Guinea-pig liver testosterone 17 β -dehydrogenase (NADP⁺) and aldehyde reductase exhibit benzene dihydrodiol dehydrogenase activity

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We have kinetically and immunologically demonstrated that testosterone 17 β -dehydrogenase (NADP⁺) isoenzymes (EC 1.1.1.64) and aldehyde reductase (EC 1.1.1.2) from guinea-pig liver catalyse the oxidation of benzene dihydrodiol (*trans*-1,2-dihydroxycyclohexa-3,5-diene) to catechol. One isoenzyme of testosterone 17 β -dehydrogenase, which has specificity for 5 β -androstanes, oxidized benzene dihydrodiol at a 3-fold higher rate than 5 β -dihydrotestosterone, and showed a more than 4-fold higher affinity for benzene dihydrodiol and $V_{\max}$ value than did another isoenzyme, which exhibits specificity for 5 α -androstanes, and aldehyde reductase. Immunoprecipitation of guinea-pig liver cytosol with antisera against the testosterone 17 β -dehydrogenase isoenzymes and aldehyde reductase indicated that most of the benzene dihydrodiol dehydrogenase activity in the tissue is due to testosterone 17 β -dehydrogenase.

A soluble NADP⁺-dependent dehydrogenase that converts benzene dihydrodiol (*trans*-1,2-dihydroxycyclohexa-3,5-diene) into catechol (Ayengar *et al.*, 1959) has been shown to decrease the mutagenicity of carcinogens such as benzo[*a*]pyrene (Glatt *et al.*, 1979) and benz[*a*]anthracene (Glatt *et al.*, 1981) when tested in the Ames assay. Furthermore, the mutagenic diol epoxide *t*-(10,11-epoxy)-8,9,10,11-tetrahydro-*r*-8,*t*-9-dihydroxybenz[*a*]anthracene is inactivated by the dehydrogenase but not by epoxide hydrolase (Glatt *et al.*, 1982). These findings have led to the hypothesis that the enzyme plays a role in the metabolic inactivation of diol epoxides derived from polycyclic hydrocarbons. The dehydrogenase (with benzene dihydrodiol as substrate) has been purified from liver cytosol of rat (Vogel *et al.*, 1980) and mouse (Bolcsak & Nerland, 1983). The rat liver enzyme is a monomer of M_r 35000 that catalyses not only the oxidation of acenaphthenol and 3 α -hydroxysteroids but also the reduction of 3-oxosteroids and aromatic aldehydes (Vogel *et al.*, 1980, 1982). On the other hand, the mouse liver enzyme shows four multiple forms, with different M_r values and charges, three of which oxidize 17 β -hydroxysteroids and cyclohex-2-en-1-ol (Bolcsak & Nerland, 1983).

NADPH-dependent aldehyde reductase exists in a number of forms in mammalian liver (Sawada *et al.*, 1979a; von Wartburg & Wermuth, 1980; Ikeda *et al.*, 1981; Flynn, 1982; Vogel *et al.*, 1982). Several forms of the enzyme from rat (Ikeda *et al.*, 1981) and guinea pig (Sawada *et al.*, 1979a,b) resemble the benzene dihydrodiol dehydrogenases from rat (Vogel *et al.*, 1980) and mouse (Bolcsak & Nerland, 1983) respectively with respect to their M_r values and ability to oxidize 3- and 17-hydroxysteroids. Subsequently the two forms of guinea-pig liver aldehyde reductase were identified with testosterone 17 β -dehydrogenase isoenzymes (Sawada *et al.*, 1979b, 1984). The present paper shows that both testosterone 17 β -dehydrogenase (EC 1.1.1.64) isoenzymes and aldehyde reductase (EC 1.1.1.2) purified from guinea-pig liver cytosol can catalyse the dehydrogenation of benzene dihydrodiol.

Experimental

Materials

Benzene dihydrodiol was synthesized by the method of Platt & Oesch (1977). Reduced and oxidized cofactors were obtained from Oriental Yeast Co. (Tokyo, Japan), and steroids and standard proteins from Sigma Chemical Co. Liver was excised from adult male Hartley guinea pigs.

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The two isoenzymes of testosterone 17 β -dehydrogenase, with pI values of 9.1 and 8.0 (Sawada *et al.*, 1979b), which have been here designated TD1 and TD2 respectively, and aldehyde reductase (Sawada *et al.*, 1979a) were purified from liver by following the procedures described previously.

Enzyme assay

Aldehyde reductase activity was assayed spectrophotometrically with 5mM-D-glucuronate or -pyridine-4-aldehyde as substrate at 25°C (Sawada *et al.*, 1979a). Dehydrogenase activity towards benzene dihydrodiol, 17 β -hydroxysteroids and other alcohols was determined by monitoring NADPH fluorescence at 455nm (excitation at 340nm). The reaction mixture contained, in a total volume of 2.0ml, 50mM-glycine/NaOH buffer, pH9.7, 1.0mM-NADP⁺ and various concentrations of alcohols and enzyme. The reactions were started by the addition of enzyme. One unit of activity was defined as the oxidation or production of 1 μ mol of NADPH/min at 25°C. For the study of pH-dependent benzene dihydrodiol dehydrogenase activity of the enzymes, 50mM-glycine/NaOH or 50mM-Tris/HCl buffer was used at various pH values.

Product identification

For identifying the products of benzene dihydrodiol oxidation, the enzyme (0.2 unit with 1.8mM-benzene dihydrodiol as a substrate) was incubated at 25°C in the reaction mixture described above. After 1 h incubation, 10ml of ethanol was added to the mixture, which was then centrifuged at 5000g for 10min. The supernatant was concentrated by evaporating the solvent. Samples of the concentrates were applied to silica-gel thin-layer plates (Merck) and chromatographed in the solvent system chloroform/methanol/water (12:4:1, by vol.). The products were located by using I₂ vapour

and their *R_F* values were compared with that of authentic catechol (*R_F* 0.45).

Immunochemical experiments

Antibodies against aldehyde reductase (Sawada *et al.*, 1979a) and isoenzyme TD2 (Sawada *et al.*, 1984) were produced in rabbits. The antisera were tested by Ouchterlony double-diffusion analysis. Immunoprecipitation procedures with the enzymes and antisera were as follows. Constant amounts of the enzyme were incubated at 4°C overnight with an increasing range of amounts of the antiserum in 0.15M-KCl/10mM-potassium phosphate buffer, pH7.2, in a final volume of 0.2ml. After the immune complex formed it was separated by centrifugation at 6000g for 10min at 4°C. The residual activity of the enzyme in the supernatant was determined by assaying 50 μ l samples. The serum from a non-immunized rabbit was used as a control.

Results and discussion

The two isoenzymes of testosterone 17 β -dehydrogenase and aldehyde reductase oxidized benzene dihydrodiol, and the products were identified with catechol on t.l.c. Since isoenzymes TD1 and TD2, although differing in their steroid specificity, reduced pyridine-4-aldehyde, which is also a substrate for aldehyde reductase as described previously (Sawada *et al.*, 1979a,b), the dehydrogenase activities of the enzymes with benzene dihydrodiol and other alcohols were compared with their pyridine-4-aldehyde reductase activities (Table 1). Isoenzyme TD1 exhibited the greatest benzene dihydrodiol dehydrogenase activity, which was 5-fold higher than its aldehyde reductase and 17 β -hydroxysteroid dehydrogenase activities, but the dihydrodiol dehydrogenase activities

Table 1. *Dehydrogenase activity of testosterone 17 β -dehydrogenases and aldehyde reductase towards steroids and xenobiotic alcohols*

The reductase activity with pyridine-4-aldehyde as a substrate was assayed at pH6.0. For experimental details see the text. Abbreviation: N.D., not detected.

Substrate	Concn. (mM)	Activity (units/mg)		
		Isoenzyme TD1	Isoenzyme TD2	Aldehyde reductase
Pyridine-4-aldehyde	5.0	0.56	2.10	32.1
5 α -Dihydrotestosterone	0.05	0.02	3.15	N.D.
5 β -Dihydrotestosterone	0.1	1.63	0.19	N.D.
Benzene dihydrodiol	1.8	3.09	0.34	0.42
1-Acenaphthenol	1.0	3.87	1.01	N.D.
1-Indanol	1.0	2.45	1.72	N.D.
1,2,3,4-Tetrahydro-1-naphthol	1.0	2.21	1.30	N.D.
Cyclohex-2-en-1-ol	1.0	1.93	0.40	N.D.
Glycerol	75.0	N.D.	N.D.	0.32

of isoenzyme TD2 and aldehyde reductase were 6-fold and 76-fold respectively lower than their reductase activities. In addition, the enzymes differed in their reactivity towards other alcohols. Both isoenzyme TD1 and isoenzyme TD2 also oxidized aromatic alcohols, including acenaphth-enol and cyclohex-2-en-1-ol, which are described as substrates for benzene dihydrodiol dehydrogenases from other animal livers (Vogel *et al.*, 1980; Bolcsak & Nerland, 1983), whereas aldehyde reductase was inactive towards the aromatic alcohols but oxidized glycerol at a low rate.

The NADP⁺-dependent benzene dihydrodiol dehydrogenase activities of isoenzymes TD1 and TD2 gave relatively narrow pH optima of 10.8 and 10.2 respectively, which are similar to those of their 17 β -hydroxysteroid dehydrogenase activities (Sawada *et al.*, 1979b). The optimum for aldehyde reductase occurred at about pH 8.6–9.5. The apparent K_m values for benzene dihydrodiol and NADP⁺ determined under respective optimal pH conditions were 0.47 mM and 25 μ M, 6.7 mM and 14 μ M, and 5.0 mM and 18 μ M for isoenzyme TD1, isoenzyme TD2 and aldehyde reductase respectively. The corresponding V_{max} values were found to be 8.5, 2.0 and 0.79 μ mol/min per mg of protein. These kinetic constants of the enzymes are compatible with those reported with hepatic benzene dihydrodiol dehydrogenases from rat (Vogel *et al.*, 1980) and mouse (Bolcsak & Nerland, 1983).

The rat and the mouse dehydrogenases have been shown to utilize NAD⁺ as a cofactor (Vogel *et al.*, 1980; Bolcsak & Nerland, 1983). Isoenzyme TD1 exhibited relatively high NAD⁺-dependent activity, which corresponded to 40% of its NADP⁺-dependent activity at its optimal pH of 10.0, but the NAD⁺-dependent activities of the

other enzymes were less than 5% of their NADP⁺-dependent activities.

The preparation of isoenzyme TD1 was not homogeneous (Sawada *et al.*, 1979b, 1984), although isoenzyme TD2 and aldehyde reductase were obtained in a pure form. Therefore we examined the effects of several carbonyl compounds, which are substrates for the enzymes in their reverse reactions (Sawada *et al.*, 1979a,b), on the benzene dihydrodiol dehydrogenase activity of the enzymes in order to elucidate whether the same protein exhibits dehydrogenase activity towards benzene dihydrodiol and 17 β -hydroxysteroids. Although pyridine-4-aldehyde was a competitive inhibitor of all the enzymes, 5 β -androstane-3,17-dione and D-glucuronate competitively inhibited the benzene dihydrodiol dehydrogenase activity of isoenzymes TD1 and TD2 and of aldehyde reductase respectively (Fig. 1). The results indicate that the benzene dihydrodiol dehydrogenase activity is associated with the testosterone 17 β -dehydrogenase isoenzymes and aldehyde reductase. This conclusion is further supported by the immunoprecipitation experiments with the antisera against isoenzyme TD2 and aldehyde reductase (Table 2). The benzene dihydrodiol dehydrogenase activity of isoenzyme TD2 and aldehyde reductase was immunoprecipitated by the antisera against the respective enzyme antigens, and that of isoenzyme TD1 was completely immunoprecipitated by the anti-(isoenzyme TD2) serum, which reacts with both isoenzyme TD1 and isoenzyme TD2 (Sawada *et al.*, 1984), but not by the anti-(aldehyde reductase) serum.

When a similar immunoprecipitation by the antisera was carried out against guinea-pig liver cytosol, approx. 80% of the benzene dihydrodiol dehydrogenase activity was immunoprecipitated

Table 2. Immunoprecipitation of benzene dihydrodiol dehydrogenase activity of testosterone 17 β -dehydrogenases and aldehyde reductase by their antisera

The enzyme solutions containing approx. 80 munits of benzene dihydrodiol dehydrogenase activity were incubated at 4°C overnight with the amounts of the antisera indicated. The benzene dihydrodiol dehydrogenase and pyridine-4-aldehyde reductase activities in the supernatants after centrifugation of the mixtures were determined, and are given as percentages (means $\pm$ S.D., $n = 3$) relative to those obtained with control serum.

Enzyme	Antiserum	Remaining activity (%)			
		50 μ l of antiserum		100 μ l of antiserum	
		Benzene dihydrodiol dehydrogenase	Pyridine-4-aldehyde reductase	Benzene dihydrodiol dehydrogenase	Pyridine-4-aldehyde reductase
Isoenzyme TD1	Anti-(isoenzyme TD2) serum	2 $\pm$ 1	1 $\pm$ 1	0	0
Isoenzyme TD1	Anti-(aldehyde reductase) serum	99 $\pm$ 1	99 $\pm$ 1	99 $\pm$ 1	98 $\pm$ 2
Isoenzyme TD2	Anti-(isoenzyme TD2) serum	55 $\pm$ 4	54 $\pm$ 6	0	0
Isoenzyme TD2	Anti-(aldehyde reductase) serum	98 $\pm$ 1	99 $\pm$ 1	99 $\pm$ 1	98 $\pm$ 2
Aldehyde reductase	Anti-(isoenzyme TD2) serum	97 $\pm$ 3	98 $\pm$ 1	98 $\pm$ 2	99 $\pm$ 1
Aldehyde reductase	Anti-(aldehyde reductase) serum	70 $\pm$ 4	69 $\pm$ 2	42 $\pm$ 2	35 $\pm$ 4

Fig. 1. Inhibition of benzene dihydrodiol dehydrogenase activity by carbonyl compounds. Velocities (v) are expressed as munits/ml. Carbonyl compounds: $\blacktriangle$, none; $\triangle$, 1 mM-pyridine-4-aldehyde; $\square$, 1 mM-D-glucuronate; $\circ$, 5 μ M-5 β -androstane-3,17-dione. (a) Isoenzyme TD1; (b) isoenzyme TD2; (c) aldehyde reductase.

by anti-(isoenzyme TD2) serum, but anti-(aldehyde reductase) serum decreased the activity by only 5% (Fig. 2). This indicates that the testosterone 17 β -dehydrogenase isoenzymes are the major enzymes capable of oxidizing benzene dihydrodiol, although the incomplete precipitation of the activity by the antisera suggests the existence of another dehydrogenase which is immunologically distinct from the testosterone 17 β -dehydrogenases and aldehyde reductase.

It has been shown that, in guinea-pig liver, there are two types of testosterone 17 β -dehydrogenase isoenzymes, differing in their steroid specificity (Kageura & Toki, 1977; Kobayashi & Kochakian, 1978). The importance of one isoenzyme, TD2, which exhibits high activity for the physiologically active hormones testosterone and 5 α -androstanes, can be accepted because of their regulatory effect on androgen metabolism. In contrast, since the other isoenzyme, TD1, shows a preference for 5 β -

Fig. 2. Immunoprecipitation of benzene dihydrodiol dehydrogenase activity in liver cytosol by the antisera against isoenzyme TD2 and aldehyde reductase

Samples (40 μl) of the liver cytosol, containing 25 munits of benzene dihydrodiol dehydrogenase activity, were incubated with anti-(isoenzyme TD2) serum (□) or anti-(aldehyde reductase) serum (■) at 4°C overnight. The activity in the supernatant after centrifugation of each mixture was determined.

androstanes and is minor in the tissue, its physiological importance is not clear. Although both isoenzymes also catalyse the reduction of xenobiotic carbonyl compounds, the activity of isoenzyme TD1 in this respect is lower than that of isoenzyme TD2 (Sawada *et al.*, 1979a,b). In the present study we have demonstrated that both isoenzymes also catalyse the oxidation of benzene dihydrodiol and several xenobiotic alcohols. Since isoenzyme TD1 exhibited higher specific activity and affinity for benzene dihydrodiol than did isoenzyme TD2, and since the dihydrodiol dehydrogenase activity of isoenzyme TD1 was much higher than its 17β-hydroxysteroid dehydrogenase and aldehyde reductase activities, the isoenzyme may play an important role in the metabolism of xenobiotics such as dihydrodiols and dihydrodiol epoxides derived from polycyclic hydrocarbons (Glatt *et al.*, 1979, 1981, 1982; Chou & Fu, 1983) and drugs (Chang *et al.*, 1970; Jerina *et al.*, 1970; Harvey *et al.*, 1972).

Aldehyde reductase also oxidized benzene dihydrodiol. The enzyme resembles one form of the mouse liver benzene dihydrodiol dehydrogenase, DD2 (Bolcsak & Nerland, 1983), with respect to its M_r and strict substrate specificity for benzene dihydrodiol. Since aldehyde reductase is widely distributed in mammalian tissues (Vogel *et al.*, 1982), the enzyme might be partly responsible for the multiplicity of benzene dihydrodiol dehydrogenase activities reported in mouse liver (Bolcsak & Nerland, 1983).

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