Characterization of Aldose Reductase and Aldehyde Reductase from the Medulla of Rat Kidney

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Aldose reductase and aldehyde reductase from the medulla of the rat kidney have been purified to homogeneity by using affinity chromatography, gel filtration and chromatofocusing. The molecular weights of aldose reductase and aldehyde reductase by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were found to be 37000 and 39000, respectively. The isoelectric points of aldose reductase and aldehyde reductase were found to be 5.4 and 6.2 by chromatofocusing, respectively. The major differences of amino acid compositions between both enzymes were found in serine, alanine and aspartic acid. Substrate specificity studies showed that aldose reductase utilized aldo-sugars such as D-glucose and D-galactose, but aldehyde reductase did not use them. The K_m values of aldose reductase for various substrates were lower than those of aldehyde reductase. Aldose reductase utilized both reduced nicotinamide adenine dinucleotide phosphate (NADPH) and reduced nicotinamide adenine dinucleotide (NADH) as coenzymes, whereas aldehyde reductase utilized only NADPH. The presence of the sulfate ion resulted in a dramatic activation of aldose reductase whereas it did not affect aldehyde reductase activity. These enzymes were strongly inhibited by the known aldose reductase inhibtors. However, aldose reductase was more susceptible than aldehyde reductase to inhibition by the aldose reductase inhibitors.

Keywords aldose reductase; aldehyde reductase; rat renal medulla; aldose reductase inhibitor

A number of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidoreductases, catalyzing the reduction of various aldehydes to their corresponding alcohols, are distributed in various mammalian tissues.[1,2] These enzymes consist of two major groups as aldose reductase and aldehyde reductase. Aldose reductase catalyzes the conversion of aldo-sugars to their corresponding sugar-alcohols while aldehyde reductase has a poor ability to reduce aldo-sugars.

Although aldose reductase catalyzes the first step of the polyol pathway, the normal physiological role of aldose reductase is not fully understood in tissues other than the seminal vesicles.[3] On the other hand, it has been demonstrated by Heyningen that the enzyme of the polyol pathway in the lens may play an important role in the formation of sugar cataracts. Subsequently, Kinoshita has demonstrated an osmotic hypothesis of diabetic cataract formation. The cataract could be ameliorated and its onset was effectively delayed by the administration of aldose reductase inhibitors. These studies have contributed to the recent examination of the role of polyol pathway in diabetic complications with other tissues. Additionally, some aldose reductase inhibitors are found to be useful for preventing or treating diabetic complications.[4]

Since diabetic nephropathy is one of the typical diabetic complications, the relationship between its etiology and aldose reductase is of much current interest. It is not yet known whether the osmotic hypothesis can be applied to diabetic nephropathy. However, an aldose reductase inhibitor, sorbinil, normalized the biochemical changes in diabetic rats,[5] and resulted in reversing their protein urea.[6] Therefore, it suggested that aldose reductase could play some role in the pathogenesis of diabetic nephropathy. Recently, using GRB-PAP1, which is a continuous line of epithelial cells from a rabbit renal inner medulla, it was shown that aldose reductase was strongly induced by elevation of extracellular sodium chloride, and large amounts of sorbitol were accumulated in the cells under the hyperosmotic stress.[7] From these results, an interest has been focused on the role of aldose reductase in the kidney. Aldose reductase from the bovine kidney was reported recently by Grimshaw *et al.*,[8] but little information is available about the properties of aldose reductase from the mammalian kidney. Terubayashi *et al.* reported aldehyde reductase and aldose reductase of the rat kidney, as contained mainly in the cortex and the inner medulla respectively, by immunohistochemical study.[9] Sato *et al.* reported that only aldehyde reductase was isolated from the rat kidney.[10] However, there is no report regarding isolation of rat renal aldose reductase.

In this paper, we report the characterization and susceptibility to aldose reductase inhibitors of rat renal medulla aldose reductase and aldehyde reductase.

Experimental

Materials Sodium D-glucuronate was purchased from Aldrich Chemical Co., along with other aldoses, 30% hydrogen peroxide and formic acid were obtained from Wako Pure Chemical Industries Ltd. NADPH and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Oriental Yeast Co. Sephadex G-75, polybuffer 74 and polybuffer exchanger 94 were purchased from Pharmacia Fine Chemicals. Methane sulfonic acid (4 N) was purchased from Pierce Chemical Company. Molecular weight standard protein kit and protein assay kit were purchased from Bio-Rad Laboratories. Matrex gel orange A was purchased from Amicon Co. Sorbinil, tolrestat, M79175, ponalrestat (statil) and alconil were kindly donated by Dr. Kador, National Institutes of Health, U.S.A. Epalrestat was a gift of Prof. Tanaka, Osaka University of Pharmaceutical Sciences. Kidneys were removed from male Wistar rats weighing about 250 g and one kidney was about 0.8 g. Performic acid solution was prepared as follows: after mixing 1 ml of 30% hydrogen peroxide and 9 ml of formic acid, the mixture was allowed to stand for 1 h at room temperature and subsequently cooled to 0 °C.

Assay of Enzyme Activity The activities of aldose reductase and aldehyde reductase were determined at 25 °C by measuring the decrease in absorption of NADPH at 340 nm on a Hitachi 557 dual-beam spectrophotometer equipped with a temperature-controlled cuvette chamber. The assay mixture, in a 3.0 ml system contained 90 mM sodium phosphate buffer (pH 6.2), 0.135 mM NADPH and 10 mM DL-glyceraldehyde. The reaction was initiated by adding the enzyme, and the rate of NADPH oxidation was followed by recording the decrease in absorbance at 340 nm. The appropriate blanks to correct for nonspecific oxidation of NADPH were prepared. One unit of the enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of

NADPH per minute under the conditions described here.

Protein Determination The concentration of protein was determined with the Bio-Rad protein assay kit according to the Bradford method.[11]

Polyacrylamide Gel Electrophoresis Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on 12% slab gel according to the method of Laemmli.[12] Proteins were visualized with Coomassie brilliant blue R250. Standard proteins used for estimation of molecular weight were phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Amino Acid Analysis Purified enzymes were hydrolyzed *in vacuo* at 110 °C with 6 N HCl for 24, 48 and 72 h, and then were analyzed using the method of Spackman *et al.*[13] by a Hitachi automatic amino acid analyzer, Model 835. For the determination of tryptophan, lyophilized purified enzymes were hydrolyzed *in vacuo* at 115 °C for 24 h with 4 N methane sulfonic acid containing 0.2% of 3-(2-aminoethyl)indole by the method of Simpson *et al.*[14] Half-cystine was estimated as cysteic acid using the method of Moore.[15]

Determination of Kinetic Constant The initial rate values for each substrate were calculated with a computer using the programs of Cleland.[16]

Determination of IC_{50} The concentration of inhibitor giving 50% inhibition of enzyme activity (IC_{50}) was estimated from the least squares regression line of the log dose–response plot. The concentrations of both enzymes in the assay mixture were 0.7—1.0 μg/ml.

Enzyme Purification All operations were performed at 0—4 °C. All buffers contained 2 mM dithiothreitol in order to protect the enzyme from inactivation.

Extract: Rat renal medullary tissue (21 g) was dissected from kidneys and was homogenized in 42 ml of 10 mM sodium phosphate buffer (pH 7.0) with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 13000 × *g* for 30 min, and then the precipitate was discarded.

Matrex Gel Orange A Affinity Chromatography: The supernatant was diluted with 10 mM sodium phosphate buffer (pH 7.0) to a protein concentration of 5 mg/ml. The diluted supernatant was applied to a Matrex gel orange A column (1.6 × 28 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0) and column was thoroughly washed with the same buffer. The enzyme was eluted with 10 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM NADPH at a flow rate of 20 ml/h, and 3-ml fractions were collected. Fractions with enzyme activity were pooled.

Ammonium Sulfate Fractionation: The enzyme fraction obtained by affinity chromatography was brought to 70% saturation by adding solid ammonium sulfate. The precipitate was dissolved in 3 ml of 10 mM sodium phosphate buffer (pH 7.0).

Sephadex G-75 Gel Filtration Chromatography: The ammonium sulfate fraction was applied to a Sephadex G-75 column (1.9 × 95 cm) equilibrated with 25 mM imidazole–HCl buffer (pH 7.0). The enzyme was eluted with the same buffer at a flow rate of 20 ml/h, and 4 ml-fractions were collected. Fractions with enzyme activity were pooled and concentrated by ultrafiltration using a collodion bag.

Chromatofocusing: The concentrated enzyme solution was applied to a polybuffer exchanger 94 column (1 × 45 cm) equilibrated with 25 mM imidazole–HCl buffer (pH 7.0) for chromatofocusing. The elution was carried out with 1 : 8 diluted polybuffer 74 (pH 4.0) at a flow rate of 20 ml/h, and fractions of 2.0 ml were collected.

Results

Enzyme Purification Both affinity chromatography and gel filtration resulted in a single peak of enzyme activity. Chromatofocusing clearly separated into three peaks with enzyme activity, tentatively designated as peak 1, 2 and 3 which were eluted at pH 6.4, 6.2 and 5.4, respectively (Fig. 1). On the basis of the enzymatic properties of enzymes described in this paper, peak 2 was identified as aldehyde reductase, and peak 3 as aldose reductase. Although the enzymatic properties of peak 1 were the same as those of peak 2, it was not described in this paper because of the minor peak.

The results of the purification of aldose reductase and aldehyde reductase from the medulla of rat kidney are given in Table I. Aldose reductase was purified about 79-fold with a specific activity of 1.57 units/mg and the yield was 5%. Aldehyde reductase was purified about 261-fold with 32% recovery and a specific activity of 5.21 units/mg. The purified aldehyde reductase and aldose reductase were found to be homogeneous by SDS-polyacrylamide gel electrophoresis

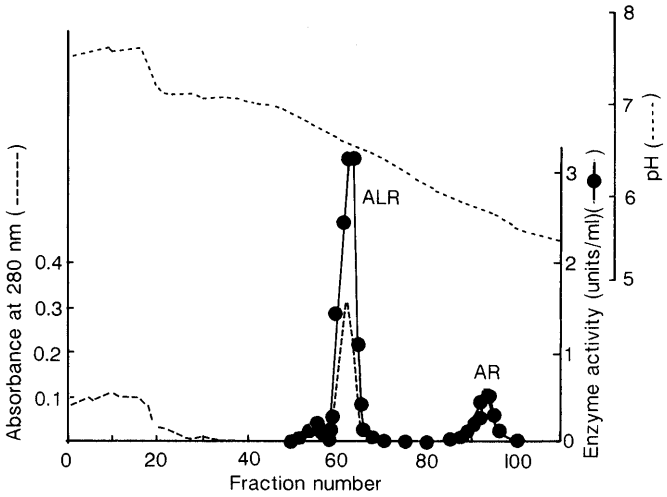

Fig. 1. Chromatofocusing of Aldose Reductase and Aldehyde Reductase from the Medulla of Rat Kidney

The pooled fraction of enzyme activity peak from a Sephadex G-75 was concentrated by ultrafiltration using a collodion bag and applied to a polybuffer-exchanger 94 column (1 × 45 cm) equilibrated with 25 mM imidazole–HCl buffer (pH 7.0) containing 2 mM dithiothreitol. The elution was carried out with 1 : 8 diluted polybuffer 74 (pH 4.0) containing 2 mM dithiothreitol. The flow rate was 20 ml/h, and fractions of 2.0 ml were collected.

TABLE I. Purification of Aldose Reductase and Aldehyde Reductase from the Medulla of Rat Kidney

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification (fold)	Recovery (%)
Extract	2937	58.7	0.020	1	100
Matrex gel orange A	42.19	58.5	1.39	70	100
70% $(NH_4)_2SO_4$ fraction	26.74	46.0	1.72	86	78
Sephadex G-75	10.95	36.2	3.31	166	62
Chromatofocusing					
Aldose reductase	1.93	3.03	1.57	79	5
Aldehyde reductase	3.55	18.5	5.21	261	32

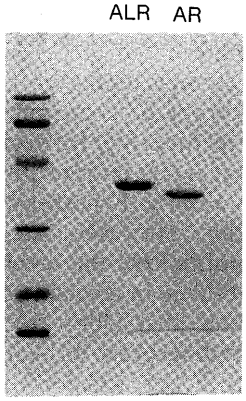

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of the Purified Aldose Reductase and Aldehyde Reductase from the Medulla of Rat Kidney

Gel electrophoresis of 4 μg of the purified enzyme was carried out on 12% polyacrylamide slab gel in the presence of SDS according to the method of Laemmli.[12] The gel was stained with coomassie brilliant blue R-250. Lane ALR: aldehyde reductase; lane AR: aldose reductase.

TABLE II. Amino Acid Compositions of Aldose Reductase and Aldehyde Reductase from the Medulla of Rat Kidney

Amino acid	Aldose reductase			Aldehyde reductase		
	Amino acid residues /mol of protein	Nearest integer /mol of protein	mol percent	Amino acid residues /mol of protein	Nearest integer /mol of protein	mol percent
Asp	34.6	35	10.5 (10.9)	28.6	29	8.0
Thr	17.1	17	5.1 (4.2)	16.7	17	4.7
Ser	18.8	19	5.7 (4.6)	29.0	29	8.0
Glu	39.7	40	12.0 (11.3)	43.6	44	12.2
Pro	23.1	23	6.9 (6.7)	24.7	25	6.9
Gly	18.8	19	5.7 (3.9)	25.4	25	6.9
Ala	22.8	23	6.9 (6.7)	30.9	31	8.6
Half-cys[a]	7.3	7	2.1 (2.8)	8.3	8	2.2
Val	27.8	28	8.4 (8.1)	28.1	28	7.8
Met	5.9	6	1.8 (1.8)	4.8	5	1.4
Ile	16.8	17	5.1 (6.3)	13.7	14	3.9
Leu	31.4	31	9.3 (9.2)	35.4	35	9.7
Tyr	10.5	11	3.3 (4.2)	11.9	12	3.3
Phe	11.4	11	3.3 (3.5)	9.0	9	2.5
Lys	25.4	25	7.5 (8.1)	21.8	22	6.1
His	8.3	8	2.4 (2.5)	11.3	11	3.0
Arg	10.0	10	3.0 (3.5)	15.8	16	4.4
Trp[b]	2.0	2	0.6 (1.8)	1.1	1	0.3
Total residues		332			361	

Values were calculated on the basis of molecular weights of 37000 for aldose reductase and 39000 for aldehyde reductase. Values for threonine and serine were obtained by extrapolation to zero time of hydrolysis. *a*) Values for half-cystine were estimated as cysteic acid after treatment of the enzyme with performic acid. *b*) Values for tryptophan were obtained by hydrolysis in 4 N methane sulfonic acid for 24 h at 115 °C. Values in parentheses are calculated on the basis of the primary sequence data[26] for the rat lens aldose reductase by complementary deoxyribonucleic acid (cDNA) analysis.

TABLE III. Substrate Specificities and Kinetic Constants of Aldose Reductase and Aldehyde Reductase from the Medulla of Rat Kidney

Substrate	Aldose reductase			Aldehyde reductase		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
DL-Glyceraldehyde	0.087	1.22	14100	8.31	8.21	989
D-Glucuronate	10.7	0.392	37.2	4.76	6.05	1270
D-Xylose	23.7	1.01	42.8	718	2.59	3.61
D-Galactose	88.6	0.678	7.66	N.D.		
D-Glucose	420	0.842	1.97	N.D.		
NADPH	0.0022	—	—	0.0025		
NADH	0.50	—	—	N.D.		

The determination of K_m values for different substrates was performed in 0.135 mM NADPH. K_m values for the NADPH and NADH were determined using 10 mM DL-glyceraldehyde as substrate. The k_{cat} values were calculated using the molecular weights of 37000 for the aldose reductase and 39000 for the aldehyde reductase. N.D.: Not detectable.

(Fig. 2).

Molecular Weights and Amino Acid Composition The molecular weights of aldose reductase and aldehyde reductase were estimated to be 37000 and 39000 by SDS-polyacrylamide gel electrophoresis, respectively (Fig. 2). The amino acid compositions of the homogeneous aldose reductase and aldehyde reductase are given in Table II. The total amino acid residues were calculated to be 332 for aldose reductase and 361 for aldehyde reductase based on the above molecular weights. The amino acid compositions of two enzymes showed a considerable resemblance to each other. Particularly, the mole percent of tyrosine, proline, glutamic acid and half-cystine was the same values between two enzymes. Major differences between two enzymes were observed in asparatic acid, serine, alanine, lysine and arginine. Tryptophan was contained one residue in aldehyde reductase and two residues in aldose reductase.

Substrate Specificity The kinetic constants of aldose reductase and aldehyde reductase determined for several substrates are given in Table III. Aldose reductase reduced aldo-sugars such as D-xylose, D-glucose and D-galactose. However, aldose reductase displayed significantly high K_m and low k_{cat}/K_m for those substrates as compared with DL-glyceraldehyde. Aldose reductase had a very low K_m value and a very high k_{cat}/K_m value for DL-glyceraldehyde. It displayed a K_m value 100 times lower and a k_{cat}/K_m value 15 times higher than aldehyde reductase. Aldose reductase also reduced D-glucuronate which gave a K_m value 120 times greater and a k_{cat}/K_m value 380 times less than DL-glyceraldehyde. Aldehyde reductase did not catalyze the reduction of aldo-sugars just as Sato *et al.* reported.[10]

Coenzyme Specificity Aldose reductase used both NADPH and NADH as coenzymes. The proportion of activity of NADH to NADPH using 10 mM DL-glyceraldehyde as the substrate was only about 20%. NADPH as coenzyme had a 230-fold higher affinity than NADH.

TABLE IV. Effects of Salts on Aldose Reductase and Aldehyde Reductase from the Medulla of Rat Kidney

Salt	Relative activity (%)			
	Aldose reductase		Aldehyde reductase	
	0.1 M	0.3 M	0.1 M	0.3 M
None	100	100	100	100
$(NH_4)_2SO_4$	230	302	106	89
Li_2SO_4	223	286	104	80
Na_2SO_4	208	247	103	91
NaCl	76	73	88	64
NH_4Cl	62	76	72	54

TABLE V. Effect of Aldose Reductase Inhibitors on Aldose Reductase and Aldehyde Reductase from the Medulla of Rat Kidney

Inhibitor	IC_{50} (μM)			
	Aldose reductase		Aldehyde reductase	
	Medulla	Testis[a)]	Medulla	Testis[a)]
M79175	0.051	0.053	0.078	0.096
Epalrestat	0.030	0.012	1.15	0.75
Tolrestat	0.0061	0.0095	0.74	0.50
Sorbinil	0.10	0.18	1.4	1.5
Alconil	0.0062	0.017	0.0081	0.012
Ponalrestat	0.0048	0.0054	0.60	0.66

a) Rat testis. Data from Kawasaki *et al.*[18] The activities of aldose reductase and aldehyde reductase were assayed with 10 mM DL-glyceraldehyde and 0.135 mM NADPH at various concentrations of inhibitors. IC_{50} values were estimated from the least squares regression line of the log dose–response plot.

In contrast, aldehyde reductase activity was displayed with only NADPH.

Effect of Salts Table IV shows the effect of salts on aldose reductase and aldehyde reductase. The aldose reductase was activated by sodium sulfate, lithium sulfate or ammonium sulfate. The presence of the salt at 0.1 and 0.3 M was increased 2.1—2.3 times and 2.5—3.0 times in the aldose reductase activity, respectively. On the other hand, sodium chloride and ammonium chloride inhibited slightly the aldose reductase activity. This indicated that sulfate ions were the activators of the aldose reductase activity. Aldehyde reductase was not activated by sulfate ions, and was inhibited by sodium chloride and ammonium chloride.

Effect of Inhibitor Aldose reductase and aldehyde reductase from rat renal medulla were inhibited potently by the known aldose reductase inhibitors, M79175, epalrestat, tolrestat, sorbinil, alconil or ponalrestat (Table V). The susceptibilities of aldose reductase to all aldose reductase inhibitors were greater than those of aldehyde reductase. Particularly, susceptibilities to tolrestat and ponalrestat were over 100 times greater. The extremely low IC_{50} values for aldose reductase were observed in the 10^{-9} M order range for ponalrestat, tolrestat and alconil.

Discussion

Purification of aldose reductase from the rat kidney has never been reported in contrast to some papers on purification of aldehyde reductase from rat kidney.[10,17] This study demonstrated that the rat renal medulla contained not only aldehyde reductase but also aldose reductase. Using dye-affinity chromatography, gel filtration and chromatofocusing, aldose reductase and aldehyde reductase were purified to homogeneity from the rat renal medulla. It was reported that the separation of aldose reductase and aldehyde reductase from rat testis was accomplished by dye-affinity chromatography.[18] However, these enzymes in rat renal medulla were not separated by this method but were separated by chromatofocusing as the final step.

On the basis of comparison of the experimental data such as the molecular weights, isoelectric point (pI), substrate and coenzyme specificities, effect of salts and susceptibilities to the aldose reductase inhibitors, it was found that rat renal medulla aldose reductase differed distinctly from rat renal medulla aldehyde reductase. The experimental data of rat renal medulla aldose reductase and aldehyde reductase resembled closely rat testis aldose reductase and aldehyde reductase, respectively.[18]

The molecular weight estimated by SDS-polyacrylamide gel electrophoresis of aldose reductase of the rat renal medulla was found to be 37000. This value was similar to those of aldose reductase from various sources.[17-25] The pI of aldose reductase by chromatofocusing was 5.4, and then was quite similar to that of rat testis aldose reductase (pI 5.3).[18] The primary sequence data for the rat lens aldose reductase by complementary deoxyribonucleic acid (cDNA) analysis have been reported by Carper *et al.*,[26] and were shown to encode 284 amino acids and to have a molecular weight of 32300. The molecular weight and total amino acid residues of rat renal medulla aldose reductase were estimated to be 37000 and 332 by SDS-polyacrylamide gel electrophoresis, respectively. Because the molecular weights and the total amino acid residues of rat lens and renal medulla aldose reductases were greatly different, comparisons of amino acid composition are difficult. The mole percent of amino acid for two enzymes showed an appreciably resemblance to each other. The mole percent of amino acid residues of rat renal medulla aldose reductase also closely resembled pig muscle aldose reductase,[27] but was unlike human erythrocyte enzyme.[28] The biggest difference between rat renal medulla and pig muscle aldose reductases was found in glycine.

A clear distinction between rat renal medulla aldose reductase and aldehyde reductase was shown on the substrate specificities. Aldose reductase had a reasonable activity with D-galactose and D-glucose. The K_m values for these substrates of rat renal medulla aldose reductase were within the same range as those of the enzymes isolated from testis,[18] placenta,[22] brain,[29] erythrocyte,[25] muscle[21,27] and lens.[23,30] The K_m values for aldo-sugars of rat renal medulla aldose reductase were much higher than those for DL-glyceraldehyde. However, aldose reductase catalyzes the aldehyde forms of aldo-sugars as pointed out by Inagaki *et al.*[31] Using the amount of the aldehyde form in aldo-sugar described by Canter *et al.*,[32] the K_m and k_{cat}/K_m values for aldehyde forms in aldo-sugars were obtained to be 0.024 mM and 42800 for D-xylose, 0.050 mM and 16400 for glucose and 0.062 mM and 10900 for D-galactose. The corrected K_m and k_{cat}/K_m values for the aldehyde forms of D-xylose, D-glucose and D-galactose were quite similar to those of DL-glyceraldehyde.

Both NADPH and NADH as coenzyme were utilized by the rat renal medulla aldose reductase, and this fact

is common to many aldose reductases.[18,23,24,27,28] Sulfate ions elevated greatly the rat renal medulla aldose reductase activity. Activation of rat renal medulla aldose reductase by sulfate ions was also similar to aldose reductase from testis,[18] brain,[33] placenta,[22] erythrocyte[25,28] and lens.[23-25] Aldose reductase from the medulla of rat kidney was enormously inhibited by the aldose reductase inhibitors, and the susceptibility to inhibition of the aldose reductase was almost the same as that of rat testis and lens aldose reductases.[10,18] However, rat renal medulla aldose reductase was inhibited more strongly than human erythrocyte aldose reductase by aldose reductase inhibitors. Especially, IC_{50} values of aldose reductase from rat renal medulla were 27 times lower than that from human erythrocytes for M79175 and ponalrestat.[25] The enzymatic properties of rat renal medulla aldehyde reductase were the quite similar to those of the rat kidney and testis aldehyde reductases as reported by Sato *et al.*[10] and Kawasaki *et al.*[18]

We demonstrated that three peaks of enzyme activity were separated by chromatofocusing as shown in Fig. 1. Peaks 2 and 3 were identified as aldehyde reductase and aldose reductase, respectively. The enzymatic properties of the minor peak 1 were the same as those of peak 2 with respect to substrate specificities, cofactor specificities, salt effect and the effect of inhibitors. The peak 1 enzyme is to be investigated further.

References

1) T. G. Flynn, *Biochem. Pharmacol.*, **31**, 2705 (1982).
2) S. K. Srivastava, N. H. Ansari, G. A. Hair and B. Das, *Biochim. Biophys. Acta*, **800**, 220 (1984).
3) H. G. Hers, *Biochim. Biophys. Acta*, **22**, 202 (1956).
4) D. Dvornik, "Aldose Reductase Inhibition," ed. by D. Porte, Biomedical Information Corporation, 1987, pp. 73—88, 326—349.
5) A. Beyer-Mears, L. Ku and M. P. Cohen, *Diabetes*, **33**, 604 (1984).
6) A. Beyer-Mears, *Metabolism*, **35**, 46 (1986).
7) S. M. Bagnasco, S. Uchida, R. S. Balaban and P. F. Kador and M. B. Burg, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 1718 (1987).
8) C. E. Grimshaw, M. Shahbaz, G. Jahangiri, C. G. Putney, S. R. Mckercher and E. J. Mathur, *Biochemistry*, **28**, 5343 (1989).
9) H. Terubayashi, S. Sato, P. F. Kader and J. H. Kinoshita, "Polyol Pathway and Its Role in Diabetic Complications," ed. by N. Sakamoto, J. H. Kinoshita, P. F. Kador and N. Hotta, Excerpta Medica, Amsterdam-New York-Oxford, 1988, pp. 330—341.
10) S. Sato, P. F. Kador and J. H. Kinoshita, "Polyol Pathway and Its Role in Diabetic Complications," ed. by N. Sakamoto, J. H. Kinoshita, P. F. Kador and N. Hotta, Excerpta Medica, Amsterdam-New York-Oxford, 1988, pp. 72—81.
11) M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
12) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
13) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
14) R. J. Simpson, M. R. Neuberger and T.-Y. Liu, *J. Biol. Chem.*, **251**, 1936 (1976).
15) S. Moore, *J. Biol. Chem.*, **238**, 235 (1963).
16) W. W. Cleland, *Nature* (London), **198**, 463 (1963).
17) N. Takahashi, T. Saito and K. Tomita, *Biochim. Biophys. Acta*, **748**, 444 (1983).
18) N. Kawasaki, T. Tanimoto and A. Tanaka, *Biochim. Biophys. Acta*, **996**, 30 (1989).
19) T. G. Flynn, J. Shires and D. J. Walton, *J. Biol. Chem.*, **250**, 2933 (1975).
20) W. F. Bosron and R. L. Prairie, *J. Biol. Chem.*, **247**, 4480 (1972).
21) J. A. Cromlish and T. G. Flynn, *J. Biol. Chem.*, **258**, 3416 (1983).
22) R. S. Clements and A. I. Winegrad, *Biochem. Biophys. Res. Commun.*, **47**, 1473 (1972).
23) S. Hayman and J. H. Kinoshita, *J. Biol. Chem.*, **240**, 877 (1965).
24) T. Tanimoto, H. Fukuda and J. Kawamura, *Chem. Pharm. Bull.*, **31** 2395 (1983).
25) T. Nakayama, T. Tanimoto and P. F. Kador, *Prog. Clin. Biol. Res.*, **290**, 265 (1989).
26) D. Carper, C. Nishimura, T. Shinohara, B. Dietzchold, G. Wistow, C. Craft, P. Kador and J. H. Kinoshita, *FEBS Lett.*, **220**, 209 (1987).
27) J. A. Cromlish and T. G. Flynn, *J. Biol. Chem.*, **258**, 3583 (1983).
28) B. Das and S. K. Srivastava, *Arch. Biochem. Biophys.*, **238**, 670 (1985).
29) M. M. O'Brien and P. J. Schofield, *Biochem. J.*, **187**, 21 (1980).
30) T. Tanimoto, H. Fukuda and J. Kawamura, *Chem. Pharm. Bull.*, **32**, 1025 (1984).
31) K. Inagaki, I. Miwa and J. Okuda, *Arch. Biochem. Biophys.*, **216**, 337 (1982).
32) S. M. Cantor and Q. P. Peniston, *J. Am. Chem. Soc.*, **62**, 2113 (1940).
33) R. A. Boghosian and E. T. McGuinness, *Biochim. Biophys. Acta*, **567**, 278 (1979).