Aldehyde reductase is a major protein associated with 3-deoxyglucosone reductase activity in rat, pig and human livers

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3-Deoxyglucosone reductase activity in the extracts of rat, pig and human livers was potently inhibited by aldehyde reductase inhibitors. The major species of 3-deoxyglucosone reductase purified from human and pig livers were biochemically and immunochemically identical with aldehyde reductase. The two enzymes and rat liver aldehyde reductase exhibited higher catalytic efficiency for 3-deoxyglucosone than for D-glucuronate, a representative substrate of aldehyde reductase.

INTRODUCTION

Glycation is the process whereby sugar molecules form a covalent adduct with protein amino groups via the Maillard reaction. This reaction causes complex changes of proteins, involving cross-linking [1–3]. The cross-linking of proteins by the Maillard reaction has been studied in connection with the aetiology of diabetic complications and aging [4,5]. 3-Deoxyglucosone (3DG) has been shown to be an active intermediate in the glucose- and fructose-mediated cross-linking of proteins [5–8]. Recently Kato *et al.* [9] have reported that 3DG administered in rats is rapidly metabolized to 3-deoxyfructose, and suggested that the 3DG-reducing enzyme, at least in part, prevents the protein modification by the Maillard reaction. More recently, NADPH-dependent 3DG reductases have been purified from chicken liver [10] and pig liver [11], and have been tentatively designated as 2-oxo aldehyde reductase because of its high reactivity to 2-oxo aldehydes such as methylglyoxal and phenylglyoxal. Although the pig liver enzyme has been reported to be distinct from aldehyde reductase [11], the physicochemical and catalytic properties of the chicken and pig liver enzymes are similar to those of aldehyde reductases of mammalian tissues, which show broad substrate-specificity for carbonyl compounds, including 2-oxo aldehydes [12–16].

In order to investigate a possible relationship between 3DG reductase and aldehyde reductase (ALR; EC 1.1.1.2) in mammalian tissues, we co-purified the two enzyme activities from human and pig livers and their biochemical and immunochemical properties were compared. The results indicate that the major active species of 3DG reductase activity in these tissues is identical with ALR, although dimeric dihydrodiol dehydrogenase (EC 1.3.1.20) [17] also exhibited low 3DG reductase activity in pig liver.

EXPERIMENTAL

Materials

Authentic 3DG was kindly supplied by Dr. H. Kato (Tokyo University, Tokyo, Japan). 3DG was also synthesized according to the method of Kato *et al.* [9], and its concentrations were determined as described previously [10]. DL-Lactaldehyde was prepared from DL-threonine by the method of Zagalak *et al.* [18]. ALRs from pig kidney [16], rat liver [14] and human liver [15]

and dimeric dihydrodiol dehydrogenases from pig liver and lens [17,19] were purified to homogeneity, and antibodies against these enzymes were raised as described previously [20].

Enzyme assay

The reductase activity was determined spectrophotometrically by measuring the oxidation rate of NADPH at 340 nm. The reaction mixture for 3DG reductase activity contained 50 mM-sodium phosphate, pH 6.5, 2 mM-3DG, 0.1 mM-NADPH and enzyme, in a total volume of 2.0 ml. For the assay of ALR, 10 mM-D-glucuronate was used as the substrate. Dihydrodiol dehydrogenase activity was determined as described previously [19]. One unit of enzyme activity was defined as the amount of enzyme catalysing the oxidation or production of 1 μ mol of NADPH/min at 25 °C. Protein concentration was determined by the method of Bradford [21], with BSA as the standard.

Preparation of tissue extract and enzyme purification

The 105000 *g* supernatants of the liver homogenates of humans, pigs and male Sprague–Dawley rats were prepared as described previously [17]. 3DG reductases from pig liver (100 g) and human liver (170 g) were purified by using a procedure for the purification of ALR from pig kidney [16]. This method consisted of the homogenization of the tissue, followed by $(\text{NH}_4)_2\text{SO}_4$ fractionation (35–70% saturation) of the 105000 *g* supernatant, gel filtration on Sephadex G-100, chromatography on Q-Sepharose, affinity chromatography on Matrex Red A and hydroxyapatite (HA-Ultrogel; LKB Produkter) column chromatography. At the final step of the hydroxyapatite column, the enzymes did not adsorb on the column and were eluted out during washing of the column with the equilibration buffer, 10 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-2-mercaptoethanol (buffer A).

Product identification

The products of the enzymic methylglyoxal reduction were analysed by a gas-chromatographic procedure as described by Peinado *et al.* [22]. The retention times of methylglyoxal, acetol and DL-lactaldehyde were 1.0, 5.2 and 7.5 min respectively. The reduction products of 3DG were detected by t.l.c. on silica-gel plates in a solvent system of chloroform/methanol/water (70:30:3, by vol.) as described previously [11].

Abbreviations used: 3DG, 3-deoxyglucosone; ALR, aldehyde reductase.

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Other methods

SDS/PAGE on a 12.5% slab gel [23] and isoelectric focusing on a 7.5% polyacrylamide disc gel [24] were performed as described previously. 3DG reductase and ALR activities in the gels were stained with 2 mM-3DG and 10 mM-D-glucuronate as the respective substrates [25], and protein in the gel was stained with Coomassie Brilliant Blue G-250. The M_r of the native enzyme was estimated by gel filtration on a Sephadex G-100 column in buffer A containing 0.15 M-KCl, and that of the denatured enzyme by SDS/PAGE standardized by the use of M_r markers. Immunodiffusion was carried out as described previously [20].

RESULTS AND DISCUSSION

Since ALR is known to be inhibited by barbital and diphenic acid [12–16], we examined the effects of these inhibitors on the activities of 3DG reductase and ALR in the 105 000 g supernatants of rat, human and pig livers (Table 1). The two reductase activities in the extracts of rat and human livers were considerably and similarly inhibited by the ALR inhibitors, whereas the inhibition of 3DG reductase activity in the pig liver extract was less than that of ALR activity. Pyrazole, an alcohol dehydrogenase inhibitor, did not affect the enzyme activities, but ascorbic acid, which inhibits dimeric dihydrodiol dehydrogenase in pig tissues [26], gave a small inhibition only of 3DG reductase activity of the pig liver extract. The result suggests that the predominant 3DG reductase activity in mammalian liver is identical with or related to ALR, and that in pig liver some 3DG reductase activity is due to the presence of dimeric dihydrodiol dehydrogenase, which can reduce several aldehydes [17].

Table 1. Effects of ALR inhibitors on the activities of 3DG reductase (DR) and ALR in the 105 000 g supernatants of rat, human and pig livers

The values are means $\pm$ S.D. from determinations with four animal and human livers. The concentrations of the inhibitors are 1 mM.

	Rat liver		Human liver		Pig liver	
	DR	ALR	DR	ALR	DR	ALR
Specific activity (munits/mg)	25 $\pm$ 5	22 $\pm$ 5	11 $\pm$ 7	17 $\pm$ 6	31 $\pm$ 10	46 $\pm$ 9
Inhibition (%) by:						
Barbital	82 $\pm$ 9	94 $\pm$ 2	87 $\pm$ 4	90 $\pm$ 8	65 $\pm$ 4	93 $\pm$ 5
Diphenic acid	78 $\pm$ 6	88 $\pm$ 7	86 $\pm$ 4	92 $\pm$ 5	64 $\pm$ 1	94 $\pm$ 4
Pyrazole	4 $\pm$ 3	5 $\pm$ 4	2 $\pm$ 2	5 $\pm$ 2	1 $\pm$ 1	2 $\pm$ 1
Ascorbic acid	5 $\pm$ 2	5 $\pm$ 4	5 $\pm$ 2	4 $\pm$ 2	15 $\pm$ 1	5 $\pm$ 4

To explore this possibility, purification of 3DG reductase from human and pig livers by a scheme based on the method described for the purification of ALR from pig kidney [16] was carried out. 3DG reductase and ALR activities of human liver co-migrated in the four column chromatographies employed (Table 2). At the first column chromatography on Sephadex G-100, 3DG reductase activity of pig liver was resolved into one minor peak containing 8 activity units and one major peak containing 57 activity units, of which the minor peak was co-eluted with dihydrodiol dehydrogenase activity at M_r 65000 and the major peak with ALR activity at M_r 34000. The two enzyme peaks were separately purified, and the results of the purification of the pig liver major enzyme form are summarized in Table 2. The data indicated that the activity ratios between 3DG reductase and ALR were essentially constant at all purification steps. The ALR specific activities of the final enzyme preparations of human and pig livers were 14.2 units/mg and 16.0 units/mg respectively, which are comparable with the values of ALRs purified from mammalian tissues [12–16]. The minor pig liver 3DG reductase was purified about 26-fold from the Sephadex G-100 fraction with 30% recovery of activity by the procedure for the purification of dimeric dihydrodiol dehydrogenase from pig tissues [17]. The specific activity of the purified enzyme was 0.4 unit/mg, and this enzyme preparation possessed a high dihydrodiol dehydrogenase activity of 10.1 units/mg.

The human liver 3DG reductase and the major pig liver enzyme showed single protein bands at pH 5.2 and 5.8 respectively on polyacrylamide gel focusing, where the respective protein bands were coincident with the enzyme activities with 3DG and D-glucuronate as substrates. The M_r values of the human liver enzyme determined by SDS/PAGE and Sephadex G-100 filtration were 38000 and 33000 respectively, and the respective values of the pig liver enzyme were 38000 and 34000. The pI and M_r values of the two enzymes are comparable with those of ALRs from human tissues [12,15] and pig liver [13]. In the immunodiffusion test the human and pig liver enzymes reacted with the antibodies against human liver and pig kidney ALRs respectively (Fig. 1). In addition, the homogeneous ALRs of human liver and pig kidney, which were separately purified by the previous methods [15,16], actively reduced 3DG (Table 3). Thus these results indicate that human liver 3DG reductase and the major form of the pig liver enzyme are identical with ALR. In addition, the homogeneous rat liver ALR showed high 3DG reductase activity (Table 3). This, together with the high susceptibility of 3DG reductase activity in the rat liver extract to the ALR inhibitors, suggests that a major species of 3DG reductase is also ALR in this animal liver.

The minor 3DG reductase of pig liver showed a single protein band with an M_r value of 35000 on SDS/PAGE and had a pI value of 7.6 on polyacrylamide gel focusing. The values are almost the same as those of dimeric dihydrodiol dehydrogenase

Table 2. Co-purification of 3DG reductase (DR) and ALR from human and pig livers

Step	Human liver				Pig liver			
	Protein (mg)	DR (units)	ALR (units)	ALR/DR ratio	Protein (mg)	DR (units)	ALR (units)	ALR/DR ratio
Crude extract	10830	94	150	1.6	11250	189	243	1.3
(NH ₄) ₂ SO ₄ fraction	6390	85	134	1.6	5590	117	148	1.3
Sephadex G-100 fraction	1090	81	109	1.3	700	57	95	1.7
Q-Sepharose fraction	50	77	93	1.2	29	54	92	1.7
Matrex Red A fraction	12	66	85	1.3	12	53	85	1.8
Hydroxyapatite fraction	5.0	54	71	1.3	4.5	43	72	1.7

Fig. 1. Immunodiffusion of 3DG reductases from human and pig livers to antibodies against ALR and dimeric dihydrodiol dehydrogenase

Upper wells contained antibodies against human liver ALR (A), pig kidney ALR (B) and pig lens dimeric dihydrodiol dehydrogenase (C). Lower wells contained the purified 3DG reductase preparations: a, the human liver enzyme; b, the major enzyme of pig liver; c, the minor enzyme of pig liver.

from pig tissues [17,19]. The enzyme reacted with the antibodies against dimeric dihydrodiol dehydrogenase of pig lens (Fig. 1). Therefore the minor 3DG reductase of pig liver appears most probably to be dimeric dihydrodiol dehydrogenase. Indeed, the homogeneous dimeric dihydrodiol dehydrogenase of pig lens also exhibited a 3DG reductase activity of 0.36 unit/mg.

The 3DG reductase activities of ALRs purified from human and pig livers were optimal around pH 6.5, which is similar to pH-dependencies of mammalian ALRs with other substrates [12–16], and the two enzymes exhibited broad substrate-specificity for various aromatic and aliphatic aldehydes, including methylglyoxal and phenylglyoxal, as reported with ALRs of mammalian tissues [12–16]. The apparent K_m values of the human and pig liver enzymes for 3DG were lower than those for D-glucuronate, the representative substrate of ALR, and the catalytic efficiency (k_{cat}/K_m) for 3DG was superior to that for D-glucuronate (Table 3). A high reactivity towards 3DG was also observed with rat liver and pig kidney ALRs. On the other hand, dimeric dihydrodiol dehydrogenase, the minor form of 3DG reductase in pig liver, showed a pH optimum around 7.5 with 3DG as a substrate, and the kinetic constants are listed in Table 3. Although the reactivity of the enzyme towards 3DG was lower than that of ALR, this enzyme may be responsible for the reductive metabolism of 3DG in pig tissues such as brain and heart, where its amount is higher than that of ALR [17].

We have here presented evidence that the major species of 3DG reductase in both human and pig livers is ALR. This conclusion is in contrast with a previous study indicating that there is a '2-oxo aldehyde reductase' distinct from 'ALR' in pig liver [11]. However, by many criteria, such as M_r , pH optimum, substrate-specificity and kinetic constants in the reduction of 3DG, the present ALR and the '2-oxo aldehyde reductase' of pig liver are indistinguishable. As shown in this purification, ALRs from mammalian tissues do not adsorb on the hydroxyapatite column but are retained on anion-exchange resins because of their acidic nature [12–16]. In the previous purification of '2-oxo aldehyde reductase' and 'ALR' [11] the behaviour of the '2-oxo aldehyde reductase' on the two columns is similar to that of ALRs of mammalian tissues [12–16], but the 'ALR' was reported to adsorb on the hydroxyapatite column and not to reduce 3DG. Therefore the '2-oxo aldehyde reductase' and 'ALR' of ref. [11] are in fact respectively ALR and some other, uncharacterized, enzyme.

The reduced product of methylglyoxal by the present ALRs of human and pig livers was identified as acetol, not lactaldehyde, by gas chromatography. This property was also shown by the pig liver '2-oxo aldehyde reductase' purified previously [11]. When the reaction products of 3DG reduction were analysed by t.l.c., ALRs of human and pig livers formed only one product with an R_f value of 0.27, which is lower than that of 3DG (R_f 0.36) and higher than those of glucose (R_f 0.12) and fructose (R_f 0.16). The relative R_f value of the product to those of the reference sugars is similar to that of 3-deoxyfructose, which is identified as the reduced product of 3DG with pig liver '2-oxo aldehyde reductase' [11]. Although we have not chemically characterized the reduced product, the identification of the '2-oxo aldehyde reductase' as ALR suggests that the reduced product by the present ALRs is 3-deoxyfructose.

ALR is distributed in various tissues of animals [27,28] and man [29]. The enzyme has been suggested to be involved in the catabolism of *myo*-inositol [30], ascorbic acid biosynthesis [27], corticosteroid metabolism [31] and the formation of prostaglandin $F_{2\alpha}$ [32]. However, these roles do not explain the near ubiquitous distribution of ALR, and it has been considered that the enzyme must play a more general role, although direct evidence is lacking [29,33]. The present finding of a high reactivity of the enzyme towards 3DG suggests that its general role is to scavenge toxic endogenous 2-oxo aldehydes. It has been reported that pig liver '2-oxo aldehyde reductase', which was here demonstrated to be ALR, prevents propagation of the Maillard reaction *in vitro* [11]. Therefore ALR may play an important role

Table 3. Kinetic constants for 3DG reduction by ALRs and dihydrodiol dehydrogenase

The activity was assayed with 0.1 mM-NADPH as a cofactor and 0.2–2.0 mM-3DG or 1.0–10 mM-D-glucuronate, and the kinetic constants were determined from Lineweaver–Burk plots, which were linear within the substrate ranges. The k_{cat} values were calculated by using an M_r value of 38 000 for ALRs of human liver, pig liver and pig kidney and an M_r value of 39 000 for rat liver ALR and pig liver dihydrodiol dehydrogenase.

Enzyme	Substrate whose concentration was varied	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)
Human liver ALR	3DG	1.4	710	510
	D-Glucuronate	5.0	810	160
Pig liver ALR	3DG	1.8	670	370
	D-Glucuronate	4.9	890	180
Pig kidney ALR	3DG	1.7	610	360
Rat liver ALR	3DG	2.0	440	220
Pig liver dihydrodiol dehydrogenase	3DG	1.0	37	37

in protecting the tissues against possible cellular damage via the Maillard reaction [5–8].

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Received 17 July 1991/19 August 1991; accepted 28 August 1991