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PROPERTIES OF NADPH-DEPENDENT CARBONYL REDUCTASES IN RAT LIVER CYTOSOL

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Abstract—Rat liver cytosol was shown previously by us to contain multiple forms of 3α-hydroxysteroid dehydrogenase. Two (F_4-II and -III) of the seven forms were purified to homogeneity, and four (F_3-II, -III, -IV and F_4-I) of them partially purified. One of them (F_4-III) has been shown previously to catalyze the reduction of long-chain aliphatic and aromatic aldehydes or aromatic ketones as well as 3-oxosteroids [M. Ikeda *et al.*, *Biochem. Pharmac.* **30**, 1931 (1981)]. The reducing activity of such compounds was examined with the other F_4 enzymes, and it was revealed that they also reduce a number of carbonyl compounds described above. In addition, quinones were tested for the first time in this report as substrates for all the F_4 enzymes, and among them 9,10-phenanthrenequinone was found to be the best substrate for them, followed by hydrindantin and 2,6-dichlorophenolindophenol, while menadione was a poor substrate. The F_4 enzymes did not catalyze the reduction of the oxo group at the 9-position of the prostaglandins of the E and A class with NADPH or NADH. On the basis of this evidence, the identity of ketone reductases (F_4-I–III) in the rat liver is proposed to be 3α-hydroxysteroid dehydrogenase, rather than prostaglandin 9-ketoreductase, which was demonstrated to correspond to ketone reductase in human brain [B. Wermuth, *J. biol. Chem.* **256**, 1206 (1981)].

Carbonyl reductases, which catalyze the reduction of xenobiotic and endogenous aldehydes and ketones in the presence of NADPH, have been found in various tissues. This kind of enzyme preferentially utilizes NADPH as coenzyme, is located in the cytoplasm of the cell, and possesses a monomeric structure with a relatively low molecular weight (30,000–40,000 daltons). Aldehyde reductase (EC 1.1.1.2), aldose reductase (EC 1.1.1.21), succinic semialdehyde reductase (EC 1.2.1.16), 17β-hydroxysteroid dehydrogenase (EC 1.1.1.64), and prostaglandin 9-ketoreductase are demonstrated to be members of the "aldehyde-ketone reductase family" [1, 2].

From rat liver Felsted *et al.* [3] purified one of them to homogeneity, which is identical with the previously classified aldehyde reductase (EC 1.1.1.2), while Pietruszko and Chen [4] suggested that an aldehyde-ketone reductase in the rat liver might be 3α-hydroxysteroid dehydrogenase.

On the other hand, we had studied the *in vitro* metabolism of trichloroethylene and chloral hydrate by rat liver [5]. In the course of the study, we purified two NADPH-dependent chloral hydrate-reducing enzymes from rat liver cytosol, one of which was identical with the previously classified aldehyde reductase (EC 1.1.1.2) and the other of which appeared to be 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) [6]. In a previous paper [7], we described characteristics of the 3α-hydroxysteroid dehydrogenase in the rat liver cytosol, and also discussed a possible role in bile acid metabolism. Thereafter, we found the presence of multiple forms of 3α-hydroxysteroid dehydrogenase in rat liver cytosol, separated these into seven enzymes, and purified two of them (F_4-II and -III) to homogeneity and four of them (F_3-II, -III, -IV and F_4-I) partially by successive DEAE-cellulose, CM-Sephadex C-50, chromatofocusing and Red-Sepharose 4B column chromatographies [8]. Steroid substrate specificity was examined in detail for these enzymes, and was summarized in a previous paper [8].

F_4-III (corresponding to F_4 in Ref. 6) had been shown to catalyze the reduction of long-chain aliphatic and aromatic aldehydes or aromatic ketones as well as 3-oxosteroids. The present paper describes the catalytic properties of F_4-I, -II and -III as aldehyde-ketone reductase. In general, aldehyde-ketone reductase is distinguishable from aldose and aldehyde reductase by its partiality for quinones [9]. Therefore, we tested several quinones as substrates for the F_4 enzymes. The relationship between the aldehyde-ketone reductase and prostaglandin 9-ketoreductase, which was demonstrated to function as carbonyl reductase in human brain as reported by Wermuth [9], is also discussed.

MATERIALS AND METHODS

Chemicals. Pyridine nucleotides were purchased from the Oriental Yeast Co. Ltd. (Tokyo, Japan). 9,10-Phenanthrenequinone, rutin, quercetin and Cibacron blue 3G-A were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). *n*-Decylaldehyde, aromatic aldehydes and ketones, and phenylglyoxal were obtained from the Tokyo Chemical Ind. Ltd. (Tokyo). Prostaglandins (PGA_1, A_2, E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$) were supplied by the Ono Pharmaceutical Co. Ltd. (Osaka), and GSH was from the Yamanouchi Pharmaceutical Co. Ltd. (Tokyo). Other chemicals (all analytical grade) were purchased from Wako Pure Chemicals Ind. Ltd. (Osaka). Materials used

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for enzyme preparation were the same as previously described [8].

Enzyme assay. Reaction mixtures consisted of 50 mM buffer (pH 4.0 to 5.5, acetate buffer; pH 5.5 to 8.0, potassium phosphate buffer; pH 8.0 to 9.0, Tris/phosphate buffer; pH 9.0 to 11.0, glycine/NaOH buffer), 0.16 mM NADPH, enzyme solution and substrate at various concentrations, as indicated in the tables and figures, with a total volume of 1.0 ml. Reactions were initiated by the addition of substrate, and the decrease in absorbance at 340 nm was monitored with a Shimadzu UV-180 spectrophotometer at 25°. 3α-Hydroxysteroid dehydrogenase activity was measured as described in a previous paper [8], except that pH of the buffer was 9.9. One unit of enzyme activity was defined as international units (1 unit = 1 μmole substrate degraded/min at 25°).

Purification of the F_4 enzymes. The separation and purification of the F_4 enzymes were performed as described in a previous paper [8]. The final preparations were stored at −20° as 25% (v/v) glycerol solution, and used for kinetic studies.

Protein concentration was assayed as previously described [8].

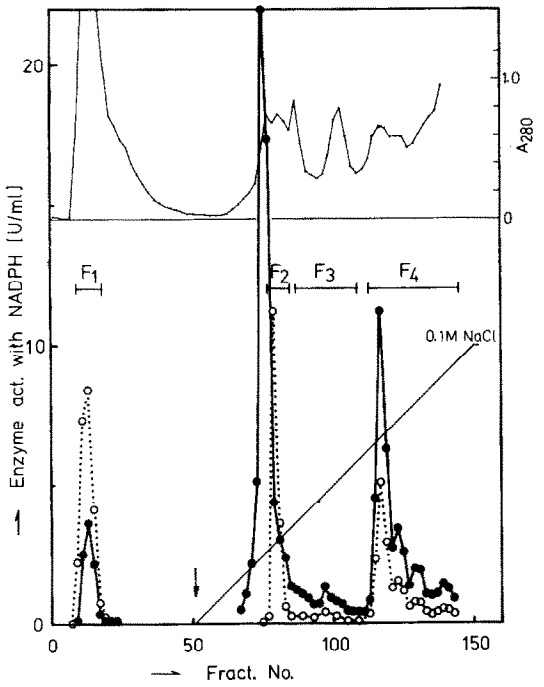

Fig. 1. Separation of enzyme activity with the various substrates on a DEAE-cellulose column. Rat liver cytosol corresponding to 150 g of wet weight of liver was treated in the same manner as described under Materials and Methods in Ref. 6, and applied to a DE-52 column (45 × 3 cm) [8]. Flow rate, 40 ml/hr. The first 50 fractions represent the effluent with the medium containing 5 mM Tris/phosphate buffer (pH 8.0) and 5 mM 2-mercaptoethanol. The arrow indicates the start of a linear salt gradient consisting of the above medium (1 liter) and the medium containing 0.1 M NaCl (1 liter). The enzyme activity in each fraction (20 ml) was assayed at pH 7.0 for the reduction of 1 mM 4-nitrobenzaldehyde (○-----○) and 0.025 mM 9,10-phenanthrenequinone (●——●) with NADPH (0.16 mM).

RESULTS

Purification of the enzymes. The F_4 enzymes were prepared as previously described by checking the activity with androsterone as substrate [8]. At the same time the reducing activities of 4-nitrobenzaldehyde, a representative of aromatic aldehydes, and 9,10-phenanethrenequinone, that of quinones, were followed at each purification step. Figure 1 shows that the reducing activities of 4-nitrobenzaldehyde and 9,10-phenanethrenequinone were separated into the four fractions on a DE-52 column, three of which completely overlapped each other. The total and specific activities for 4-nitrobenzaldehyde, 9,10-phenanethrenequinone and androsterone at the purification steps are summarized in Table 1.

pH Optima and buffer effects. The effects of pH on the enzyme activities are shown in Fig. 2. When 4-nitrobenazldehyde was used as substrate, pH optima of all the F_4 enzymes were around 6.5, while on using 9,10-phenanthrenequinone pH optima were around 8.5. pH Optima on using 4-nitroacetophenone, a representative of aromatic ketones, were about 5.5 (data not shown). The optima with 3-oxosteroid and 3α-hydroxysteroid were around 9.6 and 10.2, as previously described [8].

Substrate specificity. Several xenobiotic substances and naturally occurring compounds were tested as substrate for the F_4 enzymes. The results are summarized in Table 2. A small difference between substrate specificities of the F_4 enzymes was observed, but they showed very similar specificities. However, the reducing activities of PGA_1, E_1 and E_2 were not observed for the F_4 enzymes even if GSH was present (see Ref. 9).

Inhibitors. Table 3 lists a number of compounds that were tested as inhibitors of the F_4 enzymes using 4-nitrobenzaldehyde as substrate.

Flavonoids, good inhibitors of aldose reductase [10], inhibited all the F_4 enzymes. Lithium salts are known to activate aldose reductase [11], but Li_2SO_4 inhibited F_4-I and -III, and was without effect on F_4-II. The strongest inhibitor was Cibacron blue 3G-A, which is believed to interact at the nucleotide fold. Phenobarbital, a potent inhibitor of aldehyde reductase, showed little inhibitory effect on the enzyme activities. *p*-Chloromercuribenzoate inactivated all the F_4 enzymes by more than 90%.

DISCUSSION

Chloral-hydrate reducing activity in the rat liver cytosol was separated into four fractions by a DEAE-cellulose column chromatography, which were designated as F_1 to F_4 in order of their elution [6]. F_2 was identified as the previously classified aldehyde reductase (EC 1.1.1.2) [6], and F_4 appeared to be 3α-hydroxysteroid dehydrogenase because of its highest affinity for steroids among xenobiotic and naturally occurring aldehydes and ketones [6, 7]. F_3 also catalyzed the oxido-reduction of 3α-hydroxysteroids, although the activity was much lower than that of F_4 [7]. Subsequently, we have separated F_3 into the four

Table 1. Enzyme activities during purification*

Step	Enzyme activity		
	Substrate		
	4-Nitrobenz-aldehyde	9,10-Phenane-threnequinone	Androsterone
I. Crude extract	665† (0.107)‡	1278 (0.0206)	245 (0.039)
	214§ (0.034)	440 (0.071)	213 (0.034)
II. 40–70% $(NH_4)_2SO_4$ fr.	607 (0.125)	1384 (0.286)	216 (0.045)
	195 (0.040)	476 (0.098)	188 (0.039)
III. DE-52 column			
F_1	178	50.9	0
F_2	90.7	443	0
F_3	17.9	55.1	18.6
F_4	136 (0.247)	288 (0.523)	123 (0.223)
IV. CM-Sephadex column			
F_4-I	7.37 (0.031)	24.1 (0.101)	8.05 (0.034)
F_4-II	9.12 (0.691)	26.3 (1.99)	9.79 (0.742)
F_4-III	87.7 (0.504)	169 (0.971)	71.0 (0.408)
V. Chromatofocusing			
F_4-I	4.80	10.7	4.02
F_4-II	6.14	17.0	6.82
F_4-III	60.1	137	62.8
VI. Red-Sepharose column			
F_4-I	1.53 (0.107)	3.06 (0.214)	2.45 (0.171)
F_4-II	2.80 (2.28)	8.23 (6.69)	2.78 (2.26)
F_4-III	31.7 (2.31)	68.3 (4.99)	33.8 (2.47)
VII. ω-Aminooctyl Sepharose column (F_4-I)	0.80 (0.656)	1.50 (1.23)	1.21 (0.99)
VIII. Final preparation			
F_4-I	0.87 (0.744)	1.74 (1.49)	1.39 (1.19)
F_4-II	1.86 (2.21)	5.50 (6.54)	1.92 (2.28)
F_4-III	21.1 (2.35)	45.5 (5.06)	28.8 (3.21)

* Enzyme activities were measured at pH 7.0 for the hydrogenation of 4-nitrobenzaldehyde (1 mM) and 9,10-phenanethrenequinone (0.025 mM) with NADPH (0.16 mM), and at pH 9.7 for the dehydrogenation of androsterone (100 μM) with $NADP^+$ (0.5 mM).

† Total activity (units).

‡ Specific activity (units/mg protein).

§ The total activity was multiplied by the "fraction" of F_4 activity (0.322 for 4-nitrobenzaldehyde, 0.344 for 9,10-phenanethrenequinone, and 0.869 for androsterone) found in the DE-52 column chromatography.

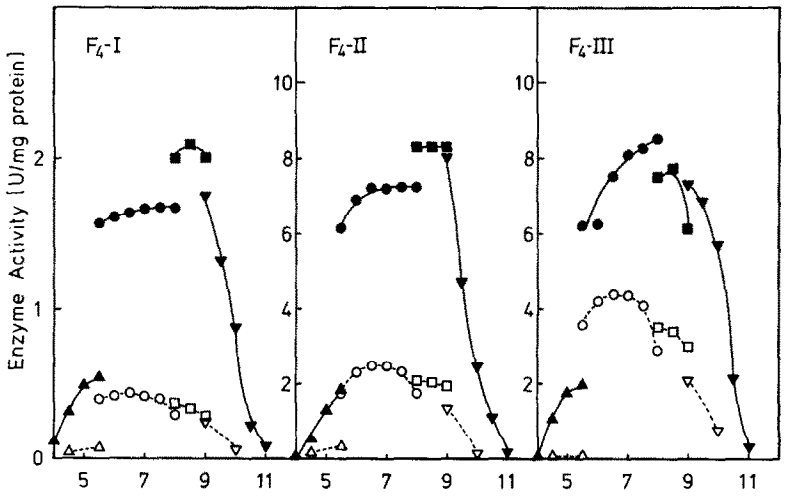

Fig. 2. Enzyme activities for the reduction of 4-nitrobenzaldehyde and 9,10-phenanthrenequinone as a function of pH and buffer composition. The activities of F_4-I–III for the reduction of 4-nitrobenzaldehyde (-----) (1 mM) and 9,10-phenanethrenequinone (———) (0.025 mM) were determined in 50 mM buffer with NADPH (0.16 mM). Key: sodium acetate buffer (△▲), potassium phosphate buffer (○●), Tris/phosphate buffer (□■) and glycine/NaOH buffer (▽▼). Activities are given in units/mg protein.

Table 2. Substrate specificity of the F_4 enzymes*

Substrates	Conc (mM)	Relative reaction rate (%)			K_m (μM)		
		F_4-I	F_4-II	F_4-III	F_4-I	F_4-II	F_4-III
Hydrindantin	0.5	207	169	177	250	162	110
9,10-Phenanethrene-quinone	0.025	412	429	408	16.8	20.5	16.6
Menadione	0.5	6.3	4.8	9.2			
2,6-Dichlorophenol-indophenol	0.0625	21.0	10.1	15.3	70.0	324	24.6
4-Nitroacetophenone	0.5	44.1	47.6	49.1	607	608	953
4-Chloroacetophenone	2.5	23.3	12.9	15.5			
Cyclohexanone	0.5	13.2	6.0	7.6			
17β-Hydroxy-5α-andro-stan-3-one	0.025	26.9	12.6	41.1	4.85	3.69	29.9
Dehydronorlithocholic acid	0.025	88.4	58.8	109	2.77	4.70	10.6
Dehydrolithocholic acid	0.025	69.6	48.2	60.6	2.55	5.58	13.0
Phenylglyoxal	0.5	51.7	24.3	27.2	258	980	541
4-Nitrobenzaldehyde	0.5	100	100	100	97.1	314	168
3-Nitrobenzaldehyde	0.5	45.6	33.3	37.7	228	985	1240
4-Carboxybenzaldehyde	0.5	10.0	2.9	0.94			
n-Decylaldehyde	0.5	20.0	13.3	12.9	275	398	181
PGA_1	0.5	ND†	ND	ND			
PGA_1-GSH‡	0.5, 0.1	ND	ND	ND			
PGE_1	0.5	ND	ND	ND			
PGE_2	0.5	ND	ND	ND			

* Enzyme activities were measured at pH 7.0 and at 25°, except for 3-oxosteroids at pH 9.6 (glycine/NaOH buffer).
† Not detectable.
‡ PGA_1 and GSH were incubated in the assay mixture for 10 min before the enzymes were added (see Ref. 9).

(F_3-I–IV) and F_4 into the three fractions (F_4-I–III) by subsequent CM-Sephadex C-50 column chromatography. Differences between the F_3 and F_4 enzymes were distinguishable only by their isoelectric points [8].

In the present paper, the properties of the F_4 enzymes were investigated for the reduction of several carbonyl compounds. As shown in the results, their catalytic properties and inhibitor sensitivities were very similar, and the enzymes were demonstrated to reduce not only aldehydes but also ketones. According to the classification of Felsted and Bachur [1], these enzymes are defined as ketone reductase. They were also disinguishable from aldose and aldehyde reductase based on their partiality for quinones and the inhibitor sensitivities.

Table 3. Effects of inhibitors on the enzyme activities*

Inhibitor	Conc (mM)	% Inhibition		
		F_4-I	F_4-II	F_4-III
Rutin	0.001	20.0	13.8	18.3
	0.005	44.4	40.0	37.5
Quercetin	0.001	35.0	27.6	36.7
	0.005	44.4	52.0	62.5
Cibacron blue 3G-A	0.005	90.0	97.7	96.7
$NADP^+$	0.1	20.2	17.2	20.0
Li_2SO_4	10	5.0	0	3.4
p-Chloromercuri-benzoate	0.1	100	98.2	90.3
Phenobarbital	1.0	4.5	5.3	9.7

* Inhibitor studies were carried out using 4-nitrobenzaldehyde (1 mM) and NADPH (0.16 mM) at pH 7.0 as described in the legend of Table 4 in Ref. 5.

Quinones are known to play important roles as oxidation–reduction catalysts in many biological processes. The cytoplasmic NAD(P)H dehydrogenase (EC 1.6.9.22, quinone reductase, DT-diaphorase) is demonstrated to reduce vitamin K in rat liver [12]. It appears to differ by several criteria, however, from the F_4 enzymes presented here. Namely, the NAD(P)H dehydrogenase uses FAD for full activity, possesses one molecule of FAD per molecule (55,000 daltons mol. wt), and consists of two identical subunits with a molecular weight of about 27,000 daltons. Menadione (vitamin K_3) was a poor substrate for the F_4 enzymes, which prefer 9,10-phenanethrenequinone, hydrindantin and 2,6-dichlorophenolindophenol. Figure 1 shows that one of the 9,10-phenanethrenequinone-reducing activities was eluted just before F_2. As previously described [6], F_1 was supposed to be alcohol dehydrogenase, F_2 was identified as aldehyde reductase, and F_3 and F_4 were supposed to be 3α-hydroxysteroid dehydrogenase. Therefore, the quinone-reducing activity eluted before F_2 may be due to NAD(P)H dehydrogenase, although this was not experimentally confirmed.

Wermuth [9] recently reported the relationship between prostaglandin 9-ketoreductase and xenobiotic ketone reductase in human brain. The reduction of the oxo group at position 9 and the oxidation of the hydroxy group at position 15 of the prostaglandins were catalyzed by carbonyl reductase purified from human brain. Above all, the GSH adduct of PGA_1 was preferred by the enzyme among the prostaglandins tested, and Wermuth suggested that a GSH conjugate of the prostaglandins may be

a natural substrate for the enzyme. The enzyme was also shown to catalyze the reduction of 3-oxosteroids, e.g. 5α-dihydrotestosterone and 5β-dihydrotestosterone. The F_4 enzymes were, therefore, examined for prostaglandin 9-ketoreductase and 15-hydroxyprostaglandin dehydrogenase activities. Table 2 reveals that the F_4 enzymes did not utilize the prostaglandins even in the presence of GSH. NADPH production was not observed in the presence of 1 mM PGA_1, a substrate of 15-hydroxyprostaglandin dehydrogenase [13].

The data presented here supports the proposal that natural substrates for the F_4 enzymes are 3-oxo- and 3α-hydroxysteroids, and the identity of ketone reductase in the rat liver is proposed to be 3α-hydroxysteroid dehydrogenase. In the present paper, the catalytic properties of the F_3 enzymes as carbonyl reductase are not studied, because of instability of the enzymes. However, the activity of fraction F_3 with androsterone on a DE-52 column was demonstrated to be much lower in female rats than male (see Ref. 8). The F_3 enzymes preferred a C_{19} steroid to C_{24}. These data strongly suggest that the F_3 enzymes participate in steroid hormone metabolism.

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REFERENCES

1. R. L. Felsted and N. R. Bachur, in *Enzymatic Basis of Detoxication* (Ed. W. B. Jakoby), Vol. 1, pp. 281–92. Academic Press, New York. (1980).
2. J-P. von Wartburg and B. Wermuth, in *Enzymatic Basis of Detoxication* (Ed. W. B. Jakoby), Vol. 1, pp. 249–60. Academic Press, New York (1980).
3. R. L. Felsted, M. Gee and N. R. Bachur, *J. biol. Chem.* **249**, 3672 (1974).
4. R. Pietruszko and F-F. Chen, *Biochem. Pharmac.* **25**, 2721 (1976).
5. M. Ikeda, Y. Miyake, M. Ogata and S. Ohmori, *Biochem. Pharmac.* **29**, 2983 (1980).
6. M. Ikeda, M. Ezaki, S. Kokeguchi and S. Ohmori, *Biochem. Pharmac.* **30**, 1931 (1981).
7. M. Ikeda, S. Hayakawa, M. Ezaki and S. Ohmori, *Hoppe-Seyler's Z. physiol. Chem.* **362**, 511 (1981).
8. M. Ikeda, H. Hattori, N. Ikeda, S. Hayakawa and S. Ohmori, *Hoppe-Seyler's Z. physiol. Chem.* **365**, 377 (1984).
9. B. Wermuth, *J. biol. Chem.* **256**, 1206 (1981).
10. S. D. Varma, A. Mizuno and J. H. Kinoshita, *Science* **195**, 205 (1977).
11. D. R. P. Tulsiani and O. Touster, *J. biol. Chem.* **252**, 2545 (1977).
12. R. Wallin, O. Gebhardt and H. Prydz, *Biochem. J.* **169**, 95 (1978).
13. C. Westbrook, Y. M. Lin and J. Jarabak, *Biochem. biophys. Res. Commun.* **76**, 943 (1977).