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MODULATION OF 3α-HYDROXYSTEROID DEHYDROGENASE ACTIVITY BY THE REDOX STATE OF GLUTATHIONE

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3α-Hydroxysteroid dehydrogenase (EC 1.1.1.50), purified to homogeneity from rat liver, was strongly inactivated by incubation with a disulfide such as GSSG, *L*-cystine or *L*-cystamine, as well as an SH-reagent such as DTNB (5,5′-dithiobis(2-nitrobenzoic acid)), NEM (N-ethylmaleimide) or iodoacetic acid. The inactivation advanced with incubation time. Coenzyme ($NADP^+$) completely protected the enzyme from this inactivation by disulfides, but neither of the substrates (androsterone and benzenedihydrodiol) did. The activity of inactivated enzyme was restored by treatment with thiols such as DTT (dithiothreitol) or GSH. In the GSH/GSSG redox buffer, the enzyme existed in an equilibrium between active (reduced) and inactive (oxidized) forms.

KEY WORDS: 3α-hydroxysteroid dehydrogenase, dihydrodiol dehydrogenase, GSSG, GSH, thiol/disulfide exchange reaction, active site.

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INTRODUCTION

Recently 3α-hydroxysteroid dehydrogenase, a well-known steroid metabolizing enzyme catalyzing the conversion of androsterone to androstanedione, has been noted for its ability to catalyze the oxidation of *trans*-dihydrodiol to *o*-quinone in detoxication of aromatic hydrocarbons.[1–5] These findings suggested that this enzyme plays an important role in suppression of the anti-diol formation of polycyclic aromatic hydrocarbons.[1–3] In addition, it is well-known that aromatic hydrocarbons induce depletion of cellular GSH[6] in the metabolic process, and that this might be one reason for induction of carcinogenesis.[7] Furthermore, it has been reported that hydroxyl radicals are generated in the course of benzene metabolism.[8] These reports, taken together, suggest that an oxidative stress may be imposed on the cell by the metabolism of polycyclic aromatic hydrocarbons.

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Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; DTT, dithiothreitol; cAMP, 3′,5′-cyclic adenosine monophosphate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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It has been suggested that the thiol/disulfide exchange reaction between proteins and low-molecular-weight disulfides is dependent mainly on the concentration ratio of cellular thiols and disulfides,[9] e.g., reduced and oxidized glutathione ([GSH]/[GSSG]). In view of the presence of accessible thiols or disulfides in many proteins, post-translational modification might lead to alteration of redox state protein.[10] Thus, there can be a control mechanism by which many metabolic enzymes vary their activities through thiol/disulfide exchanges according to the redox state.

3α-Hydroxysteroid dehydrogenase is known to be strongly inhibited by SH-reagents.[1,11,12] The protective effect of GSH against *o*-quinone inactivation and the result of affinity labeling experiments suggested that this enzyme has an essential thiol group.[4,13] In the present work, we demonstrated that the activity of rat liver 3α-hydroxysteroid dehydrogenase is modulated by the [GSH]/[GSSG] ratio.

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MATERIALS AND METHODS

Materials

GSSG, *L*-cystamine, *L*-homocystine and *S*-(+)-indanole were purchased from Sigma Chemicals. *L*-cystine, GSH and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Wako Pure Chemical Industries, Ltd. and iodoacetic acid, *N*-ethylmaleimide (NEM) and dithiothreitol were from Nacalai Tesque. NAP-column[R] was commercially available from Pharmacia-LKB Biotechnology. *S*-Sulfocysteine was prepared according to the method of Segal *et al.*[14] Benzenedihydrodiol (*trans*-1,2-dihydro-benzene-1,2-diol) and *trans*-naphthalenedihydrodiol were synthesized by the method of Platt and Oesch.[15,16] Other reagents used were of the highest grade commercially available.

Enzyme Activity

Unless otherwise specified, 3α-hydroxysteroid dehydrogenase activity was measured under the following conditions as described by Penning *et al.*[2]; 100 μM androsterone, 67 μM $NADP^+$, and 100 mM glycine/NaOH buffer, pH 9.0 (standard assay system). The assay of the reductase activity was carried out in the following mixture; 100 μM androstanedione, 67 μM NADPH, and 100 mM sodium phosphate buffer, pH 7.0. The dihydrodiol dehydrogenase activity was measured by using 2 mM dihydrodiol compound in place of androsterone in the same mixture. Activities of aldehyde reductase (EC 1.1.1.2) and DT-diaphorase (EC 1.6.99.2) were measured by the spectrophotometric method as reported by Flynn[17] and Wallin[18] using D-glucuronate and menadione, respectively. All the assays were initiated by addition of the enzyme solution to the reaction mixture and monitoring the absorbance at 340 nm at 25°C. One unit is defined, for all the enzymes, as the activities which consume 1 μmole substrate per min.

Enzyme Preparation

3α-Hydroxysteroid dehydrogenase and aldehyde reductase were purified from the liver cytosol of 7-week old rats essentially following the method previously described by Penning *et al.*[2] DT-diaphorase was purified from the rat liver cytosolic fraction

RIGHTSLINK

by the method of Wallin.[18] These three enzyme preparations showed single bands on 12.5% SDS-PAGE with silver staining.

Treatment of 3α-Hydroxysteroid Dehydrogenase with Disulfides and SH-reagents

Prior to treatment with SH-reagent or disulfide, the enzyme was reduced by 1 mM DTT for 30 min at 25°C. Then the enzyme solution was gel-filtered by using a NAP-column^R for elimination of excess DTT. Reduced enzyme was subjected to the treatment with SH-reagent (5 mM iodoacetic acid, 0.5 mM NEM or 0.1 mM DTNB) or disulfide (5 mM GSSG, 5 mM *L*-cystamine, 5 mM *S*-sulfocysteine, 1 mM *L*-cystine or 1 mM *L*-homocystine). Aliquots of the reaction mixture were taken for measurement of the enzyme activity at appropriate intervals.

Treatment of 3α-Hydroxysteroid Dehydrogenase with Redox Buffer System

DTT-treated enzyme was added to redox buffer, which was made by changing the ratio of [GSH]/[GSSG] (from 0 to 50), fixing the total gluthione concentration at 10 mM. Then, the mixture was incubated at 25°C for 24 h under nitrogen gas. Throughout the incubation under this condition the [GSH]/[GSSG] ratio did not alter.

RESULTS

Characterization of Purified Rat Liver 3α-Hydroxysteroid Dehydrogenase

The enzyme was purified 300 fold from the cytosolic fraction of rat liver. Homogeneity of the final preparation was indicated by SDS-PAGE as shown in Figure 1. Its specific activity was 6 units/mg protein using androsterone as the substrate. The relative

Figure 1 SDS-Polyacrylamide Gel Electrophoresis of 3α-Hydroxysteroid Dehydrogenase. Purified 3α-hydroxysteroid dehydrogenase (1 μg) was subjected to 12.5% SDS-PAGE. After electrophoresis, the protein band was visualized by the silver staining.

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Table 1 Substrate specificity of 3α-hydroxysteroid dehydrogenase

Substrate	Conc. (mM)	Relative velocity (%)	K_m (μM)	V_{max} (units/ml)
Dehydrogenase activity				
Androsterone	0.1	100	1.60±0.25	1.89±0.22
Testosterone	0.1	0	—	—
Benzenedihydrodiol	1.0	39	960±100	0.93±0.093
Naphthalenedihydrodiol	1.0	2.5	1510±270	0.16±0.027
Indanole	2.0	5.2	2440±230	0.16±0.027
Reductase activity				
Androstanedione	0.1	241	2.0±0.23	3.97±0.53
4-Benzoylpyridine	1.0	67	—	—
4-Nitroacetophenone	0.5	675	19±2.4	8.03±2.1
Phenanthrenequinone	0.1	988	79±12	13.5±1.9
Camphorquinone	0.1	1470	1.1±0.21	20.4±2.0
1,4-Benzoquinone	0.1	7	—	—
4-Nitrobenzaldehyde	0.1	554	154±32	7.67±1.5
D-Glucuronate	3.3	7	—	—

—, Not determined. Assays were carried out as described in the text. K_m and V_{max} values are the mean (±S.D.) of 3 or 4 determinations.

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molecular mass of purified enzyme was estimated to be 32 kD, and thus the enzyme consisted of monomer. The substrate specificity of this enzyme was as summarized in Table 1. The enzyme had an ability to reduce various carbonyl compounds including androstanedione, 4-nitroacetophenone, 4-benzoylpyridine, camphorquinone, phenanthrenequinone and 4-nitrobenzaldehyde with NADPH. But the enzyme could not reduce *p*-benzoquinone (1,4-benzoquinone) and D-glucuronate efficiently. Alternatively the enzyme was able to catalyze the dehydrogenation of androsterone and benzenedihydrodiol, but not testosterone, in the presence of $NADP^+$. Apparent K_m values for androsterone and benzenedihydrodiol were estimated to be about 1.6 and 960 μM. These K_m values were very similar to those previously reported.[11,19] The substrate specificity and relative molecular mass suggested that this enzyme was identical with 3α-hydroxysteroid/dihydrodiol dehydrogenase reported by Smithgal *et al.*[20]

Inhibition of 3α-Hydroxysteroid Dehydrogenase by SH-Reagents and Disulfides

The enzyme was incubated with SH-reagent for 15 min and the remaining activity measured. The results in Table 2 showed that alkylating reagents (NEM and iodoacetic acid) were potent inhibitors of the enzyme. Disulfides such as GSSG, *L*-cystamine and DTNB, were also very effective inhibitors. When benzenedihydrodiol was used as substrate, 3α-hydroxysteroid dehydrogenase was inactivated in the same manner as that for androsterone (data not shown). During the 15 min disulfide treatment, the enzyme lost about 50% of its original activity, and this lost activity was restored fully (about 94 or 95%, respectively) by the addition of 10 mM GSH or DTT. These results suggested that 3α-hydroxysteroid dehydrogenase contained essential cysteine

RIGHTSLINK

Table 2 Inactivation of 3α-hydroxysteroid dehydrogenase, aldehyde reductase and DT-diaphorase by disulfides and SH-reagents

Compound	Conc. (mM)	Remaining activity (%) HSD	ALR	DT-diaphorase
None	—	100.0	100.0	100.0
GSSG	5.0	46.7±2.9	99.5±0.8	101.6±1.5
L-cystamine	5.0	49.9±2.5	—	—
S-Sulfocysteine	5.0	54.5±2.1	—	—
Iodoacetic acid	5.0	43.8±2.6	95.0±4.1	90.3±3.4
N-Ethylmaleimide	0.5	26.3±3.6	—	—
DTNB	0.1	9.3±2.6	—	—

—, Not determined. Purified 3α-hydroxysteroid dehydrogenase (HSD), aldehyde reductase (ALR) and DT-diaphorase were incubated with disulfide or SH-reagent as specified for 15 min at 25°C, and then the enzyme activity was measured using androsterone, D-glucuronate or menadione, respectively, as described in the text. The values are the mean (±S.D.) of 3 or 4 determinations.

residue(s) for the activity and also that the modification of this cysteine residue(s) by SH-reagents or disulfides caused inactivation of the enzyme. However, aldehyde reductase and DT-diaphorase are not sensitive to these reagents (Table 2), suggesting that these enzymes did not have essential cysteine residue(s) at or in the vicinity of their active sites.

Effects of Coenzyme and Substrate on Inactivation of 3α-Hydroxysteroid Dehydrogenase

Table 3 shows the results of inactivation of 3α-hydroxysteroid dehydrogenase with 5 mM GSSG at 25°C in varying incubation times with or without coenzyme (100 μM $NADP^+$) or substrate (100 μM androsterone or 1 mM benzenedihydrodiol). As shown in Table 3, the coenzyme ($NADP^+$) was an effective protector of the enzyme from inactivation, but neither of the substrates protected the enzyme. Considering the specificity of coenzyme and the specific protective effect of coenzyme on inactivation, it was deduced that cysteine residue(s) of the enzyme might be located at or in the vicinity of the coenzyme binding site.

Table 3 Effect of substrate and coenzyme on inactivation of 3α-hydroxysteroid dehydrogenase

Compound	Conc. (mM)	Remaining activity (%) 10 min	15 min	30 min
No addition		63.2±2.2	49.8±1.0	22.1±1.9
Androsterone	0.1	65.1±3.2	51.4±2.9	24.1±4.4
Benzenedihydrodiol	1.0	61.4±2.1	47.8±3.3	22.5±1.6
$NADP^+$	0.1	94.5±3.1	95.9±5.3	92.7±3.4

The purified enzyme was incubated with 5 mM GSSG at 25°C in the absence or presence of 100 μM androsterone, 1 mM benzenedihydrodiol or 100 μM $NADP^+$. The values are the mean (±SD.) of 3 or 4 determinations of remaining activity at indicated time points.

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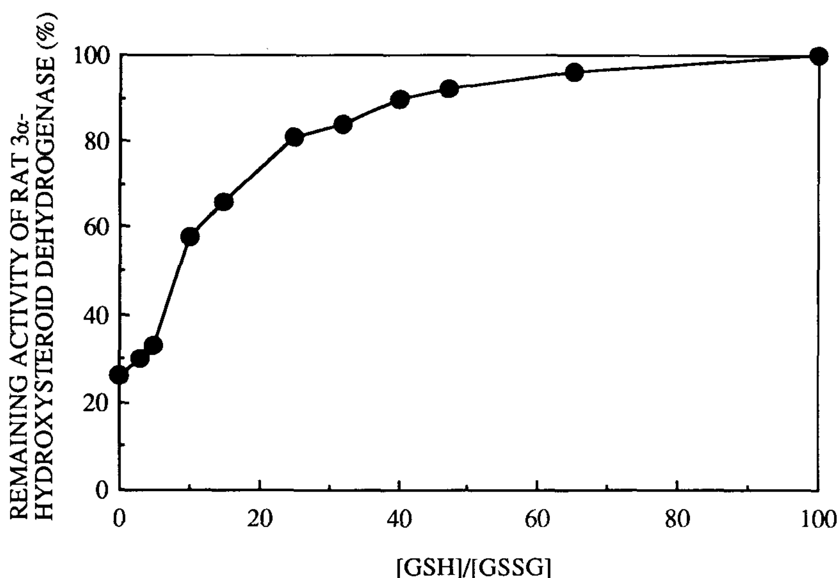

Figure 2 Redox Equilibration of 3α-Hydroxysteroid Dehydrogenase Activity with [GSH]/[GSSG] ratio. Purified 3α-hydroxysteroid dehydrogenase was incubated in the buffer containing various ratios of [GSH]/[GSSG] for 24 h at 25°C under anaerobic conditions. After incubation, the enzyme activity was measured. The remaining activity was expressed as a percentage of the original activity for the average of 2 experiments.

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Change of 3α-Hydroxysteroid Dehydrogenase Activity in Redox Buffer System

The above result show that the enzyme is inactivated by GSSG and its activity is restored by GSH. In order to investigate the possible alteration of the enzyme activity under physiological conditions, the enzyme was incubated in redox buffer systems with varying [GSH]/[GSSG] ratio. The enzyme activity decreased with a decrease in [GSH]/[GSSG] ratio in the range from 0–15. By contrast, slight alteration of the enzyme activity was still observed in the buffer system with higher ratios (>15) (Figure 2). After 24 h incubation under anaerobic condition (N_2 gas), the [GSH]/[GSSG] ratio remained unaltered as measured by glutathione reductase [EC 1.6.4.2] and NADPH. The activity of bovine liver 3α-hydroxysteroid dehydrogenase was also modulated as markedly as that of the rat liver enzyme under the same conditions (data not shown).

DISCUSSION

The thiol/disulfide exchange reaction is a remarkable regulation system for various enzymes such as phosphofructokinase,[20,21,22] pyruvate kinase[21,22] and glutathione *S*-transferase.[23,24,25] The present study demonstrates that rat liver cytosolic 3α-hydroxysteroid dehydrogenase is also modulated by the thiol/disulfide exchange reaction.

RIGHTSLINK

It is well-known that in the living cell there are many thiols including low-molecular-weight compounds and proteins. For example, intracellular concentrations of GSH, L-cysteine and L-cysteamine have been reported as 7–8, 0.25 and 0.1–0.2 mM, respectively.[26] In contrast, the concentrations of disulfides were much lower than those of thiols; concentrations of GSSG and proteins carrying mixed disulfides were about 0.16 and 1.7 mM, respectively. Furthermore, both L-cystine and L-cystamine appear to make insignificant contribution to the thiol/disulfide exchange reaction, as judged by their trace amounts. Thus, the thiol/disulfide exchange reaction between GSH and GSSG would be the most important in the alteration of the [GSH]/[GSSG] ratio. Gilbert[9,20] proposed a "third messenger" theory from the viewpoint that this alteration could be induced by changing the level of cAMP. In addition to cAMP, various factors causing the imbalance of [GSH]/[GSSG] ratio have been reported: they are termed oxidative stress, including radical formation, lipid peroxidation and GSH conjugation.[26,27] Carbonyl compounds are also inducers of [GSH]/[GSSG] imbalance,[4,27] because radicals produced by their autooxidation consume GSH in the living cell and induce oxidative stress.

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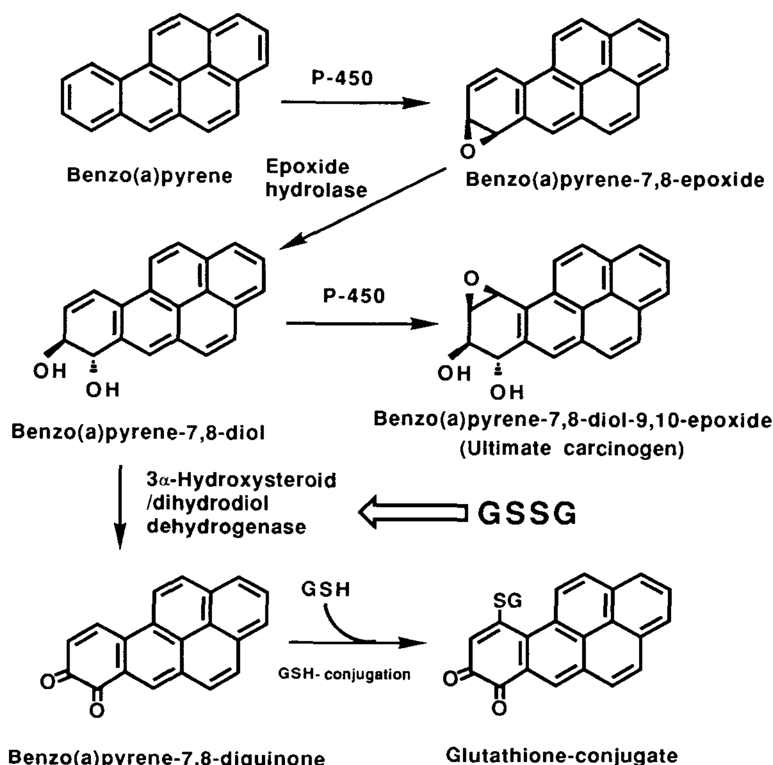

Scheme A Possible Role of Modulation of 3α-Hydroxysteroid Dehydrogenase Activity in Polycyclic Aromatic Hydrocarbon Metabolism. ⇐ = inhibition.

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Aldo-keto reductase is a generic name for enzymes which catalyze the reduction of various carbonyl compounds using NADPH.[11,12,28–32] Our present results suggest that rat liver 3α-hydroxysteroid dehydrogenase belongs to the aldo-keto reductase family as well as bovine liver 3α-hydroxysteroid dehydrogenase.[11] Recently, Penning and coworkers suggested that this enzyme plays an important role in the formation of 7,8-dicarbonyl benzo(a)pyrene from its 7,8-dihydrodiol form by oxidation, and proposed a benzo(a)pyrene detoxication pathway as shown in the Scheme.[2]

Our present study suggested that a suppression of the activity of 3α-hydroxysteroid dehydrogenase under oxidative stress might reduce the dicarbonyl formation and thus facilitate the accumulation of ultimate carcinogen. In fact, it has been reported that the addition of 3α-hydroxysteroid dehydrogenase in the Ames' test reduces the mutagenic activity of benzo(a)pyrene.[2,3]

On the other hand, it has been reported that the [GSH]/[GSSG] ratio is in the range between 20–300 under physiological conditions[26] suggesting little alteration of the enzyme activity (Figure 2). Whether the [GSH]/[GSSG] ratio is smaller under certain abnormal conditions[33] so that its suppression could make the carcinogen more toxic remains to be investigated.

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