respect to the native form, with a concomitant decrease in the catalytic efficiency of only approximately 3 times. This dramatic change in stereospecificity may account for the reported apparent cooperative behavior exhibited also by highly purified electrophoretically homogeneous preparations of aldose reductase.

The intracellular accumulation of sorbitol in hyperglycemia conditions has been proposed as the initial event in the etiology of several ocular lesions, such as diabetic cataract and corneal and retinal diseases, as well as peripheral neuropathies occurring in diabetic subjects (1–4). The formation of this metabolite is catalyzed, through a NADPH-dependent reduction of glucose, by aldose reductase (alditol:NADP$^+$ oxidoreductase, EC 1.1.1.21). This enzyme has a broad catalytic efficiency toward several aldo sugars and a variety of aromatic and aliphatic aldehydes. Because of its involvement in cellular damage, many inhibitors of aldose reductase have been studied and proposed as therapeutic agents for the prevention of diabetic complications. Lens aldose reductase has been purified to homogeneity by several authors (5–9). Despite the large abundance of reports on the characterization of the enzyme, its susceptibility to inhibition by special chemicals, which appears to be dependent on the different degree of purification (10, 11), and its apparent cooperative behavior with respect to glyceraldehyde (6, 7, 12–16), are still a matter of debate. Recently, we have shown that systems known to generate oxygen radical species lead to a modification of bovine lens aldose reductase and that the native and modified enzyme forms exhibit a markedly different sensitivity to inhibition by Sorbinil, a classical aldose reductase inhibitor (17, 18). To explain the apparent cooperative behavior of aldose reductase, models of cooperativity for monomeric proteins (6) or the autoxidation of the substrate (7, 13) have been proposed. Without invoking the above reported explanations, in this paper we show that the modification of bovine lens aldose reductase brings about a dramatic change in stereospecificity of the enzyme toward the glyceraldehyde enantiomers and that this property alone may well account for the reported singular kinetic behavior of aldose reductase.

EXPERIMENTAL PROCEDURES

Materials—NADPH was obtained from Sigma; DL-, D-, and L-glyceraldehyde were purchased from Fluka. Ammonium sulfate and all inorganic chemicals were of reagent grade from BDH. Calf eyes were obtained from freshly slaughtered animals at ACM Slaughterhouse, Reggio Emilia, and the lenses were removed and kept frozen until needed.

Preparation of the Native (ARb)[1] *and Modified (ARa) Forms of Aldose Reductase*—After incision of the capsula, frozen lenses were suspended in 50 mM sodium phosphate, pH 6.8, supplemented with 5 mM 2-mercaptoethanol and 2 mM dithiothreitol (100 g of tissue/350 ml), and stirred in an ice-cold bath for 1 h. The suspension was then centrifuged at 40,000 × *g* at 4 °C for 40 min, and the supernatant was processed as previously described (18) with the only exception that the Orange Matrex purification step preceded the Sephadex G-75 chromatography. The modified form of aldose reductase (ARa) was prepared incubating the purified "*b*-form" (0.03 mg/ml) in 85 mM sodium phosphate, pH 6.8, containing 5 mM 2-mercaptoethanol, 0.42 M ammonium sulfate, 0.1 mM $FeSO_4$, and 0.3 mM EDTA (17, 18). The modification process was monitored by following the activation of aldose reductase at different times of incubation, using DL-glyceraldehyde as substrate. Fully activated enzyme was usually obtained after 2 h of incubation. ARa was dialyzed by Amicon YM5 membranes against 10 mM sodium phosphate, pH 7. Both enzyme preparations, which appeared homogeneous on sodium dodecyl sulfate gel electrophoresis, were stored at 4 °C under sterile conditions. As previously described, ARa and ARb can be separated by anion-exchange chromatography by a fast protein liquid chromatography system on a Mono Q HR5/5 column, with retention times of 10.5 and 12 min, respectively (18). Therefore, before the kinetic analysis, in order to obtain the native form of aldose reductase free of any trace of the modified form and vice versa, the enzyme preparations were chromatographed on the Mono Q column and then dialyzed and concentrated by Amicon YM5 membranes to approximately 1 mg of protein/ml. The specific activities were 2.2 and 0.9 units/mg for ARa and ARb, respectively.

Enzyme Activity Measurement—The assay for the enzyme activity was performed with the proper substrates as previously described (17, 18) by following the decrease in absorbance at 340 nm, which parallels the NADPH oxidation. The standard assay was performed with 4.7 mM DL-glyceraldehyde. One unit of enzyme activity is the amount of enzyme which catalyzes the oxidation of 1 μmol of NADPH/min.

Other Methods—Protein was determined by the Coomassie Blue binding assay (19), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The purified *a* and *b*-forms of bovine lens aldose reductase exhibited, with respect to both D- and L-glyceraldehyde, a hyperbolic behavior, as shown by the double-reciprocal plots

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[1] The abbreviations used are: ARb, native form of aldose reductase; ARa, modified form of aldose reductase.

of Figs. 1 and 2. This result seems to be inconsistent with previously reported kinetic studies on bovine lens aldose reductase (6, 7, 12, 13, 15, 16). In fact, besides nonhomogeneous preparations (15, 16), also highly purified bovine lens aldose reductase, purposely prepared to define the kinetic behavior of the enzyme, exhibited concave downward curvatures in double-reciprocal plots with respect to glyceraldehyde concentrations (6, 7, 12, 13). This was ascribed for this monomeric protein either to an allosteric-type behavior (6) or to an effect of the autoxidation products of glyceraldehyde on the enzyme activity measurement (7, 13). However, the analysis of the kinetic parameters of both forms of bovine lens aldose reductase (Table I) indicates a feasible explanation for the apparent cooperativity of the enzyme reported so far. In fact, the enzyme modification is accompanied by a dramatic change in stereospecificity toward the two enantiomers of the substrate so that L-glyceraldehyde, for which a K_m of 5 μM with respect to the native form of aldose reductase was evaluated, became a very poor substrate for ARa with a K_m over 150 times higher and a decrease in the catalytic efficiency of over 200 times. By contrast, for the D-enantiomer an increase of 7 times in K_m with respect to the native form was evaluated, with a concomitant decrease in the catalytic efficiency of only approximately 3 times. It must be noted, in fact, that the modified form of aldose reductase exhibits a k_{cat} for D-glyceraldehyde 2.5 times higher than the native form. This property may explain the activation of the enzyme, previously described by us (17), which was the first indication of aldose reductase modification. It is evident that the simultaneous presence of ARa and ARb gives rise to nonlinear kinetics in double-reciprocal plots and that the major contribution to this kinetic behavior stems from the L-enantiomer for which the two enzyme forms exhibit the greatest difference in K_m. This is confirmed by the concave downward curvature of the double-reciprocal plot obtained when L-glyceraldehyde was used as the substrate of a preparation of ARa containing approximately only 10% of ARb (Fig. 3, *inset*), as judged by measuring the activity with DL-glyceraldehyde, the most commonly used substrate for aldose reductase. A satisfactory fitting of the experimental data with a computer-simulated curve was observed, when appropriate parameters were inserted in an equation applying to a system of two enzymes acting on the same substrate (20) (Fig. 3). We have already shown that the aldose reductase modification is greatly stimulated by high ionic strength and that the native and modified forms of the enzyme exhibit the same molecular weight on sodium dodecyl sulfate gel electrophoresis (17, 18). Therefore, since most procedures for the purification of bovine lens aldose reductase involved an ammonium sulfate fractionation step and no precautions were taken to protect the enzyme against oxidative stress (6, 7, 12), it is highly possible that even the purified electrophoretically homogeneous preparations used in previous kinetic studies on aldose reductase were in fact a mixture of the two enzyme forms. In light of our results, the dramatic change in stereospecificity with respect to L-glyceraldehyde, which accompanies the process of modi-

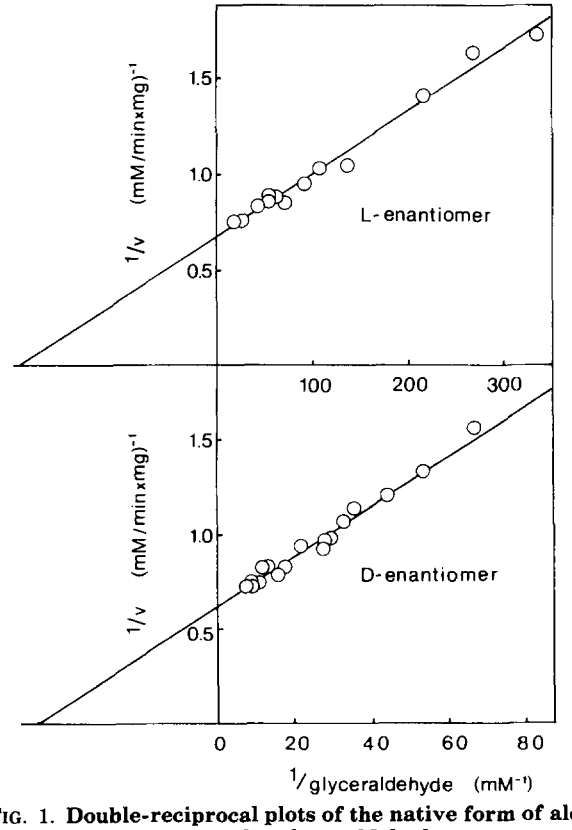

FIG. 1. **Double-reciprocal plots of the native form of aldose reductase (ARb) for D- and L-glyceraldehyde.**

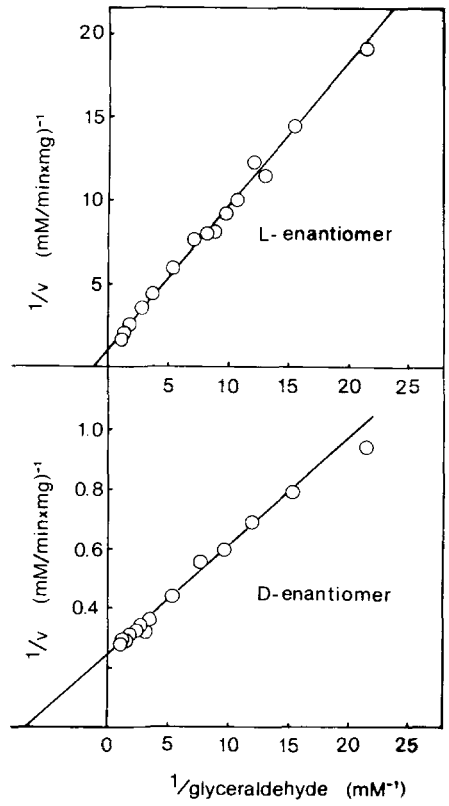

FIG. 2. **Double-reciprocal plots of the modified form of aldose reductase (ARa) for D- and L-glyceraldehyde.**

TABLE I

Kinetic parameters of the native (b-form) and modified (a-form) forms of bovine lens aldose reductase

Substrate	*a*-form			*b*-form		
	K_m	k_{cat}[a]	k_{cat}/K_m	K_m	k_{cat}[a]	k_{cat}/K_m
	mM	s^{-1}		*mM*	s^{-1}	
L-Glyceraldehyde	0.820	300	370	0.005	450	90,000
D-Glyceraldehyde	0.147	1,200	8,160	0.021	490	23,300

[a] Evaluated on the basis of a M_r = 34,000.

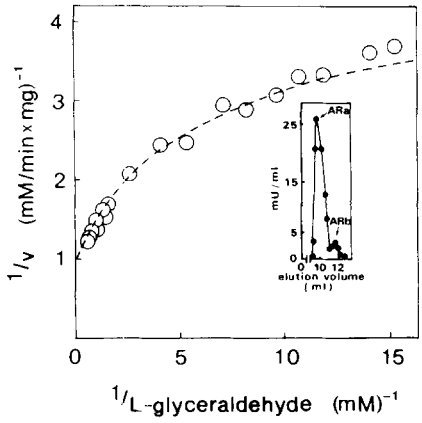

FIG. 3. **Double-reciprocal plot for L-glyceraldehyde of a mixture of the native and modified forms of aldose reductase.** The *inset* shows the chromatographic analysis of the enzyme mixture by fast protein liquid chromatography on a Mono Q column using DL-glyceraldehyde as a substrate for the enzyme activity measurement. When evaluated with L-glyceraldehyde as substrate, the relative contribution of ARa and ARb to the total activity of the enzyme mixture was 72 and 28%, respectively. The *dotted line* refers to a computer-simulated curve obtained using an equation of two enzymes acting on the same substrate (20). To solve the equation, apparent V_{max} values of 0.72 and 0.28 for ARa and ARb, respectively, and the K_m for L-glyceraldehyde of the two enzyme forms (Table I) were used.

fication of aldose reductase, may well account for the reported singular kinetic behavior of the enzyme.

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