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RAT LIVER 5β-CHOLESTANE-3α,7α,12α,26-TETROL DEHYDROGENASE AS A LIVER ALCOHOL DEHYDROGENASE

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SUMMARY

5β-Cholestane-3α,7α,12α,26-tetrol:NAD oxidoreductase in a soluble fraction of rat liver was studied.

1. Rat liver 5β-cholestane-3α,7α,12α,26-tetrol:NAD oxidoreductase was purified by ammonium sulfate fractionation, gel filtration, hydroxylapatite column chromatography and ion-exchange column chromatography. The overall purification attained was about 70-fold.

2. 5β-Cholestane-3α,7α,12α,26-tetrol:NAD oxidoreductase was always accompanied by liver alcohol:NAD oxidoreductase (EC 1.1.1.1) activity, and the ratio of these two enzyme activities was constant throughout the purification procedures.

3. Both 5β-cholestane-3α,7α,12α,26-tetrol:NAD oxidoreductase and liver alcohol:NAD oxidoreductase behaved similarly to thermal inactivation and also to inactivation caused by *p*-chloromercuribenzoate (PCMB).

4. The K_i value for pyrazole of 5β-cholestane-3α,7α,12α,26-tetrol:NAD oxidoreductase was found to be about 80 times that of liver alcohol:NAD oxidoreductase.

5. The possibility was discussed that both 5β-cholestane-3α,7α,12α,26-tetrol:NAD oxidoreductase and liver alcohol:NAD oxidoreductase activities may be due to a single enzyme protein.

INTRODUCTION

It was reported in the previous paper that in rat liver, 5β-cholestane-3α,7α,12α,26-tetrol (TeHC), which is an intermediate in the conversion of cholesterol to cholic acid, is dehydrogenated to 5β-cholestane-3α,7α,12α-triol-26-oic acid (THCA) via 5β-cholestane-3α,7α,12α-triol-26-al (THAL). These two dehydrogenation reactions are catalyzed by TeHC:NAD oxidoreductase and THAL:NAD oxidoreductase, respectively, which are separable by gel filtration on Sephadex G-100. Furthermore, it was also shown that TeHC:NAD oxidoreductase activity is accompanied with that of

Abbreviations: TeHC, 5β-cholestane-3α,7α,12α,26-tetrol; THAL, 5β-cholestane-3α,7α,12α-triol-26-al; THCA, 5β-cholestane-3α,7α,12α-triol-26-oic acid; PCMB, p-chloromercuribenzoate.

ethanol:NAD oxidoreductase and THAL:NAD oxidoreductase activity is accompanied with that of acetaldehyde:NAD oxidoreductase[1-3].

In this paper the further purification of rat liver TeHC:NAD oxidoreductase will be described and some evidence which favors the identity of TeHC:NAD oxidoreductase with rat liver alcohol:NAD oxidoreductase will be given.

MATERIALS AND METHODS

Tritium-labeled and unlabeled TeHC, THAL and THCA were prepared according to the method of OKUDA AND DANIELSSON[4]. NAD, NADH, and PCMB were purchased from Sigma Chemical Co. (St. Louis, Mo). Sephadex G-100 and SE-Sephadex were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxylapatite was prepared according to the method of LEVIN[5].

THAL reductase was assayed according to the chromatographic method described previously[2].

Liver alcohol:NAD oxidoreductase was assayed by the following two methods. (1) Alcohol dehydrogenase activity: to each of two cuvettes 1.8 ml of 0.1 M glycine buffer (pH 10.0), 50 μl of 20 mM NAD, 10 μl of enzyme solution and an appropriate amount of water to make the final volume 3.0 ml were added. Then 50 μl of 0.5 M ethanol was added to one cuvette and the absorbance was recorded at 340 nm by a Hitachi spectrophotometer Model 356. The enzyme unit was defined by μmoles of NADH formed per min. (2) Aldehyde reductase activity: to each of two cuvettes, 1.0 ml of Tris buffer (pH 8.0), 50 μl of 5 mM NADH, 25 μl of enzyme solution and an appropriate amount of water to make the final volume 3.0 ml were added. Then 50 μl of 0.05 M acetaldehyde was added to one cuvette, and the absorbance was recorded as described above. The enzyme unit was defined by μmoles of NADH oxidized per min.

Disc electrophoresis was performed according to the procedure of DAVIS[6]. 0.1-ml volumes of the enzyme solutions were run directly without making sample gel. Electrophoresis was carried out in an apparatus made of polystyrene with buffer chambers equipped with platinum electrodes and run in 0.04 M Tris-γ-aminobutyric acid buffer (pH 8.3) with a constant current of 2.5 mA per tube for 3–5 h in the cold. Upon completion of the run, the gels were removed from the tubes and assayed for enzymatic activities. The alcohol dehydrogenase activities were stained by incubating the gel in a medium which contained 10 mg of NAD, 5 mg of nitro blue tetrazolium, 0.5 mg of phenazine methosulfate and 20 μl of ethanol in 30 ml of 0.1 M carbonate buffer (pH 10.0). Excess stain was removed by washing in changes of dilute acetic acid. THAL reductase activities were assayed by the conventional chromatographic method after slicing and extracting the gels.

Purification of TeHC:NAD oxidoreductase: the enzyme was prepared from livers of white male rats (Wistar strain). 24 g of livers were homogenized in a Potter-Elvehjem homogenizer with 48 ml of 0.1 M phosphate buffer (pH 7.0). The homogenate was then centrifuged at 100 000 $\times$ g in a Hitachi preparative ultracentrifuge Model 55P-2 for 1 h. The supernatant (39 ml) was fractionated with $(NH_4)_2SO_4$. The precipitate obtained from the 0.3–0.6 $(NH_4)_2SO_4$ saturation was dissolved in 0.035 M phosphate buffer (pH 7.8) and dialyzed against 0.007 M phosphate buffer (pH 7.8) for 20 h. 16.4 ml of the dialyzed solution was put on the Sephadex G-100

column (3.0 × 140 cm) and eluted with 2 l of 0.01 M phosphate buffer (pH 7.8). Fractions of 10 ml were collected at a flow rate of about 15 ml per h and were tested for TeHC:NAD oxidoreductase and liver alcohol:NAD oxidoreductase activities. The fractions from 380 ml to 440 ml were combined and placed on a hydroxylapatite column (1.7 × 15 cm). The column was eluted with 0.05 M phosphate buffer (pH 7.8), 0.1 M phosphate buffer (pH 7.8) and 0.15 M phosphate buffer (pH 7.8), successively. Both TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase activities were found in the eluate of 0.1 M phosphate buffer. This eluate was dialyzed against 0.01M Tris buffer (pH 7.5) for 20 h, and then put on the column (1.7 × 15 cm) of SE-Sephadex equilibrated with 0.01 M Tris buffer (pH 7.5). The column was washed with 60 ml of 0.01 M Tris buffer (pH 7.5) and then eluted with 70 ml of 0.1 M Tris buffer (pH 7.5) and 70 ml of 0.1 M Tris buffer (pH 8.5). Both enzyme activities were found only in the initial washings.

RESULTS

TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase in the gel filtration eluate: Both enzyme activities in the gel filtration eluate of the 0.3–0.6 ammonium sulfate fraction of rat liver extract were found in the same fraction and the profiles of both activities coincided with each other in accordance with the previous reports[2,3].

TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase in the eluate from the hydroxyl apatite column: Fig. 1 shows the chromatogram obtained from the hydroxylapatite column chromatography. Both TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase activities were observed only in the eluate with 0.1 M phosphate buffer. Neither activity was found in the eluate with 0.01 M, 0.05 M and 0.15 M phosphate buffer.

TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase in the eluate from

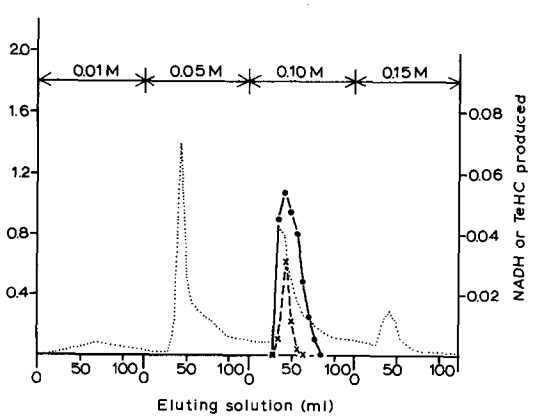

Fig. 1. Column chromatography of TeHC:NAD oxidoreductase on hydroxylapatite. The eluate of Sephadex G-100 which contained both THAL reductase and alcohol dehydrogenase activities was applied to a hydroxylapatite column (1.7 × 15 cm). 7-ml fractions were collected at a rate of 15 ml per h. Protein was measured by reading absorbance at 280 nm, and enzymatic activities were assayed as described in the text. ·····, protein concentration; ●—●, THAL reductase (μmoles/3 min./10 μl of fraction); ×— — —×, alcohol dehydrogenase (μmoles/min./10 μl of fraction).

TABLE I

SUMMARY OF ENZYME PURIFICATION

Step	*Volume (ml)*	*Total units of alcohol dehydrogenase*	*Specific activity (units/mg protein)*	*Total units of THAL reductase*	*Specific activity (units/mg protein)*	*Alcohol dehydrogenase/ THAL reductase*
Initial extract	39.0	180.6	0.08	—	—	—
$(NH_4)_2SO_4$ ppt.	16.4	141.5	0.15	—	—	—
Sephadex G-100	66.7	141.4	1.40	48.6	0.48	2.9
Hydroxylapatite	44.3	106.8	3.81	31.4	1.12	3.4
SE-Sephadex	41.5	95.9	5.78	29.3	1.77	3.3

the cation-exchange column: When cation-exchange column chromatography was carried out at pH 7.5, both enzyme activities were found only in the initial washings. A change of the pH of the column to lower values (*i.e.* pH 6.0–7.0) or a change of molarity of the buffer to lower values (*i.e.* 0.005 M) seriously reduced the recovery of the activities. Use of DEAE-Sephadex or QAE-Sephadex gave results similar to those obtained with the cationic exchanger, at least within the pH range used (pH 6.0–8.0).

The extent of purification and the ratios of TeHC:NAD oxidoreductase activity to alcohol:NAD oxidoreductase activity at each purification step: The specific activities and yields of both enzyme activities at each purification step are shown in Table I. THAL reductase activities of initial extract and $(NH_4)_2SO_4$ fraction were not assayed because coenzyme-linked dismutation, as described previously[3], might disturb the results.

As shown in the table, the overall purification was 72-fold. The ratio of TeHC: NAD oxidoreductase and ethanol:NAD oxidoreductase activities was almost constant as shown in the table throughout the purification procedures.

Changes of TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase activities by inactivation treatment: (1) Heat inactivation. The enzyme solution was heated at 50°, 60°, and 70° for 10 min and both enzyme activities were then assayed. As shown in Table II, TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase activities were slightly affected by heating at 50° for 10 min, whereas they were considerably reduced at 60° and were completely lost at 70°. Furthermore, both enzyme activities

TABLE II

CHANGES OF THAL REDUCTASE AND ALCOHOL DEHYDROGENASE ACTIVITIES BY THERMAL TREATMENT

No.	*Temperature*	*THAL reductase*		*Alcohol dehydrogenase*	
		unit × 10^3	*%*	*unit* × 10^3	*%*
1	2°	5.32	100	3.76	100
2	50°	4.04	76	2.90	77
3	60°	0.92	17	0.70	19
4	70°	0	0	0	0

TABLE III

CHANGES OF THAL REDUCTASE AND ALCOHOL DEHYDROGENASE ACTIVITIES BY PCMB TREATMENT

No.	Concn. of PCMB in preincubation mixture (μM)	THAL reductase		Alcohol dehydrogenase	
		unit × 10^3	%	unit × 10^3	%
1	0	5.00	100	3.28	100
2	1.0	4.18	84	2.85	87
3	2.5	1.50	30	0.82	25
4	5.0	0	0	0	0

were reduced to similar extents at each temperature. From these results it may be concluded that both enzyme activities behave similarly to thermal treatment.

(2) Inactivation by PCMB. The enzyme solutions were preincubated with various amounts of PCMB for 10 min and the solutions then diluted to the appropriate amount, and both TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase were assayed at the same time. The results are shown in Table III. Both enzyme activities were slightly affected by treatment of 1 μM PCMB, whereas they were completely abolished by 5 μM PCMB. The extents of reduction of both enzyme activities were similar at each PCMB concentration. These results seem to suggest that TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase behave similarly to PCMB treatment.

K_i values for pyrazole obtained by both THAL reductase and acetaldehyde reductase assays: According to DIXON AND WEBB[7], it is a useful criterium of enzyme substrate specificities to compare K_i values for a competitive inhibitor obtained using different substrates. K_i values of both enzymes were obtained by the graphical method of DIXON *et al.* (Fig. 2 and Fig. 3). The K_i value for pyrazole of acetaldehyde reductase was 0.25 μM, whereas that of THAL reductase was 20 μM. Apparently the K_i values obtained by two different methods were different.

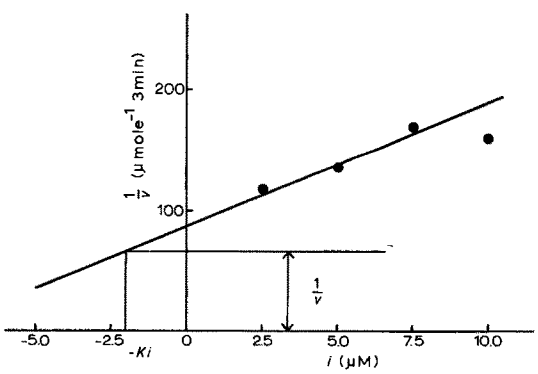

Fig. 2. K_i value for pyrazole of THAL reductase. The incubation mixture contained 0.06 μmole of THAL, 50 μmoles of Tris buffer (pH 8.0), 0.5 μmole of NADH, 0.5 μl of enzyme solution (the eluate of hydroxylapatite column) and varying amount of pyrazole in a final volume of 1.5 ml. The reaction was carried out for 3 min at 37°.

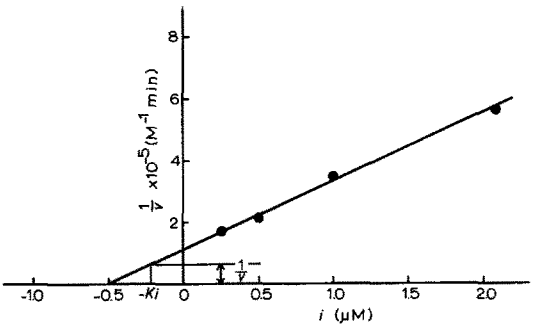

Fig. 3. K_i value for pyrazole of acetaldehyde reductase. The incubation mixture contained 1.0 ml of Tris buffer (pH 8.0), 0.25 μmole of NADH, 2.5 μmole of acetaldehyde, 25 μl of enzyme solution (the eluate of hydroxylapatite column) and varying amount of pyrazole in a final volume of 3.0 ml. Changes of absorbance at 340 nm were recorded by a spectrophotometer equipped with recorder.

Disc electrophoresis of TeHC:NAD oxidoreductase and alcohol:NAD oxidoreductase: The eluates from Sephadex G-100, hydroxylapatite and SE-Sephadex columns were subjected to disc electrophoresis, and the enzyme activities were located by either staining for alcohol:NAD oxidoreductase activity (ethanol dehydrogenase activity) or by assaying TeHC:NAD oxidoreductase activity (THAL reductase activity) after eluting the sliced gel with a small amount of 0.01 M phosphate buffer (pH 7.0). As a result it was found that each eluate gave a single band on staining for alcohol dehydrogenase activity where THAL reductase activity was also observed, while neither of the two enzyme activities was found anywhere else.

DISCUSSION

On the basis of the findings that the activity of THAL reductase and that of liver alcohol dehydrogenase showed a similar profile on gel filtration, it was suggested in the previous paper that these enzyme activities are due to the same enzyme[2,3]. However, at the time the possibility was not excluded that two different enzymes having a similar molecular weight or size are merely mixed together. In the present experiment it was attempted to purify the enzyme and to verify whether this is the case.

As described in the preceding section, throughout the purification procedures such as ammonium sulfate fractionation, gel filtration, hydroxylapatite and SE-Sephadex column chromatography, THAL reductase activity was always accompanied by liver alcohol dehydrogenase activity, and the latter activity was not observed anywhere else. The ratio of THAL reductase activity to that of liver alcohol dehydrogenase was constant throughout the purification steps, and each of the eluates from Sephadex G-100, hydroxyl apatite and SE-Sephadex column revealed a single band on disc electrophoresis located by the two enzyme activities. Furthermore, both enzyme activities were reduced to similar extents by heating and PCMB treatment. These results seem to suggest that THAL reductase and acetaldehyde reductase activities are due to the same enzyme. The reason why the K_i values for pyrazole obtained by the two enzyme assays are different from each other can not be answered

at present. However, if we take into account the facts that the chemical structure of THAL is quite different from that of acetaldehyde except for the aldehyde group, and that the physical significance of K_i in the case of a coenzyme–substrate reaction is more complex than in the case of a single substrate reaction, the present results do not necessarily seem to be incompatible with the above conclusion.

In 1965 WALLER *et al.*[8] reported that horse liver alcohol dehydrogenase could oxidize 3β-hydroxy-5β-cholan-24-oic acid to 3-keto-5β-cholan-24-oic acid, and it could also oxidize the bile alcohol having a primary alcohol group such as TeHC, although they did not identify the reaction product(s).

PIETRUSZKO *et al.*[9] separated a commercial liver alcohol dehydrogenase preparation by gel electrophoresis and ascribed the steroid dehydrogenase activity of liver alcohol dehydrogenase preparation to some steroid active fractions which move faster than the ethanol-active one. Soon after that, THEORELL *et al.*[10] isolated and crystallized a basic subfraction of liver alcohol dehydrogenase ($LADH_S$) which possessed both 3β-hydroxy-5β-cholan-24-oic acid dehydrogenase and ethanol dehydrogenase activities, while the ethanol-active fraction (which is the conventional liver alcohol dehydrogenase and was described as $LADH_E$) was free from 3β-hydroxy-5β-cholan-24-oic acid dehydrogenase activity. In 1969 PIETRUSZKO *et al.*[11] reported that horse liver alcohol dehydrogenase occurs in multiple, electrophoretically separable forms; $LADH_E$ (EE) consists of two identical halves (E, for activity with ethanol), and $LADH_S$ (ES) is a hybrid consisting of one subunit E and one subunit S (S, for activity on 3β-hydroxysteroid).

Recently it was found in our laboratory that TeHC is oxidized not only by $LADH_S$ but also by $LADH_E$ in the presence of NAD, while 3β-hydroxy-5β-cholan-24-oic acid is oxidized only by $LADH_S$ and not by $LADH_E$ in accordance with THEORELL's observation[10]. This shows that TeHC resembles ethanol as substrate for liver alcohol dehydrogenase ($LADH_E$ and $LADH_S$) whereas it does not resemble 3β-hydroxy-5β-cholan-24-oic acid. (The symbols $LADH_E$ and $LADH_S$ or EE and SS are now confusing. Therefore, they might be changed.)

In the present experiment we could not find isoenzyme(s) of rat liver alcohol dehydrogenase, at least in the experimental conditions applied. When disc electrophoresis was performed with conventional glycine buffer, the alcohol dehydrogenase did not move from the starting point. A buffer which has a pK_a value higher than glycine (pK_a 9.78) was sought, and γ-aminobutyric acid (pK_a 10.56) was found to give a satisfactory result. When this buffer was used the alcohol dehydrogenase moved sufficiently, but isoenzyme(s) was not observed. It is concluded, therefore, that if there is any isoenzyme in rat liver alcohol dehydrogenase, it may have similar properties to the alcohol dehydrogenase presently described.

Our present results are relevant to the recent findings obtained in our laboratory with horse liver LADH. It may well be suggested from these results that one of the natural substrates of liver alcohol dehydrogenase is TeHC, and the enzyme is functioning on cholesterol metabolism.

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