OXIDATION OF ω-HYDROXYLATED FATTY ACIDS AND STEROIDS BY ALCOHOL DEHYDROGENASE

Ingemar Björkhem, Hans Jörnvall and Eila Zeppezauer

Kemiska Institutionen, Karolinska Institutet, Stockholm and

Kemiska Institutionen, Lantbrukshögskolan, Uppsala, Sweden

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SUMMARY

Recrystallized alcohol dehydrogenase from horse liver was found to oxidize 17-hydroxystearic acid into 17-oxostearic acid, the 17-L-enantiomer faster than the 17-D-enantiomer. Alone at high pH or in combination with aldehyde dehydrogenase, the alcohol dehydrogenase also catalyzed conversion of 18-hydroxystearic acid into 1,18-octadecadioic acid and 5β-cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid. All the activities as well as the ethanol dehydrogenase activity disappeared after specific carboxymethylation of a single cystein residue at the active site of alcohol dehydrogenase. These results conclusively show that alcohol dehydrogenase itself has ω-hydroxyfatty acid dehydrogenase activity and ω-hydroxysteroid dehydrogenase activity.

In a previous communication (1) it was shown that preparations of alcohol dehydrogenase (ADH, ethanol:NAD oxidoreductase, EC 1.1.1.1) from horse liver (HLADH) and rat liver (RLADH) were able to catalyze oxidation of ω2-hydroxylated long-chain fatty acids into the corresponding ω2-oxofatty acids. HLADH and RLADH, either alone at high pH or in combination with aldehyde dehydrogenase (1,2), were also found to catalyze conversion of long-chain ω1-hydroxylated fatty acids into the corresponding dicarboxylic acids. The mechanism of formation of a carboxylic acid in experiments where only ADH was used, probably involves dismutation of a primarily formed aldehyde (cf. 3). As ethanol inhibited oxidation of ω-hydroxyfatty acids by RLADH and vice versa it seems most probable that the ω-hydroxyfatty acid dehydrogenase activity was due to ADH itself and not to contami-

nating enzymes. In order to prove this conclusively, the ω-hydroxyfatty acid dehydrogenase activity of HLADH, specifically carboxymethylated at the active site cystein residue (4-6) was studied. A loss of this activity should then prove that HLADH itself has ω-hydroxyfatty acid dehydrogenase activity and that ω-hydroxyfatty acids bind to the enzyme at the same site as ethanol. In the present work, therefore, 17-hydroxystearic acid and 5β-cholestane-3α,7α,12α,26-tetrol were incubated with native recrystallized HLADH as well as with HLADH specifically carboxymethylated at the active site. 5β-Cholestane-3α,7α,12α, 26-tetrol was included as substrate as it has been reported that the ω-hydroxyl group in the side chain in this steroid is oxidized by HLADH (7,8).

EXPERIMENTAL PROCEDURE

HLADH (Boehringer Mannheim GmbH, Germany (mainly EE-isoenzyme (6)) was dissolved (10 mg/ml) in 0.05 Tris-Cl, pH 8.4, 0.5 M KCl, and crystallized by dialysis against 0.05 Tris-Cl containing 30% ethanol. The crystalline material was dialyzed against 5 mM imidazole, pH 7.8 in order to stimulate carboxymethylation (5). The latter was performed by addition of 75 µl 0.05 M ^{14}C-iodoacetate to 10 ml dialysis buffer per 10 mg protein (15-fold molar excess of iodoacetate per protein subunit). After 48 hours at 4°C the material was dialyzed against several changes of 0.05 Tris-Cl, pH 8.0. The specificity of carboxymethylation was checked by tryptic digestion and autoradiography, which showed that the only cystein residue alkylated was the cys-46 in HLADH (6). Noncarboxymethylated samples of HLADH were treated similarily, except that no iodoacetate was added. The activity of the carboxymethylated HLADH against ethanol was less than 5% of the corresponding activity of the native HLADH.

18-Hydroxystearic acid, 17-D,L-hydroxystearic acid, 9D,L-hydroxydecanoic acid and 10-hydroxydecanoic acid were the same compounds as used previously (1). 17-L- and 17-D-hydroxystearic acids were generous gifts by Professor Tulloch (Prairie Regional Laboratory, Saskatchewan, Canada). Tritium-labeled 5β-cholestane-3α,7α,12α,26-tetrol (specific radioactivity 8 μC/mg) was prepared by exposure of unlabeled 5β-cholestane-3α,7α,12α, 26-tetrol to tritium gas according to Wilzbach (9). ω1-Hydroxyfatty acid dehydrogenase activity (18-hydroxystearic acid as substrate), ω2-hydroxyfatty acid dehydrogenase activity (17-L-hydroxystearic acid as substrate) and ethanol dehydrogenase activity were assayed as described previously (1) and aldehyde dehydrogenase was added in the case of determination of ω1-hydroxyfatty acid dehydrogenase activity. In some incubations with ω1-hydroxyfatty acid, however (cf. Results), no aldehyde dehydrogenase was added and in these experiments a 0.1 M glycine buffer, pH 10.0, was used instead of 0.1 M Tris-Cl, pH 9.5. ω-Hydroxysteroid dehydrogenase activity was assayed with tritium-labeled 5β-cholestane-3α,7α,12α,26-tetrol as substrate and the incubation conditions were the same as those with 18-hydroxystearic acid, both with and without aldehyde dehydrogenase. The incubation mixture was extracted with ether, the ether extract treated with diazomethane and analyzed by radio-gas chromatography after trimethylsilylation (1). In all cases conversions were calculated from experiments in which linearity between dehydrogenation and enzyme concentration was observed. In some experiments the products from the incubations (methyl esters of 17-oxostearic acid and 1,18-octadecadioic acid, methyl ester-trimethylsilyl ether of 3α,7α,12α-trihydroxy-5β-cholestanoic acid) were identified with combined gas chromato-

graphy-mass spectrometry using the LKB 9000 instrument equipped with a 1.5% SE-30 column. The Lowry method was used for determination of protein (10).

RESULTS

Oxidation of 17-L- and 17-D-hydroxystearic acid

The HLADH was found to catalyze oxidation of 17-L-hydroxystearic acid efficiently. Under the conditions employed (4 µg HLADH and 50 µg substrate) the conversion was linear with time up to 20 min. With an incubation time of 15 min the conversion was linear with enzyme up to 6 µg of HLADH. The K_m for the reaction varied in different experiments with different preparations of HLADH between 1.0×10^{-5} M and 2.5×10^{-5} M. The capacity of the HLADH to catalyze oxidation of 17-L-hydroxystearic acid was about 0.5 µmoles/mg protein/min. The rate of oxidation of 17-L-hydroxystearic acid was about ten times faster than the rate of oxidation of 17-D-hydroxystearic acid and about twice faster than the rate of oxidation of 17-D,L-hydroxystearic acid. The oxidation of 17-L-hydroxystearic acid by HLADH was inhibited by about 50% after addition of ethanol at a concentration of 0.7 mM. The rate of oxidation of 17-L-hydroxystearic acid by carboxymethylated HLADH was less than 5% of that obtained with native HLADH.

Oxidation of 9-D,L-hydroxydecanoic acid and 10-hydroxydecanoic acid

When 9-D,L-hydroxydecanoic acid and 10-hydroxydecanoic acid were tested as substrates for HLADH, the rate of oxidation was less than 1% of the rate of oxidation obtained with 17-L-hydroxystearic acid and 18-hydroxystearic acid.

Oxidation of 18-hydroxystearic acid

HLADH catalyzed oxidation of 18-hydroxystearic acid into

1,18-octadecadioic acid when combined with rat liver aldehyde dehydrogenase as described previously (1). The rate of conversion in the presence of optimal amounts of aldehyde dehydrogenase was about 1 μmole/mg protein/min. HLADH alone was found to catalyze oxidation of 18-hydroxystearic acid into 1,18-octadecadioic acid when the reaction was performed in a glycine buffer of pH 10.0. The rate of conversion was considerably slower under these conditions, about 0.3 μmoles/mg protein/min. When the native HLADH was substituted with carboxymethylated HLADH, the rate of oxidation was only about 5% of that obtained with native HLADH.

Oxidation of 5β-cholestane-3α,7α,12α,26-tetrol

HLADH in combination with rat liver aldehyde dehydrogenase was found to catalyze oxidation of 5β-cholestane-3α,7α, 12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid. The rate of oxidation was about 0.3 μmoles/mg protein/min. HLADH alone was found to catalyze oxidation of 5β-cholestane-3α,7α,12α,26-tetrol into the corresponding acid provided that the reaction was carried out in glycine buffer of pH 10.0 and that a large amount of enzyme was used. Under these conditions the rate of conversion was about 0.01 μmoles/mg protein/min. With 150 μg of HLADH and 50 μg of substrate, the reaction was linear with time up to 20 min. With an incubation time of 15 min the conversion was linear with enzyme up to about 300 μg of HLADH. Under the conditions employed the enzyme was saturated with substrate and the apparent K_m for conversion of 5β-cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid was about 2×10^{-5} M (cf. 7). When native HLADH was substituted with carboxymethylated HLADH the rate of oxidation was less than 5% of the rate of oxidation with native HLADH.

DISCUSSION

The present work conclusively shows that ADH exhibits ω-hydroxyfatty acid dehydrogenase activity and ω-hydroxysteroid dehydrogenase activity. This conclusion is based on the fact that a specific carboxymethylation of a single cystein in the enzyme preparation inhibited the ω-hydroxyfatty acid dehydrogenase activity, ω-hydroxysteroid dehydrogenase activity and ethanol dehydrogenase activity to about the same extent. Since different preparations of HLADH (1) did not have higher activity than the HLADH used in the present work and since no S-chain peptides (11) were detected in peptide maps of the preparation it is likely that isoenzyme $LADH_{EE}$ is responsible for the main part of the ω-hydroxyfatty acid dehydrogenase activity and ω-hydroxysteroid dehydrogenase activity in HLADH (cf. 8).

The properties of the ω-hydroxyfatty acid dehydrogenase activity now established to be intrinsic to HLADH, were similar to those previously detected (1). Thus, 17-L-hydroxystearic acid was oxidized at a much faster rate than 17-D-hydroxystearic acid and hydroxyfatty acids with a chain length of 10 carbon units were not oxidized to a significant degree (cf. 1). The K_m value for oxidation of 17-L-hydroxystearic acid was similar to the corresponding value obtained from experiments with RLADH. The concentration of ethanol needed to inhibit oxidation of 17-L-hydroxystearic acid by HLADH was about ten-fold lower than the concentration of ethanol needed to inhibit oxidation of 17-D,L-hydroxystearic acid by a preparation of RLADH (1). The difference might be due to the possibility that the K_m for oxidation of ethanol by HLADH (K_m = 0.5-2mM (12)) is lower than the K_m for oxidation of ethanol

by RLADH (K_m = 2-40 mM (13,14)). The specific activity (μmoles ethanol oxidized per mg protein per min) was also more than 20-fold higher in the HALDH than in the RLADH (1). X-Ray crystallographic studies on the tertiary structure of HLADH (15) show that the size of the substrate binding site, at least before addition of cofactor, permits much larger molecules than ethanol as substrates. This is to be expected since compounds like retinol (16), some 3β-hydroxylated 5β-steroids (7) as well as ω-hydroxylated steroids and fatty acids ((1), (7), (8), and this study) are efficiently oxidized by HLADH. The K_m values for oxidation of all these compounds by ADH are also considerably lower than the K_m for oxidation of ethanol by ADH (1,7,8,16).

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