Biochimica et Biophysica Acta, 341 (1974) 172–177

BBA 67168

GUINEA PIG LIVER 3-HYDROXYHEXOBARBITAL DEHYDROGENASE AS A 17β-HYDROXYSTEROID DEHYDROGENASE

ECHIKO KAGEURA and SATOSHI TOKI

Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Fukuoka (Japan)

(Received August 14th, 1973)

SUMMARY

In order to confirm the identity of guinea pig liver 3-hydroxyhexobarbital dehydrogenase with 17β-hydroxysteroid dehydrogenase (17β-hydroxysteroid: $NADP^+$ 17-oxidoreductase, EC 1.1.1.64), several experiments were attempted.

1. During the purification steps, the ratios of the two enzyme activities were almost constant after another newly discovered 17β-hydroxysteroid dehydrogenase was eliminated by DEAE-cellulose DE-32 column chromatography.

2. The two enzymes showed the same susceptibility to thermal inactivation, and were activated similarly by phosphate ion when NAD^+ was used as cofactor.

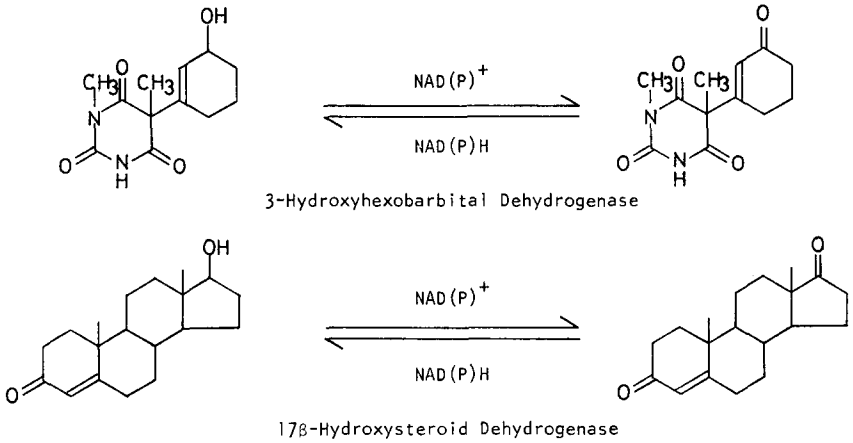
3. K_i values for *p*-chloromercuribenzoate were almost the same between the two enzymes.

4. Results of mixed substrate method indicated that 3-hydroxyhexobarbital and testosterone were metabolized by a single enzyme, and kinetic studies gave the information that 3-hydroxyhexobarbital and testosterone were inhibited competitively each other, and that the active sites of the two enzymes were identical.

These results indicated that 3-hydroxyhexobarbital dehydrogenase is identical with 17β-hydroxysteroid dehydrogenase in guinea pig liver.

INTRODUCTION

As described in the previous paper [1], 3-hydroxyhexobarbital dehydrogenase, which catalyzes the reversible oxidation of 3-hydroxyhexobarbital to 3-oxohexobarbital, has been purified as a single homogeneous protein from the soluble fraction of guinea pig liver. It was also demonstrated that the purified enzyme participated in the dehydrogenation of C_{19}-17β-hydroxysteroids such as testosterone, 4-androstene-3β,17β-diol, 5α-androstan-17β-ol-3-one and 5α-androstane-3β,17β-diol. Furthermore, partially purified 17β-hydroxysteroid (testosterone) dehydrogenase (17β-hydroxysteroid:$NADP^+$ 17-oxidoreductase, EC 1.1.1.64), which was obtained from the same origin following the method of Joshi et al. [2], was able to catalyze the dehydrogenation of 3-hydroxyhexobarbital [3]. These findings strongly suggested that the oxidation of 3-hydroxyhexobarbital and 17β-hydroxysteroids must be catalyzed by the



same enzyme. The present investigation was initiated to verify the identity of 3-hydroxyhexobarbital dehydrogenase with 17β-hydroxystreoid dehydrogenase.

MATERIALS AND METHODS

Materials

NAD^+ and $NADP^+$ were obtained from Oriental Yeast Co. (Tokyo, Japan); Sephadex G-100 was from Pharmacia Fine Chemicals AB (Uppsala, Sweden); DEAE-cellulose DE-32 was from H. Reeve Angel and Co. (N.J., U.S.A.); hydroxylapatite was from Seikagaku Kogyo Co. (Tokyo, Japan). 3-Hydroxyhexobarbital was prepared by the method of Takenoshita and Toki [4]. Testosterone was supplied from Teikoku Hormone MFG. Co. (Tokyo, Japan).

Enzyme assay

Enzyme activity was determined by measuring spectrophotometrically the rate of NAD(P)H formation in a Shimadzu QV-50 spectrophotometer at 340 nm. Assays were carried out at 25 °C in a cuvette with a light path of 1 cm. 3-Hydroxyhexobarbital was dissolved in 1.1 equivalent of 0.1 M NaOH, then diluted with water to give a 15 mM solution. Testosterone was dissolved in methanol in a concentration of 1.5 mM solution. The analytical cuvette contained 1.5 μmoles of 3-hydroxyhexobarbital, 0.5 μmole of $NADP^+$ (or 1.5 μmoles of NAD^+), enzyme preparation (0.05–0.1 ml) and 0.1 M NaH_2PO_4–Na_2HPO_4–$Na_4P_2O_7$ buffer (pH 8.9) to make 1.5 ml. For the measurement of the enzyme activity for testosterone, 0.15 μmole of this compound was added instead of 3-hydroxyhexobarbital and 0.1 M Na_2HPO_4–NaOH buffer (pH 10.7) (or pH 10.1 in the case of NAD^+) was used. 1 unit was defined as 1 μmole of NAD(P)H formed per min at 25 °C. Specific activity was expressed as units of enzyme per mg of protein. Protein was determined by the method of Lowry et al.[5], after the protein was precipitated from a solution by sodium tungstate and H_2SO_4.

Enzyme purification

All the procedures of the purification of 3-hydroxyhexobarbital dehydrogenase were carried out as described previously [1]. 105 000 $\times$ g supernatant fraction of

guinea pig liver was purified by $(NH_4)_2SO_4$ precipitation followed by first and second Sephadex G-100 gel filtration, DEAE-cellulose DE-32 column chromatography and hydroxylapatite column chromatography.

RESULTS

The ratios of 3-hydroxyhexobarbital dehydrogenase activity to 17β-hydroxysteroid dehydrogenase activity at each purification step

The ratios of the two enzyme activities in each purification step were calculated based on the specific activity under the condition of NAD^+ or $NADP^+$ was used as cofactor. As shown in Table I, the ratios were almost constant in all the sub-

TABLE I

THE SPECIFIC ACTIVITIES OF 3-HYDROXYHEXOBARBITAL DEHYDROGENASE AND 17β-HYDROXYSTEROID DEHYDROGENASE, AND THE RATIOS OF THE TWO ENZYME ACTIVITIES IN EACH PURIFICATION STEP

Abbreviations: H, 3-hydroxyhexobarbital; T, testosterone.

Fraction	Spec. act. (munits/mg protein)					
	Cofactor NAD^+ with		Ratio H/T	Cofactor $NADP^+$ with		Ratio H/T
	H	T		H*	T*	
First Sephadex G-100	42.7	37.3	1.14	262.0	120.9	2.17
Concentrated	42.6	39.5	1.08	261.8	118.4	2.21
Second Sephadex G-100	51.1	49.7	1.03	353.5	143.8	2.46
Concentrated	49.1	46.6	1.05	347.4	147.3	2.36
DEAE-cellulose DE-32 (Fraction b)	469.1	295.3	1.59	3330.7	1104.8	3.01
Concentrated	452.4	284.9	1.59	3061.2	1040.3	2.94
Hydroxylapatite	1317.6	822.4	1.60	9384.0	3103.0	3.02

sequent steps including DEAE-cellulose DE-32 fraction after another 17β-hydroxysteroid dehydrogenase was eliminated by DEAE-cellulose DE-32 column chromatography.

Effect of phosphate ion

Phosphate ion stimulated the activity of 3-hydroxyhexobarbital dehydrogenase only when NAD^+ was used as cofactor, and the activity was increased in accordance with the molar concentration of phosphate buffer [1]. The same effects of the phosphate ion were also observed in the case that testosterone was added instead of 3-hydroxyhexobarbital and incubated with NAD^+.

Inactivation studies

The effects of temperature and *p*-chloromercuribenzoate on the activities of 3-hydroxyhexobarbital dehydrogenase and 17β-hydroxysteroid dehydrogenase were studied. The two enzyme activities were decreased the same degree by heat treatment and finally abolished at 55 °C. *p*-Chloromercuribenzoate inhibited compe-

TABLE II

TEST WITH COMBINED SUBSTRATES

The reaction mixture contained $NADP^+$ (0.5 μmole), enzyme solution (0.05 ml), substrate and buffer in a total volume of 1.5 ml, and incubated for 5 min. To compensate the effect of methanol which was used to dissolve testosterone, 0.1 ml of methanol was also added to the incubation mixture with 3-hydroxyhexobarbital alone.

Substrate	Concn (M)	NADPH produced (nmoles/ml/min)
3-Hydroxyhexobarbital	$2.0 \cdot 10^{-2}$	648.0*
Testosterone	$2.5 \cdot 10^{-4}$	38.4*
Combined substrates		312.0*
Testosterone	$2.5 \cdot 10^{-4}$	78.2**
3-Hydroxyhexobarbital	$5.0 \cdot 10^{-3}$	40.8**
Combined substrates		64.8**

* 0.1 M NaH_2PO_4–Na_2HPO_4–$Na_4P_2O_7$ buffer (pH 8.9).
** 0.1 M Na_2HPO_4–NaOH buffer (pH 10.7).

titively either enzyme activity. The K_i values for 3-hydroxyhexobarbital dehydrogenase and 17β-hydroxysteroid dehydrogenase obtained by the method of Dixon [6] were $3.0 \cdot 10^{-5}$ and $1.4 \cdot 10^{-5}$ M, respectively. Thus, both K_i values were almost the same.

Test with combined substrates

Since the optimal pH conditions for the dehydrogenation of 3-hydroxyhexobarbital and testosterone were not the same, two different systems, 0.1 M NaH_2PO_4–Na_2HPO_4–$Na_4P_2O_7$ buffer (pH 8.9) and 0.1 M Na_2HPO_4–NaOH buffer (pH 10.7), were employed. The substrate concentrations used were sufficient to saturate the enzyme when added separately. As shown in Table II, the total rate of the reaction was less than the sum of the rates of the reactions measured separately.

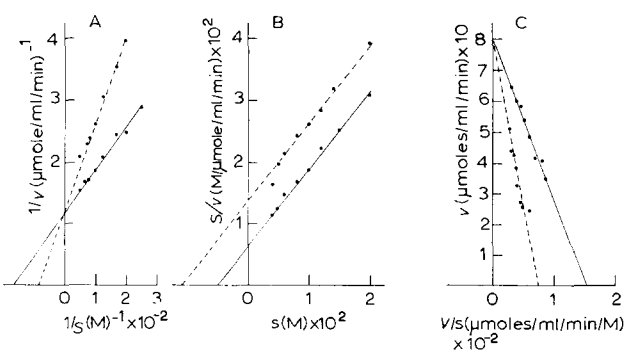


Fig. 1. Kinetic studies for the effect of testosterone on the 3-hydroxyhexobarbital dehydrogenase activity. The enzyme was preincubated with testosterone ($5 \cdot 10^{-5}$ M), or methanol in the absence of testosterone, in 0.1 M NaH_2PO_4–Na_2HPO_4–$Na_4P_2O_7$ buffer (pH 8.9) for 2 min at 25 °C. Then 3-hydroxyhexobarbital was added and left for another 2 min. The reaction was initiated by the addition of $NADP^+$ and the reaction rate was recorded. ●---●, 3-hydroxyhexobarbital dehydrogenase activity in the presence of testosterone; ●—●, 3-hydroxyhexobarbital dehydrogenase activity in the absence of testosterone; A, Lineweaver–Burk plot; B, Hofstee plot; C, Eadie plot.

Competitive inhibition of 3-hydroxyhexobarbital dehydrogenase by testosteron

The enzyme was incubated with 3-hydroxyhexobarbital in the presence and absence of testosterone ($5 \cdot 10^{-5}$ M), at pH 8.9 which is the pH optimum for 3-hydroxyhexobarbital dehydrogenase. Under these conditions, the oxidation of testosterone was so slow that the activity of 3-hydroxyhexobarbital dehydrogenase would not be affected by the activity of 17β-hydroxysteroid dehydrogenase.

The effect of testosterone to 3-hydroxyhexobarbital dehydrogenase activity was expressed by the three ways of plotting: Lineweaver–Burk plot, Hofstee plot, and Eadie plot (Fig. 1). These results indicated that the 3-hydroxyhexobarbital dehydrogenase activity was competitively inhibited by the addition of testosterone, and that the active sites of the two enzymes were identical.

DISCUSSION

During the course of an investigation of the properties of 3-hydroxyhexobarbital dehydrogenase, it was presumed that the enzyme would be concerned with not only foreign substrates such as 3-hydroxyhexobarbital, the microsomal oxygenation product of hexobarbital, but also with the metabolism of some sort of body constituents or natural compounds which are utilized frequently in the body. In other words, dehydrogenation of 3-hydroxyhexobarbital was assumed to be catalyzed by a certain enzyme participating in the metabolism of body constituents and having a wide substrate specificity.

Recently, it was demonstrated that most of C_{19}-17β-hydroxysteroids served as the substrate of purified 3-hydroxyhexobarbital dehydrogenase, and this fact was further confirmed by the experiments with electrophoresis and electrofocusing [1]. Accordingly, it was quite possible that 3-hydroxyhexobarbital dehydrogenase and 17β-hydroxysteroid dehydrogenase are identical, and that 3-hydroxyhexobarbital is metabolized by 17β-hydroxysteroid dehydrogenase.

In the preliminary study [3], we reported that partially purified 17β-hydroxysteroid dehydrogenase obtained by the method of Joshi et al. [2] was able to catalyze the dehydrogenation of 3-hydroxyhexobarbital. In the present work, the identity of guinea pig liver 3-hydroxyhexobarbital dehydrogenase with 17β-hydroxysteroid dehydrogenase was proved from the results of several experiments. In addition, kinetic studies indicated that 3-hydroxyhexobarbital and testosterone were metabolized at the same active site of the enzyme.

3-Hydroxyhexobarbital dehydrogenase was also purified from rabbit liver in this laboratory [4], and it was shown that, in the case of rabbit, the enzyme activity to dehydrogenate testosterone was separated from 3-hydroxyhexobarbital dehydrogenase during the purification steps, and that testosterone did not serve as the substrate of rabbit liver 3-hydroxyhexobarbital dehydrogenase. Moreover, 3-hydroxyhexobarbital dehydrogenases obtained from the two different origins, guinea pig liver and rabbit liver, possessed very different properties (e.g. cofactor requirement, substrate specificity, electrophoresis, optimum pH, and molecular weight).

In the studies of 3-hydroxyhexobarbital dehydrogenase, we demonstrated [3] that guinea pig liver contains another 17β-hydroxysteroid dehydrogenase which does not show the activity to 3-hydroxyhexobarbital. Studies on the purification and properties of the new 17β-hydroxysteroid dehydrogenase, especially in connection

with the role of the enzyme in the metabolism of steroids and foreign compounds, are under way in this laboratory.

REFERENCES

1 Kageura, E. and Toki, S. J. Biol. Chem., submitted
2 Joshi, S. G., Duncan, E. L. and Engel, L. L. (1963) Steroids 1, 508–527
3 Kageura, E. and Toki, S. (1971) Life Sci. 10, 469–474
4 Takenoshita, R. and Toki, S. J. Biol. Chem., submitted
5 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
6 Dixon, M. (1953) Biochem. J. 55, 170–171