3-Deoxyglucosone Reductase in Dog Adrenal Glands. Identification as Aldose Reductase

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3-Deoxyglucosone is one of the major cytotoxic intermediates in the Maillard reaction. Adrenal glands showed the highest NADPH-linked 3-deoxyglucosone reductase activity of dog tissues. The enzyme was purified to homogeneity from the adrenal glands, and demonstrated to be structurally, functionally and immunologically identical with aldose reductase, which comprises about 6% of the soluble adrenal proteins.

Key words adrenal gland; aldose reductase; 3-deoxyglucosone; glycation; β-keto acid ester

3-Deoxyglucosone has been reported to be a major intermediate in the glucose-mediated cross-linking of proteins under physiological conditions.[1] 3-Deoxyglucosone has been detected in human serum and levels are elevated in patients with diabetic nephropathy.[2] In addition, 3-deoxyglucosone has been shown to be rapidly metabolized to a less reactive, reduced product, 3-deoxyfructose, in rat[3] and man.[4]

3-Deoxyglucosone reductase (3DGR) has been purified from the liver of chickens[5] and several other mammals,[6] and the major forms of the enzyme in rat, pig and human liver have been identified as aldehyde reductase (ALR, EC 1.1.1.2).[6b,c] In addition to ALR, dimeric dihydrodiol dehydrogenase (DD, EC 1.3.1.20) and aldose reductase (AR, EC 1.1.1.21) act as major and minor forms, respectively, of 3DGR in monkey kidney.[7] Dimeric DD is also a major form of 3DGR in dog liver.[8] These findings suggest that several distinct enzymes act in the detoxication of the reactive dicarbonyl compound depending on the species and/or tissues. Therefore, it is important from the point of view of the regulation of glycation to elucidate the distribution and multiplicity of 3DGR in mammalian tissues. In this study, we examined the tissue distribution of 3DGR in the dog and found that the adrenal glands exhibited significantly high 3DGR activity. This paper describes the purification of the adrenal enzyme and its identity with AR.

MATERIALS AND METHODS

Materials Acetoacetyl CoA was obtained from Sigma Chemical Co., 3-hydroxybutyric acid esters were from Aldrich Chemicals, and β-keto acid esters were from Tokyo Kasei Organic Chemicals. Rabbit antibody against dog kidney AR was kindly donated by Dr. M. Ohta (National Institutes of Hygienic Sciences, Japan). Other chemicals used in this study are as specified elsewhere.[7,8]

Enzyme Assay 3DGR activity was determined by measuring the oxidation rate of NADPH at 340 nm, in a standard assay mixture (2.0 ml) which contained 0.1 M potassium phosphate buffer, pH 7.0, 2 mM 3-deoxyglucosone, 0.1 mM NADPH and enzyme. AR activity was assayed with 0.2 M D-glucose as the substrate in 0.1 M potassium phosphate buffer, pH 6.5. One unit of enzyme activity was defined as the amount catalyzing the oxidation of 1 μmol NADPH per min at 25 °C.

Preparation of Tissue Extract The adrenal glands and other tissues of male beagle dogs were homogenized as described previously,[8] and the 105000 × *g* supernatants were analyzed for protein[9] and enzyme activity. The activity is expressed as the average (munits/mg of protein) of determinations on tissues from two dogs.

Enzyme Purification 3DGR was purified from the 105000 × *g* supernatant of dog adrenal glands (10 g) by using a procedure reported for the purification of AR from monkey kidney[7] with minor modification. The enzyme preparation, obtained by the final purification step, HA-Ultrogel chromatography, was further purified by chromatofocusing. The enzyme solution was applied to a PBE 94 (Pharmacia) column (0.7 × 15 cm) that had been equilibrated with 25 mM histidine–HCl, pH 6.2, and was eluted at a flow rate of 10 ml/h with 140 ml of diluted Polybuffer 74 (Pharmacia) (1 : 8), pH 4.0. The enzyme active fractions were concentrated by ultrafiltration through an Amicon YM-10 membrane, and the concentrate was dialyzed against 10 mM Tris–HCl, pH 7.5, containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol, and stored at 4 °C.

Other Methods The methods for amino acid sequence determination, including reductive pyridylethylation of the enzyme, digestion with lysylendopeptidase, isolation of peptides by HPLC and Edman degradation using a 473A Protein Sequencer (Applied Biosystems Japan) were carried out as described previously.[7] The molecular mass of the enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[10] standardized using molecular mass markers. Western blot analysis was performed using peroxidase-conjugated goat anti-(rabbit IgG) IgG (Bio-Rad) as the secondary antibody.[11]

RESULTS

Tissue Distribution 3DGR activity was detected in all the dog tissues tested. Adrenal glands howed the highest specific activity of 88 munits/mg, and the values for kidney, liver, small intestine, testis, stomach, muscle, heart, spleen and lung were 31, 26, 25, 9.7, 8.7, 6.3, 5.3, 4.4, 2.9 and

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Table 1. Co-purification of 3DGR and AR from Dog Adrenal Glands

Step	Protein (mg)	(A) 3DGR activity			(B) Total AR activity (units)	Ratio of A/B
		Total (units)	Specific activity (units/mg)	Yield (%)		
Crude extract	315	27.6	0.088	100	10.7	2.6
Ammonium sulfate	177	24.7	0.14	89	9.39	2.6
Sephadex G-100	44.3	22.0	0.497	80	8.60	2.6
Q-Sepharose	19.3	20.5	1.06	76	7.75	2.6
Matrex Red A	15.0	20.1	1.34	73	7.44	2.6
HA-Ultrogel	10.8	15.6	1.44	56	6.20	2.5
Chromatofocusing	7.4	10.8	1.45	39	4.25	2.5

2.7 munits/mg, respectively. The value for dog adrenal glands is higher than those of pig, rat and human livers,[6] and comparable with that of monkey kidney, in which ALR and dimeric DD are two major forms of 3DGR.[7] No significant inhibition of 3DGR activity in dog adrenal extract was observed by the addition of 1 mM isoascorbic acid, an inhibitor specific to dimeric DD,[7,8] and 1 mM diphenic acid, an inhibitor specific to ALR,[12a] but more than 83% of the activity was inhibited by 10 μM AR inhibitors such as sorbinil and tolrestat.

Purification and Properties of Adrenal 3DGR The enzyme was co-purified with AR from dog adrenal glands to investigate the identity of the two enzymes. The results are summarized in Table 1. The activities of 3DGR and AR were co-eluted in a single peak in the chromatography, and the activity ratios of the two enzymes were constant at each purification step.

The 3DGR and AR activities were co-eluted as a single peak at pH 5.7 in the chromatofocusing purification step, and the final preparation of the enzyme showed a single protein band (M_r 36000) on SDS-PAGE. The isoelectric point and molecular mass of the purified enzyme are similar to those of ARs from other tissues of mammals including dog.[12] Western immunoblot analysis using the antibody against dog kidney AR showed a single immunopositive band against the purified adrenal enzyme. Furthermore, the sequences of ten peptides from the adrenal enzyme were similar to regions in the amino acid sequence deduced from cDNA for human placental AR (Table 2).[13]

The 3DGR activity of the adrenal enzyme was increased about 2-fold by the addition of 0.2 M ammonium sulfate, a known activator of AR,[12] and inhibited by AR inhibitors, such as tolrestat ($IC_{50}=30$ nM), AL1756 ($IC_{50}=70$ nM) and sorbinil ($IC_{50}=90$ nM). The enzyme reduced representative substrates for AR (Table 3), and the kinetic constants for the substrates are also similar to those of dog kidney AR.[12b] In addition, the enzyme reduced acetoacetic acid esters, including acetoacetyl CoA, and slowly oxidized (*S*)-isomers of 3-hydroxybutyric acid esters.

Table 2. Sequences of Ten Peptides Derived from Dog Adrenal 3DGR and Their Alignment with the Amino Acid Sequence Deduced from cDNA for Human AR

Peptide sequence[a]	Amino acid residue position[b]	Sequence identity (%)
MPILGLGTWK	13—22	100
VAIDLGYRHIDCAHVYQNENEVGLAIQEK	34—62	93
LWCTYHEK	79—86	100
LDYLDLYLIHWPTGFK	102—117	100
EYFPLDGEGNVIPSDTSFVDTXXAMEQLVDXGLVK	121—155	71
YXPAVNQIECHPYLTQEK	178—195	94
QYCQAK	198—203	83
GIVVTAYXPLGSPDRPXAK	204—222	83
TTAQVLIRFPM	244—254	100
DYPFTEEF	309—316	88

a) An unidentifiable phenylthiohydantoin-amino acid is expressed as X. *b*) The value represents the region of the human AR sequence which could be aligned with each peptide sequence of dog adrenal 3DGR.

Table 3. Substrate Specificity of Adrenal 3DGR

Substrate	K_m (mM)	V_{max} (units/mg)
Reductase activity[a]		
3-Deoxyglucosone	0.070	1.48
Pyridine-3-aldehyde	0.003	1.69
DL-Glyceraldehyde	0.014	1.37
D-Glucuronate	4.2	0.69
D-Glucose	119	0.88
Methyl acetoacetate	1.2	0.59
Ethyl acetoacetate	0.36	0.94
n-Butyl acetoacetate	0.12	1.39
n-Hexyl acetoacetate	0.068	1.68
n-Octyl acetoacetate	0.014	1.24
Acetoacetyl CoA	0.12	0.65
Ethyl 3-keto-*n*-valerate	1.3	0.87
Dehydrogenase activity[a]		
(*S*)-Methyl 3-hydroxybutyrate[b]	95	0.04
(*S*)-Ethyl 3-hydroxybutyrate[b]	49	0.50

a) The reductase and dehydrogenase activities were assayed at pH 7.0 with 0.1 mM NADPH and 0.5 mM $NADP^+$, respectively, as coenzymes. *b*) The (*R*)-isomers of the substrates were not oxidized.

DISCUSSION

The tissue distribution of 3DGR in dogs has revealed that the enzyme is present in virtually all tissues, as previously reported in monkeys.[7] However, the distribution is different between the two species. In monkeys, kidneys show the highest enzyme activity which results from the presence of high amounts of ALR and dimeric

DD, and AR is a minor form of kidney 3DGR. In dogs, adrenal glands exhibited greater activity than other tissues, and the purification and characterization of adrenal 3DGR have demonstrated that the high 3DGR activity in this tissue is due to AR, but not to ALR and/or dimeric DD. Since the K_m value of dog AR for 3-deoxyglucosone is similar to that of monkey kidney AR[7] and is lower than those of ALR and dimeric DDs from mammalian liver and kidney,[6-8] AR may act as an important scavenger of 3-deoxyglucosone not only in dog adrenal gland but also other tissues where AR is expressed more highly than ALR and dimeric DD.

AR has been shown to be localized in the cortex of dog adrenal glands.[14] The present purification data indicate that the content of AR in this tissue is about 6% of the soluble proteins. A similarly high level of AR has also been reported in an adrenal gland from a patient with adrenocarcinoma.[15] Of the postulated physiological substrates for AR, isocorticosteroids formed from aldol intermediates in a metabolic pathway of corticosteroids have been shown to be the best substrates.[12,16] AR in the cortex of dog adrenal glands might be involved in the metabolism of isocorticosteroids rather than in preventing glycation, although further study is necessary to understand the precise physiological role of the enzyme highly expressed in this tissue.

The present specificity of dog AR for acetoacetic acid esters and hydroxybutyric acid esters indicates that the enzyme reduces the carbonyl group at the C-3 on the acetoacetyl group to a hydroxy group with an (*S*)-configuration. Although the physiological significance of this is unclear, the broad substrate specificity of AR, which has not been recognized previously, suggests its functional relationship to the 20α-hydroxysteroid dehydrogenase[17] and L-stereospecific carbonyl reductase[18] of microorganisms, which are also structually related to AR.

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