Identity of dihydrodiol dehydrogenase and 3α-hydroxysteroid dehydrogenase in rat but not in rabbit liver cytosol

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Dihydrodiol dehydrogenase and 3α-hydroxysteroid dehydrogenase activity in rat and rabbit liver cytosol have been analyzed by isoelectric focussing and subsequent activity staining. Identity of the two enzymes in rat liver cytosol is demonstrated. At least 4 main enzyme forms possessing dihydrodiol dehydrogenase activity can be detected in rabbit liver cytosol. However, in this species, only one of these forms has measurable activity towards 3α-hydroxysteroids.

Dihydrodiol dehydrogenase *3α-Hydroxysteroid dehydrogenase* *Aldo/keto reductase* *Activity staining* *Medroxyprogesterone acetate*

1. INTRODUCTION

In 1959, authors in [1] reported on an enzyme which converts benzene dihydrodiol to catechol. We have recently purified this $NADP^+$-dependent dihydrodiol dehydrogenase from rat liver cytosol [2]. The enzyme reduces the mutagenicity of metabolically activated benzo(*a*)pyrene [3] as well as that of the ultimate reactive metabolite *anti*-benz(*a*)anthracene 8,9-dihydrodiol 10,11-oxide [4], probably by conversion of the corresponding dihydrodiols to catechols, and thus may play an important role in detoxification of ultimate carcinogens of polycyclic aromatic hydrocarbons.

By its physico-chemical and catalytic properties [2], rat liver dihydrodiol dehydrogenase is closely related to aldo/keto reductases (review [5,6]). Here we show that rat liver dihydrodiol dehydrogenase is in all respects investigated, identical with 3α-hydroxysteroid dehydrogenase which recently has been shown to belong to the group of aldo/keto reductases [7,8]. However, in contrast to rat, in rabbit liver several isoenzyme forms of dihydrodiol dehydrogenase exist, which do not possess 3α-hydroxysteroid dehydrogenase activity.

Enzymes: dihydrodiol dehydrogenase (EC 1.3.1.20); 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50); aldehyde reductase (EC 1.1.1.2); aldose reductase (EC 1.1.1.21); carbonyl reductase (EC 1.1.1.184)

Nomenclature: The following systematic names are given to compounds referred to by trivial names: benzene dihydrodiol, *trans*-1,2-dihydroxycyclohexa-3,5-diene; medroxyprogesterone acetate, 17α-acetoxy-6α-methyl-4-pregnene-3,20-dione

2. MATERIALS AND METHODS

Preparation of liver cytosol from male Sprague-Dawley rats (200 g) and New Zealand white rabbits (2.5 kg), purification of rat liver dihydrodiol dehydrogenase and synthesis of benzene dihydrodiol have been carried out as in [2,9]. When indicated, the following protease inhibitors were included for the preparation of rat liver cytosol: 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), 0.1 mM Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 1 mM benzamidine, 4 μg/ml leupeptin, 4 μg/ml antipain, and 4 μg/ml pepstatin A. Isoelectric focussing on thin layer agarose gels was performed as recommended by the manufacturer [10] with pH gradients of 3–10.

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To reveal enzymatic activity, the agarose sheets were rinsed at the end of the run with 0.1 M glycine–NaOH (pH 9.5) for 5 min, then incubated in the dark at 37°C in 0.1 M glycine–NaOH containing 0.25 mM $NADP^+$, 8 μg/ml phenazine methosulfate, 0.2 mg/ml nitro blue tetrazolium chloride, and either 1 mM benzene dihydrodiol or 75 μM androsterone, and 2% acetone. After 10–20 min, enzymatic activity was detected by the appearance of purple bands.

3. RESULTS

3.1. *Analysis of dihydrodiol dehydrogenase and 3α-hydroxysteroid dehydrogenase activities by isoelectric focussing*

The cytosolic fractions from rat and rabbit liver, as well as purified rat liver dihydrodiol dehydrogenase have been analyzed by isoelectric focussing and subsequent activity staining for dehydrogenases, using benzene dihydrodiol and androsterone as substrates. The results are shown in fig.1. Firstly, several forms of dihydrodiol dehydrogenase differing in their isoelectric points were present in rat liver cytosol (lane 3). The most intensely stained band had an isoelectric point of 5.9. In addition, some minor bands with isoelectric points of 5.1, 5.4, 5.6 and 6.1 were observed. These forms were not separated from each other during our usual purification procedure for dihydrodiol dehydrogenase [2] but were present in our purified enzyme preparations (lane 1). However, when the stained bands from the cytosol and the purified proteins were compared, differential intensities were observed. No differences in the staining pattern could be detected whether protease inhibitors were included in the buffers for the preparation of the cytosolic fraction (lane 2) or not (lane 3). To detect 3α-hydroxysteroid dehydrogenase activity, staining was performed with androsterone as substrate. A very similar staining pattern was obtained, the only difference being that the staining of the minor bands was more intense (lane 4–6).

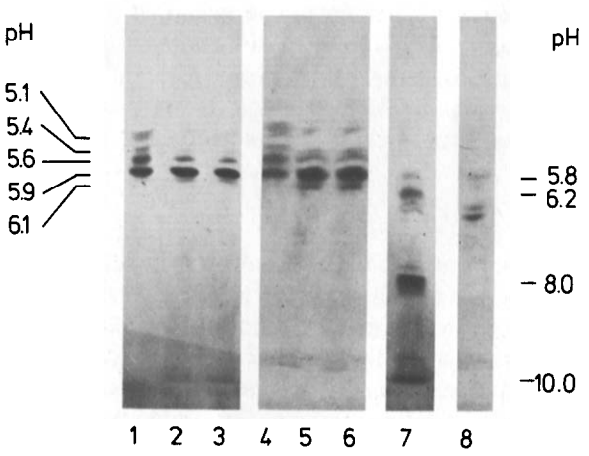

Fig.1. Dihydrodiol dehydrogenase and 3α-hydroxysteroid dehydrogenase activity in rat and rabbit liver cytosol. Purified rat liver dihydrodiol dehydrogenase (5 μg, lane 1,4), rat liver cytosol (200 μg) prepared in the presence (lane 2,5) or absence (lane 3,6) of protease inhibitors, and rabbit liver cytosol (300 μg, lane 7,8) were subjected to isoelectric focussing (pH 3–10) and subsequent activity staining for dihydrodiol dehydrogenase (lane 1–3,7) or 3α-hydroxysteroid dehydrogenase (lane 4–6,8).

With rabbit liver cytosol, the situation was more complex. There was a wider variability of forms with respect to isoelectric point and substrate specificity. At least 4 main bands were stained, using benzene dihydrodiol as substrate, with isoelectric points of 5.8, 6.2, 8.0 and 10, respectively (lane 7). Two additional bands with isoelectric points of 6.5 and 6.7 were also stained in the absence of any substrate and therefore were not further considered. Apart from the unspecifically stained bands only one band, with an isoelectric point of 5.8, was stained using androsterone as substrate (lane 8).

3.2. *Substrate specificity and inhibitors of dihydrodiol dehydrogenase*

It has already been shown [2] that 3α-hydroxysteroids and 3-ketosteroids are substrates for rat liver dihydrodiol dehydrogenase and that steroids with A/B *cis* configuration are metabolized at a faster rate than the A/B *trans* isomers. Table 1 shows that in addition to this, several carbonyl group-containing xenobiotics are substrates for this enzyme. Surprisingly, both 1,2-quinones tested have a very high affinity towards the enzyme. The K_m value of phenanthrene 9,10-quinone was 2 μM and that of acenaphthenequinone was below 10 μM (accurate determination not possible). D-Glucuronate and D-xylose, which are characteristic substrates for two other aldo/keto reductases (aldehyde reductase and aldose reduc-

Table 1

Substrate specificity of rat liver dihydrodiol dehydrogenase

Substrates	Concentration	K_m^{app} (mM)	k_{cat} (s^{-1})
4-Nitrobenzaldehyde		0.1	2.1
3-Nitrobenzaldehyde		0.9	2.4
Chloral hydrate		18	2.7
Phenylglyoxal		0.5	1.9
9,10-Phenanthrenequinone		0.002	3.3
Acenaphthenequinone[a]	10 μM	–	2.2
D-Glucuronate[a]	0.1 M	–	0
D-Xylose[a]	0.1 M	–	0
5α-Dihydrotestosterone[a]	10 μM	–	0.6

[a] Molar catalytic activities with indicated concentrations of these substrates are presented

K_m and k_{cat} values were calculated from Hanes plots [14]. Initial rates of the reaction were determined by measuring the oxidation rate of NADPH (0.2 mM) at 37°C in 50 mM sodium phosphate buffer (pH 7.0) for different substrate concentrations

tase), were not measurably metabolized by dihydrodiol dehydrogenase.

Δ^4,3-Ketosteroids have been reported to be very potent inhibitors of 3α-hydroxysteroid dehydrogenase [11–13]. Interestingly, these compounds also proved to be very potent inhibitors of purified rat liver dihydrodiol dehydrogenase: fig.2 shows the effect of medroxyprogesterone acetate on dihydrodiol dehydrogenase in a Hanes plot [14,15]. From this plot, it can be seen that medroxyprogesterone acetate causes a mixed inhibition. Inhibition constants of 0.11 μM (K_{ic}) and 0.04 μM (K_{iu}) were calculated from replots of intercepts and slopes (not shown). Dihydrodiol dehydrogenase was also inhibited by a large number of other Δ^4,3-ketosteroids including testosterone, progesterone, and prednisolone. However, no inhibition was detected in the presence of corticosterone (30 μM, not shown).

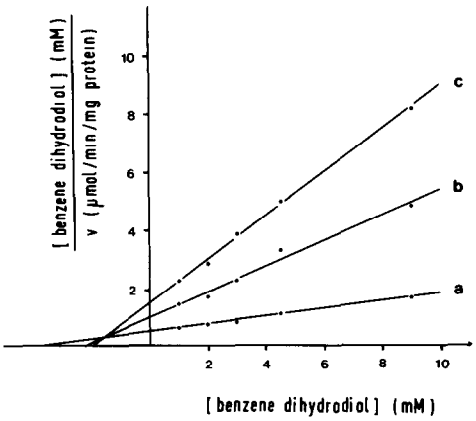

Fig.2. Inhibition of rat liver dihydrodiol dehydrogenase by medroxyprogesterone acetate. Reaction rates were determined by measuring the reduction of $NADP^+$ (2.3 mM) at 37°C in 50 mM Tris–HCl (pH 9.0) for various concentrations of benzene dihydrodiol in the presence of: (a) 0; (b) 0.1 μM; (c) 0.2 μM medroxyprogesterone acetate.

4. DISCUSSION

Rat liver dihydrodiol dehydrogenase has been shown to be involved in the control of dihydrodiol epoxides from polycyclic aromatic hydrocarbons, by both sequestration of the precursor dihydrodiols and inactivation of dihydrodiol epoxides [3,4,16]; the latter constituting ultimate carcinogens of polycyclic aromatic hydrocarbons [17]. Apart from its role in dihydrodiol metabolism, dihydrodiol dehydrogenase also catalyzes the reversible oxidation of 3α-hydroxysteroids [2].

In the search for an inhibitor of rat liver dihydrodiol dehydrogenase which would allow us to study the role of this enzyme in in vivo systems, we found that medroxyprogesterone acetate and other Δ^4,3-ketosteroids are very potent inhibitors of dihydrodiol dehydrogenase. Δ^4,3-Ketosteroids have also been reported to be inhibitors of 3α-hydroxysteroid dehydrogenase from rat liver [13,18], rat testis [11], and prostate [12]. Together with the finding that dihydrodiol dehydrogenase copurifies with 3α-hydroxysteroid dehydrogenase activity [2,18], this prompted us to consider that dihydrodiol dehydrogenase and 3α-hydroxysteroid dehydrogenase might be identical enzymes. The differential inhibition patterns with medroxyprogesterone acetate observed here (mixed type) in comparison to [11] (competitive type) may be explained by the use of different substrates; i.e., benzene dihydrodiol and androsterone.

To confirm identity of dihydrodiol dehydrogenase and 3α-hydroxysteroid dehydrogenase, rat liver cytosol has been analyzed by isoelectric

focussing. Dihydrodiol dehydrogenase segregated into multiple forms which reflects charge heterogeneity. Both a main form with an isoelectric point of 5.9, and several minor forms were observed. Whether these forms constitute genetically different enzyme species (isoenzymes), are formed as a result of post-transcriptional modifications or are due to secondary effects such as, for example, binding of small charged molecules to a part of the enzyme population, cannot be definitively answered. Proteolytic degradation seems unlikely because the same pattern is obtained when protease inhibitors were included in the buffers. Most forms were also present in our pure enzyme preparation, but the activity of the more acidic forms was slightly increased. When 3α-hydroxysteroid dehydrogenase activity was analyzed, nearly the same staining pattern was obtained as for dihydrodiol dehydrogenase. Although staining of the minor band with an isoelectric point of 6.1 was enhanced, all forms were active towards both substrates, benzene dihydrodiol and androsterone. Thus, dihydrodiol dehydrogenase constitutes a large part of 3α-hydroxysteroid dehydrogenase activity in rat liver cytosol. These results would tend to support previous evidence for the existence of isoenzymes of 3α-hydroxysteroid dehydrogenase which has been presented by several investigators ([19,20], personal communication).

Dihydrodiol dehydrogenase has been shown to catalyze the oxidation/reduction of various xenobiotics [1,2,21,22]. This has also been shown for 3α-hydroxysteroid dehydrogenase. In 1976, it was demonstrated [23] that one of two rat liver cytosolic aldehyde reductases also catalyzes the reduction of 3-ketosteroids to the 3α-hydroxy-isomers. This has been confirmed in [7,8], a study on chloral hydrate-reducing enzymes. Two of the 4 chloral hydrate-reducing isoenzymes, F_2 and F_4, were identified as aldo/keto reductases [7]. In a subsequent study [8], isoenzyme F_4 has been shown to be identical to the rat liver 3α-hydroxysteroid dehydrogenase described in [24,25] as judged from substrate specificity. Apart from 3α-hydroxysteroids, chloral hydrate reductase F_4 is also able to reduce a wide variety of physiological and xenobiotic carbonyl compounds. As expected, chloral hydrate reductase F_4 and dihydrodiol dehydrogenase are closely related to each other with respect to their substrate specificity and their physico-chemical properties; both are monomeric enzymes with a relative M_r of 32500 (chloral hydrate reductase F_4) [7,8] and 35000 (dihydrodiol dehydrogenase) [2]. Recently, authors in [18] isolated a 3α-hydroxysteroid dehydrogenase (M_r 33000; pI 5.7) which comprises most dihydrodiol dehydrogenase and aromatic ketone reductase activity of rat liver cytosol.

Based on its catalytic properties, dihydrodiol dehydrogenase (chloral hydrate reductase F_4, 3α-hydroxysteroid dehydrogenase) is related to aldo/keto reductases from other species; e.g., carbonyl reductase from human brain [26], rabbit liver aldehyde reductase F_3 [27] and chicken carbonyl reductases [28]. It differs, however, from rat aldo/keto reductases 1 (aldehyde reductase) and 2 (aldose reductase) by the fact that D-glucuronate and D-xylose are not substrates.

Thus, we conclude that dihydrodiol dehydrogenase and 3α-hydroxysteroid dehydrogenase from rat liver cytosol are the same enzyme species, with respect to all criteria tested.

In contrast to rat liver cytosol, where dihydrodiol dehydrogenase isoenzymes oxidize both substrates (benzene dihydrodiol and androsterone), in rabbit liver cytosol only one (minor) enzyme form (isoelectric point, 5.8) was observed which can metabolize both substrates. The other (including the major) forms of dihydrodiol dehydrogenase showed no appreciable ($NADP^+$-dependent) oxidation of androsterone. Authors in [27] purified four aldo/keto reductases from rabbit liver, one of which (F_3) is a 3α-hydroxysteroid dehydrogenase with an isoelectric point of 5.8. This is in agreement with our results. One other form (F_4) is heterogeneous with respect to its isoelectric point. Although it might be possible that this form is related to our basic enzyme forms, we think that this is unlikely because these forms differ in their substrate specificity (not shown). For example, the form with an isoelectric point of about 10 is stained with benzyl alcohol as a substrate, whereas the other form with an isoelectric point of 8.0 is not. Recently, authors in [29] purified multiple forms of dihydrodiol dehydrogenase from mouse liver cytosol. Three isoenzymes were active towards 17β-hydroxysteroids whereas another form showed no activity towards the steroids tested.

Thus, the ability to metabolize dihydrodiols is not restricted to 3α-hydroxysteroid dehydro-

genases. In different species, a variety of other enzymes may contribute to dihydrodiol metabolism.

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