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Characterization of Aldose Reductases Ia and Ib from Rabbit Lens

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The properties of two aldose reductases (Ia and Ib) from rabbit lens were investigated. Both enzymes showed similar substrate specificity, and were capable of reducing various aldoses and aldehydes. On the basis of apparent K_m, V_{max} and second-order rate constant (k_{cat}/K_m) values, both enzymes had the highest reductive efficiency toward aromatic aldehydes such as *p*-nitrobenzaldehyde. Among the aldoses tested, the aldose reductases exhibited a high affinity for DL-glyceraldehyde (K_m of 31 μM for Ia and 32 μM for Ib) and a low affinity for D-glucose (K_m of 92 mM for Ia and 126 mM for Ib). Aldose reductase I's could utilize both reduced nicotinamide adenine dinucleotide phosphate (NADPH) and reduced nicotinamide adenine dinucleotide (NADH) as coenzymes, but NADH was less effective than NADPH. The K_m values for NADPH (1.4 μM for Ia and 1.3 μM for Ib) were much smaller than those for NADH (420 μM for Ia and 270 μM for Ib). Aldose reductase I's were strongly activated by sulfate ion and their K_m and V_{max} values for substrate and coenzyme were increased. Aldose reductase I's were inhibited strongly by aldose reductase inhibitors: about 80% by 0.3 μM quercitrin, 65% by 1.6 μM quercetin and about 70% by 8.0 μM 3,3-tetramethyleneglutaric acid. $NADP^+$ and adenosine 2′,5′-diphosphate (2′,5′-ADP) were strong competitive inhibitors of both aldose reductase I's with respect to the coenzyme. The K_i values for 2′,5′-ADP were about 30 μM, and those for $NADP^+$ were about 70 μM.

Keywords——aldose reductase; rabbit lens; substrate specificity; coenzyme specificity; kinetic constant; K_m value; V_{max} value; inhibition; activation

Aldose reductase [EC 1.1.1.21] is an enzyme which catalyzes the reduction of aldoses to the corresponding alcohols, and it catalyzes the first step of the sorbitol pathway. A number of aldose reductases have been isolated from various mammalian sources such as brain,[1] liver,[2] placenta,[3] kidney[4] and lens.[5] However, in many cases its metabolic significance is not yet well understood. In recent years, interest in this enzyme has increased because of its possible role in the pathogenesis of diabetic complications.[6] Aldose reductase in lens was first reported by van Heyningen,[7] and it has been suggested that it plays a leading role in the etiology of diabetic cataract. In the previous paper,[8] we described the presence of aldose reductase in rabbit lens and the participation of this enzyme in the accumulation of intracellular sorbitol in alloxan-diabetic rabbit lens. Subsequently, we obtained evidence for the existence of aldose reductase in multiple forms in rabbit lens, and we separated four species termed aldose reductases Ia, Ib, IIa and IIb by means of chromatofocusing.[9]

This paper deals with the substrate specificities, kinetic properties and aspects of the inhibition and activation of aldose reductases Ia and Ib.

Experimental

Materials——Glycolaldehyde, D-erythrose and D-glucuronic acid (sodium salt) were purchased from Aldrich Chemical Co., and other aldoses and aldehydes were obtained from Wako Pure Chemical Industries Ltd. Reduced nicotineamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Oriental Yeast Co., and NAD^+, $NADP^+$, β-nicotinamide adenine dinucleotide 3′-

phosphate, reduced form (3′-NADPH), nicotinamide hypoxanthine dinucleotide phosphate, reduced form (d-NADPH) and other nucleotides were purchased from Sigma Chemicals. Quercetin and quercitrin were purchased from Wako Pure Chemical Industries Ltd., and 3,3-tetramethyleneglutaric acid was obtained from Aldrich Chemical Co.

Preparation of Aldose Reductases Ia and Ib——The method used for the preparation of aldose reductase I's from normal rabbit lens was described in the previous paper.[9] Briefly, a 25—55% ammonium sulfate fraction was subjected to gel filtration on Sephadex G-200, followed by three column chromatographic steps, *i.e.*, affinity chromatography using Mātrex gel Orange A, gel filtration on Sephadex G-100 and chromatofocusing using polybuffer exchanger 94 and polybuffer 74. Aldose reductases Ia and Ib were purified about 800-fold and 1100-fold, respectively. The purified enzymes were each homogeneous on polyacrylamide gel electrophoresis. The enzymes were stored at 5 °C with 2 mM dithiothreitol.

Standard Assay of Aldose Reductase——Aldose reductase activity was determined at 25 °C by measuring the decrease in absorption of NADPH at 340 nm on a Union High-Sens SM-401 spectrophotometer equipped with a National X-Y recorder. The assay mixture contained 100 mM sodium phosphate buffer (pH 6.2), 10 mM DL-glyceraldehyde, 0.15 mM NADPH and an appropriate amount of the enzyme in a final volume of 3.0 ml. The reaction was initiated by adding the enzyme, and the decrease of absorption at 340 nm was followed for 200 s. 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.

Kinetics——The initial rate determination of each substrate was carried out in duplicate at seven substrate concentrations ranging from appreciably below to appreciably above the predetermined apparent K_m value. Aldose reductase I's from rabbit lens display a very low K_m value for NADPH (about 1.3 μM) and therefore, care was taken to ensure that all kinetic studies were carried out at an adequate coenzyme concentration. Under these conditions, the enzyme reaction followed the Michaelis–Menten equation. Kinetic constants were determined by fitting the data to the Michaelis–Menten equation by using the weighing procedure of Wilkinson.[10] The values of K_m and V_{max} and their standard errors were calculated with a computer using the programs of Cleland.[11] Under conditions where $S \ll K_m$, the Michaelis–Menten equation reduces to the following equation.

$$v = \frac{k_{cat}}{K_m} \cdot E_0 \cdot S$$

Here k_{cat} is the overall catalytic rate constant, and E_0 is the enzyme concentration. E_0 was calculated using a molecular weight of 35000.[9] Relative pseudo-second order rate constants were calculated as k_{cat}/K_m.

Protein Concentration——The concentration of protein was determined by a dye-binding assay method according to Bradford[12] using the Bio-Rad protein assay kit. Lyophilized bovine gamma globulin was used as a standard protein for assay.

Results

Substrate Specificity

A comparative study of the substrate specificities of aldose reductases Ia and Ib was carried out with various aldoses and aldehydes. The kinetic parameters for these enzymes are shown in Tables I and II. These enzymes reacted with many common substrates. The K_m values of aldose reductase Ia for all substrates tested were very similar to those of aldose reductase Ib. Aldose reductase I's exhibited high affinity for DL-glyceraldehyde (K_m of 31 μM for Ia and 32 μM for Ib) and low affinity for D-glucose (K_m of 92 mM for Ia and 126 mM for Ib). The K_m values of both enzymes for aldoses tended to increase with increasing chain length, though the K_m value for triose was exceptionally the same as that for tetrose. On the other hand, the K_m values for aliphatic aldehydes decreased markedly with increasing chain length. For aliphatic aldehydes with 4 or 5 carbon atoms, both enzymes exhibited very much smaller K_m values than those for acetaldehyde and propionaldehyde. Among the substrates tested, *p*-nitrobenzaldehyde displayed the lowest K_m value for both enzymes. As shown by the second-order rate constant (k_{cat}/K_m), both enzymes appear to be catalytically most efficient with respect to *p*-nitrobenzaldehyde. D-Glucuronolactone had a lower K_m and was reduced more rapidly than D-glucuronic acid.

Coenzyme Specificity

Aldose reductases Ia and Ib could use both NADPH and NADH as coenzymes (Table III), though NADPH was preferred by both enzymes. The activities found with 0.15 mM

TABLE I. Kinetic Constants for Substrates of Aldose Reductase Ia from Rabbit Lens

Substrate	$K_m \pm$S.E. (mM)	$V_{max} \pm$S.E. (μmol·min^{-1}·mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	Relative V_{max} (%)
DL-Glyceraldehyde	0.0309±0.0026	0.791±0.025	0.461	14900	100
D-Erythrose	0.0251±0.0041	0.827±0.041	0.482	19200	104.6
D-Xylose	7.64±0.85	0.728±0.036	0.425	55.6	92.0
D-Ribose	8.96±0.33	0.648±0.011	0.378	42.2	81.9
D-Glucose	92.4±9.0	0.645±0.021	0.376	4.07	81.5
D-Galactose	46.5±3.2	0.661±0.022	0.386	8.30	83.6
D-Glucuronolactone	1.40±0.13	0.806±0.036	0.470	336	101.9
D-Glucuronic acid	9.14±0.70	0.609±0.027	0.355	38.8	77.0
Glycolaldehyde	0.736±0.186	0.694±0.067	0.405	550	87.7
Acetaldehyde	35.2±2.2	0.758±0.022	0.442	12.6	95.9
Propionaldehyde	3.04±0.29	1.194±0.064	0.697	229	150.9
n-Butyraldehyde	0.0496±0.0036	0.754±0.024	0.440	8870	95.3
Isobutyraldehyde	0.0625±0.0022	0.865±0.011	0.505	8080	109.3
n-Valeraldehyde	0.00731±0.00122	0.791±0.039	0.461	63100	100.0
Isovaleraldehyde	0.00659±0.00034	0.765±0.010	0.446	67700	96.7
p-Nitrobenzaldehyde	0.00337±0.00054	0.942±0.007	0.550	163000	119.1

TABLE II. Kinetic Constants for Substrates of Aldose Reductase Ib from Rabbit Lens

Substrate	$K_m \pm$S.E. (mM)	$V_{max} \pm$S.E. (μmol·min^{-1}·mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	Relative V_{max} (%)
DL-Glyceraldehyde	0.0324±0.0024	1.285±0.037	0.750	23100	100
D-Erythrose	0.0234±0.0006	1.230±0.009	0.718	30700	95.8
D-Xylose	9.02±0.33	1.072±0.018	0.625	69.3	83.4
D-Ribose	6.54±0.93	0.841±0.049	0.491	75.1	65.4
D-Glucose	126±24	0.829±0.065	0.484	3.84	64.5
D-Galactose	47.2±3.4	0.770±0.028	0.449	9.51	59.9
D-Glucuronolactone	1.21±0.06	0.964±0.022	0.562	464	75.0
D-Glucuronic acid	7.61±1.32	0.718±0.069	0.419	55.1	55.9
Glycolaldehyde	0.728±0.070	0.955±0.036	0.557	765	74.3
Acetaldehyde	17.7±0.9	0.675±0.011	0.394	22.3	52.5
Propionaldehyde	1.70±0.28	0.981±0.071	0.572	336	76.3
n-Butyraldehyde	0.0628±0.0062	1.127±0.051	0.658	10500	87.7
Isobutyraldehyde	0.0632±0.0074	1.127±0.058	0.658	10400	87.7
n-Valeraldehyde	0.00992±0.00063	1.227±0.026	0.716	72200	95.4
Isovaleraldehyde	0.00931±0.00064	1.178±0.026	0.687	73800	91.7
p-Nitrobenzaldehyde	0.00519±0.00086	1.344±0.126	0.784	151000	104.6

NADH were about 40% of those found with 0.15 mM NADPH under the standard assay conditions. Analogs of NADPH, 3′-NADPH and d-NADPH, were also used as coenzymes by aldose reductase I's, but the relative activities of both enzymes with 3′-NADPH and d-NADPH were less than 10% of those with NADPH. The K_m values for NADPH of aldose reductases Ia and Ib were virtually the same (1.4 and 1.3 μM, respectively), while those for NADH were much larger (420 and 270 μM, respectively).

Inhibition by Various Nucleotides

Inhibitory effects of $NADP^+$ and compounds structurally related to the coenzyme molecule were tested (Table IV). $NADP^+$ and adenosine 2′,5′-diphosphate (2′,5′-ADP) were effective inhibitors, while 2′-AMP and 5′-AMP showed only slight inhibition. NAD^+,

Table III. Coenzyme Specificity of Aldose Reductases from Rabbit Lens

Coenzyme	Aldose reductase Ia		Aldose reductase Ib	
	Relative activity (%)	K_m (μM)	Relative activity (%)	K_m (μM)
NADPH	100	1.4	100	1.3
3′-NADPH	3	—	4	—
d-NADPH	9	—	8	—
NADH	42	420	37	270

Table IV. Inhibition of Aldose Reductases from Rabbit Lens by Nucleotides

Nucleotide	Aldose reductase Ia		Aldose reductase Ib	
	Inhibition (%)	K_i (mM)	Inhibition (%)	K_i (mM)
$NADP^+$	75	0.069	74	0.067
NAD^+	0	—	6	—
ATP	1	—	4	—
ADP	2	—	6	—
2′,5′-ADP	85	0.032	87	0.031
2′-AMP	8	>10	8	>10
3′-AMP	7	—	10	—
5′-AMP	2	>50	6	>50
c-AMP	28	—	23	—
d-AMP	1	—	5	—
NMN	1	—	6	—

Inhibition is expressed as a percentage with respect to the uninhibited control. Nucleotide concentration was 2.5×10^{-4} M.

adenosine triphosphate (ATP), ADP, 2-deoxyadenosine 5′-phosphate (d-AMP) and nicotinamide mononucleotide (NMN) hardly inhibited the enzymes at the concentration tested. $NADP^+$ and 2′,5′-ADP competed with the coenzyme but did not compete with DL-glyceraldehyde. The inhibition constants (K_i) for $NADP^+$ were estimated from Dixon plots[13] to be 69 and 67 μM for aldose reductases Ia and Ib, respectively. The K_i values for 2′,5′-ADP were 32 and 31 μM, respectively. The K_i values for 2′-AMP and 5′-AMP were very high, being more than 10 and more than 50 mM, respectively.

Activation by Sulfate Ion

As described previously,[9] rabbit lens aldose reductases Ia and Ib were activated by sulfate ion. The effect of ammonium sulfate on the apparent kinetic constants of aldose reductase I's was investigated (Table V). The addition of 0.3 M ammonium sulfate caused an increase in both apparent K_m and V_{max} values for DL-glyceraldehyde and NADPH. The K_m and V_{max} values of aldose reductase Ia for DL-glyceraldehyde increased by factors of 2.8 and 2.6, and those for NADPH increased by factors of 8.6 and 2.4, respectively. The increases of the K_m and V_{max} values of aldose reductase Ib were similar.

Effect of Aldose Reductase Inhibitors

The effects of known aldose reductase inhibitors on aldose reductases Ia and Ib were

TABLE V. Effect of Ammonium Sulfate on Kinetic Constants of Aldose Reductases from Rabbit Lens

Substrate	Ammonium sulfate	Aldose reductase Ia		Aldose reductase Ib	
		K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})
DL-Glyceraldehyde	−	29	0.785	27	1.26
	+	81	2.08	74	2.84
NADPH	−	1.4	0.845	1.3	1.15
	+	12	2.03	13	2.76

The concentration of ammonium sulfate was 0.3 M.

TABLE VI. Inhibition of Aldose Reductase from Rabbit Lens by Aldose Reductase Inhibitors

Compd.	Conc. (μM)	Inhibition (%)	
		Aldose reductase Ia	Aldose reductase Ib
Quercitrin	0.06	41	40
	0.3	81	73
Quercetin	0.3	26	29
	1.6	65	65
3,3-Tetramethylene-glutaric acid	1.6	37	36
	8.0	69	73

Inhibition is expressed as a percentage with respect to the uninhibited control.

examined, and the results are shown in Table VI. Both enzymes were affected similarly by flavonoids, quercitrin and quercetin, and 3,3-tetramethyleneglutaric acid. Quercitrin was a very potent inhibitor for both enzymes; at 0.3 μM it inhibited aldose reductases Ia and Ib by 81 and 73%, respectively. Quercetin and 3,3-tetramethyleneglutaric acid were also strong inhibitors for both enzymes. Quercetin decreased both enzyme activities by 65% at 1.6 μM, and 3,3-tetramethyleneglutaric acid inhibited the enzymes by about 70% at 8.0 μM.

Discussion

Two aldose reductases from rabbit lens, termed aldose reductases Ia and Ib, were characterized with respect to substrate and coenzyme specificities and responses to inhibitors and effectors. Aldose reductases Ia and Ib of rabbit lens showed wide substrate specificities, and were reactive with many common substrates. The substrate specificities of the enzymes were quite similar to that of calf lens aldose reductase,[5a] except that the calf enzyme reduced isovaleraldehyde very slowly. The substrate specificities of aldose reductase,[1-5] aldehyde reductase[13] and L-hexonate dehydrogenase[14] are known to overlap in many cases. In the case of L-hexonate dehydrogenase, the K_m value for D-glucuronate was similar to that for DL-glyceraldehyde, and cyclic aldoses exhibited very low affinities and much lower maximal velocities than D-glucuronate. However, rabbit lens aldose reductase I's exhibited much lower K_m values for DL-glyceraldehyde than for D-glucuronate, and the K_m values for pentoses were similar to those for D-glucuronate. In addition, relative V_{max} valûes of aldose reductase I's for aldoses were greater than those for D-glucuronate. Although it has been reported that aldehyde reductase exhibited very high K_m values for pentoses (about 100—1500 mM),[13a-d,15]

those of rabbit lens aldose reductase I's were about 7—9 mM. The K_m values of aldose reductase from other sources have also been determined as about 5—16 mM.[1a,b,d,3a,5a] On the basis of the profile of substrate specificity and kinetic properties, rabbit lens aldose reductase I's are clearly different enzymes from aldehyde reductase and L-hexonate dehydrogenase.

The K_m values of aldose reductase I's for aldoses increased in the following order: hexose > pentose > tetrose = triose. The tendency for the K_m value of an aldose to increase with chain length may be due to a requirement for a free aldehyde group in the substrate. There is an approximate agreement between the K_m values of these aldoses and the ratio of aldehyde form present in solution. For example, the K_m value of D-glucose is about 3 times and 10 times those of D-galactose and D-xylose, respectively, whereas the fraction of aldehyde form in a 0.1 M solution of glucose at pH 6.5 is one-sixth and one-eighth of those for D-galactose and D-xylose.[16]

Aldose reductase I's use both NADPH and NADH as coenzymes, but the reactions proceeded with NADH at about 40% of the rates observed with NADPH at 0.15 mM. The K_m values of aldose reductase I's for NADH were relatively large, but those for NADPH were very small as in the cases of enzymes from other sources. $NADP^+$ and 2′,5′-ADP were very strong inhibitors. The K_i values for 2′,5′-ADP were about 300 times smaller than those for 2′-AMP and over 1000 times smaller than those for 5′-AMP. Both 2′- and 5′-phosphate moieties seem to be necessary for binding to the enzymes. However, since the K_i value for 2′-AMP was over 5 times smaller than that for 5′-AMP, the 2′-phosphate group of the adenosine moiety appears to contribute more than the 5′-phosphate group in the binding to the enzymes. This is also supported by the finding that NADPH is about 200 times more effective than NADH.

Like the sheep seminal vesicle,[17] human lens,[5b] calf lens,[5a] human placenta[3a] and pig brain[1c] aldose reductases, the rabbit lens enzymes were activated by sulfate ion, though the enzyme from calf liver[2] was inhibited by sulfate ion. In the case of rabbit lens aldose reductase I's, ammonium sulfate caused not only a doubling of the V_{max} value but also a significant increase in the K_m values for DL-glyceraldehyde and NADPH. A similar effect was observed with calf lens aldose reductase. It is suggested that sulfate affects the active center of the enzyme, especially the binding site for coenzyme. Though aldehyde reductase is not activated by sulfate ion, aldose reductase is generally activated by this ion. Therefore, rabbit lens aldose reductase I's are clearly a different enzyme from either aldehyde reductase.

Rabbit lens aldose reductase I's closely resemble the enzyme in the calf lens, described by Hayman and Kinoshita,[5a] in substrate specificity, requirement for coenzyme, kinetic characteristics and the effect of sulfate ion. As described in a previous paper,[9] the rabbit lens enzymes were also similar to the calf lens enzyme in molecular weight. In addition, the rabbit lens enzymes were similar to aldose reductases from other mammalian sources with regard to substrate specificity and other properties. From the results of this study, it seems likely that aldose reductase I's from rabbit lens can best be classified as alditol : $NADP^+$ oxidoreductase [EC 1.1.1.21] (trivial name: aldose reductase), and that aldose reductases Ia and Ib are isozymes.

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