ROLE OF REDUCED GLUTATHIONE IN THE Δ^5-3-KETOSTEROID ISOMERASE REACTION OF LIVER

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SUMMARY. Partially purified rat liver Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) is profoundly and specifically activated by reduced glutathione (GSH). This stimulating effect shows normal saturating kinetics, and both K_m and V_{max} are pH-dependent. The binding of GSH is independent of the concentration of Δ^5-androstene-3,17-dione, whereas the K_m for Δ^5-androstene-3,17-dione is markedly reduced by saturating levels of GSH. The same catalytic site appears to isomerize both Δ^5-androstene-3,17-dione and Δ^5-pregnene-3,20-dione. Several steroidal inhibitors compete with Δ^5-androstene-3,17-dione, whereas S-methylglutathione competes with GSH. This activation of Δ^5-3-ketosteroid isomerase is also observed in the livers of other species (calf, guinea pig, human), and represents a hitherto unrecognized function of reduced glutathione.

INTRODUCTION

A family of widely distributed Δ^5-3-ketosteroid isomerases (EC 5.3.3.1) concerned with the conversion of $\Delta^{5(6)}$- and $\Delta^{5(10)}$-3-ketosteroids to the corresponding conjugated Δ^4-3-ketosteroids was first described in 1955 (1). The primary structure of the crystalline isomerase of *Pseudomonas testosteroni* has been established, and information on the stereochemistry and mechanism of the reaction has been reviewed (2,3). Isomerase activity has been detected in several animal tissues (1,2,4,5), but there is disagreement on the substrate specificities and the mechanisms of these enzymes (2). The adrenal isomerase of some species is profoundly activated by low levels of NAD^+ and NADH, but to a much lesser extent by $NADP^+$ and NADPH (6,7). Koerner (4) has shown that the rat liver isomerase loses activity upon salt fractionation or gel chromatography, but that this activity can be restored by a low molecular weight organic factor present in the supernatant fractions of the livers of the rat and several other species. This factor could not be re-

The abbreviations used are: GSH, reduced glutathione; DTT, dithiothreitol; isomerase, Δ^5-3-ketosteroid isomerase (EC 5.3.3.1).

placed by nicotinamide adenine nucleotides.

We have confirmed the finding of an activator of rat liver isomerase and established that this naturally occurring soluble activator can be fully and specifically replaced by GSH. The isomerase activities of crude and partially purified preparations of guinea pig, calf and human liver are all profoundly activated by GSH. The rat liver isomerase has been purified and the nature of the activating effect of GSH has been analyzed. These findings establish a novel biological role for GSH.

METHODS

Enzyme Assays. The standard spectrophotometric assay system for Δ^5-3-ketosteroid isomerase activity contained in a final volume of 3.0 ml: 25 mM Tris base, 12.5 mM K_2HPO_4, sufficient H_3PO_4 to attain a pH of 8.5, 100 μM dithiothreitol, 100 μM GSH, 68 μM Δ^5-androstene-3,17-dione (in 0.02 ml of methanol), and an appropriate quantity of enzyme which was used to initiate the reaction. The absorbance at 248 nm was measured at 25° against a blank containing all components except the steroid. One unit of enzyme is defined as the amount causing the isomerization of 1 μmole of Δ^5-androstene-3,17-dione per min to Δ^4-androstene-3,17-dione (a_M = 16,300 $M^{-1}cm^{-1}$) under these conditions. Corrections were made, when necessary, for the nonenzymatic isomerization.

Purification of Δ^5-3-Ketosteroid Isomerase. All operations were conducted at 0-4°. Livers of Sprague-Dawley rats were homogenized with 3 volumes of the following medium: 50 mM Tris-HCl, 250 mM sucrose, 5 mM $MgCl_2$, 25 mM KCl (pH adjusted with HCl to 7.4 at 0°). The homogenate was centrifuged successively at 1000 x g, 20,000 x g, and finally at 105,000 x g for 2 hours. The supernatant faction (S_{105}) contained 79% of the isomerase activity found in the original low speed supernatant fraction (Table 1). The isomerase activity was precipitated quantitatively from the S_{105} fraction between 60% and 90% saturation with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ was removed by passing the solution through a Sephadex G-25F column in 1 mM potassium phosphate - 7.2 mM 2-mercaptoethanol, at pH 7.15. The void volume eluate was then passed through a DEAE-cellulose column equilibrated with the same solution. No significant retardation of the enzyme activity was observed. At this stage GSH was added to a final concentration of 45 μM in an effort to stabilize the enzyme. Further purification was achieved by chromatography on CM-cellulose with a linear gradient from 1 mM to 200 mM potassium phosphate (containing 1 mM EDTA, 7.2 mM 2-mercaptoethanol, and 50 μM GSH), final pH 7.1. The isomerase activity was eluted by 20-30 mM potassium phosphate (Table I).

RESULTS

Properties of the Isomerase. Purification of the isomerase is accompanied by a progressively increasing dependence on added GSH for full activity. The crude S_{105} fraction was stimulated about 2-fold by GSH, and following precipitation with ammonium sulfate the activity was enhanced about 10-fold by GSH. The degree of stimulation obtained with GSH depends both upon the state

TABLE I. Purification of Δ^5-3-Ketosteroid Isomerase of Rat Liver

Step	Volume (ml)	Total Activity (units)	Specific Activity (units/mg protein)
S_{100}	101	110	0.0433
60-90% $(NH_4)_2SO_4$ precipitate	9.7	108	0.170
Combined fractions from Sephadex G-25	14.5	104	0.170
After 18 hr at 4°	14.5	69.2	0.113
Combined fractions from DEAE-cellulose	27.2	67.9	0.287
Combined fractions from CM-cellulose	94	49.3	0.545

Sprague-Dawley rat liver (51 g) was used in this purification. The Sephadex, DEAE-cellulose, and CM-cellulose columns all had diameters of 2 cm and adsorbent bed heights of 31-37 cm.

The enzyme assays are described under "Methods".

of purification and the pH of the assay system.

The isomerase has an isoelectric point of about 10. Gel filtration on Sephadex G-150 and G-200 in 50 mM potassium phosphate, pH 7.0, gave a molecular weight of about 45,000.

Specificity for Sulfhydryl Compounds. Several sulfhydryl compounds, including 2,3-dimercaptopropanol, 2-mercaptoethanol, dithiothreitol, γ-L-glutamyl-L-cysteine, and L-cysteinylglycine, at concentrations of 50 to 350 μM were substituted for GSH in the assay system. Oxidized glutathione, cyst(e)ine, homocyst(e)ine, cyst(e)amine, and oxidized and reduced coenzyme A, lipoic acid, and lipoamide were also tested in the assay system, and in incubations (at 0.5-1 mM) with enzyme at pH 8.5 for 26 hours at 25°. (DTT was omitted from the assays with disulfide compounds.) None of the compounds tested

could replace GSH, nor did prior incubation enhance the enzymatic activity measured in the presence or absence of GSH.

We conclude that the stimulating effect of GSH is highly specific. Moreover, unlike the adrenal enzyme (6,7), the isomerase activity of crude and purified liver preparations is not affected by NAD^+ or NADH.

Substrate Specificity. The isomerase is active toward C_{19} and C_{21} Δ^5-3-ketosteroids. Under the standard assay conditions, the K_m for Δ^5-androstene-3,17-dione is 16 μM. In a modified assay system containing 1 mg of neutralized bovine serum albumin and 0.24 ml of methanol, Δ^5-androstene-3,17-dione and Δ^5-pregnene-3,20-dione exhibited K_m values of 25 μM and 8.5 μM, and V_{max} values of 5.76 units/ml and 5.45 units/ml, respectively. With both substrates present, a V_{max} of 5.08 units/ml was obtained, indicating that both steroids are isomerized at the same enzymatic site.

$\Delta^{5(10)}$-3-Ketosteroids also serve as substrates. In the standard assay system, $\Delta^{5(10)}$-estrene-3,17-dione and $\Delta^{5(10)}$-estren-17β-ol-3-one, (both at 68 μM) were isomerized at rates 0.36% and 0.17%, respectively, of the rate of isomerization of Δ^5-androstene-3,17-dione (68 μM). The isomerization of Δ^5-pregnene-3,20-dione, as well as of the $\Delta^{5(10)}$-estrene derivatives was likewise stimulated markedly by GSH. Δ^5-Cholesten-3-one was not isomerized.

The enzyme preparation contained no detectable 3α-, 3β-, and 17β-hydroxysteroid dehydrogenase activities, in the presence or absence of GSH.

Nature of the Stimulatory Effect of GSH. The specific binding of GSH to the enzyme, measured by its effect on the rate of isomerization of Δ^5-androstene-3,17-dione exhibits typical saturation kinetics. All double reciprocal plots were strictly linear. The stimulatory effect of GSH is pH-dependent (Fig. 1), and the optimal activity occurs at pH 8.5 in the presence of saturating levels of GSH. At pH 6.0, there is negligible isomerase activity in the absence of GSH, whereas the V_{max} in the presence of saturating levels of GSH is about one-third of that observed at the pH optimum of 8.5. Measurements over the range from pH 5 to pH 10 have shown that the binding of GSH is also pH-

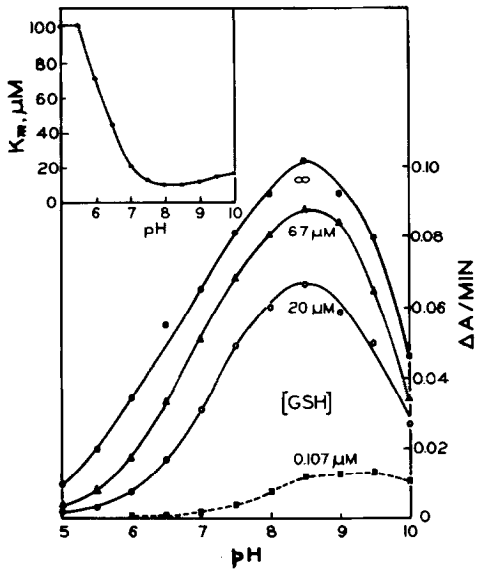

Fig. 1. Velocity of isomerization of Δ^5-androstene-3,17-dione by partially purified rat liver isomerase as a function of GSH concentration and pH. The calculated V_{max} is indicated by [GSH] = ∞ . The inset gives the calculated values of K_m for GSH as a function of pH. The standard assay system was used with [GSH] varied. The enzyme preparation (24 μg of protein per cuvette; specific activity, 0.66 unit per mg of protein). Initial velocities (right ordinate) are expressed as rate of change of absorbance per minute in cuvettes of 1 cm light path.

dependent, with the greatest change (approx. 10-fold increase) occurring between pH 5.5 (K_m = 100 μM) and pH 8.0 (K_m = 10-15 μM). A series of measurements at pH 8.5 at Δ^5-androstene-3,17-dione concentrations between 22 and 68 μM showed that the K_m for GSH was not affected by these variations in steroid concentration.

GSH increased the affinity of the enzyme for Δ^5-androstene-3,17-dione although the steroid concentration does not affect the K_m for GSH. Thus, GSH increases the rate of isomerization of Δ^5-androstene-3,17-dione both by decreasing K_m and increasing V_{max} for the steroid.

Inhibitors. 19-Nortestosterone (K_i = 31 μM) inhibits competitively with Δ^5-androstene-3,17-dione under standard assay conditions. Other reversibly inhibiting steroids include 17β-estradiol, estrone, and the antiandrogens, chlormadinone acetate, megestrol acetate, and RO-7-2340 (6α-bromo-17β-

hydroxy-17α-methyl-4-oxa-androstan-3-one). In the presence of 5 μM GSH, L-cysteinylglycine (100 μM) did not inhibit, whereas γ-L-glutamyl-L-cysteine (300 μM) inhibited only 20%. S-Methylglutathione inhibits competitively with GSH (K_i = 143 μM) and noncompetitively with Δ^5-androstene-3,17-dione (K_i = 635 μM, at a GSH concentration of 20 μM).

DISCUSSION

The nature of the stimulatory effect of GSH on the rat liver isomerase remains obscure. We have known for some time that GSH (among many peptides tested) can promote a nonenzymatic steroid isomerization reaction. Yet this activity is feeble in comparison to the enzymatic rate. The enzyme preparations contain some isomerase activity in the absence of added GSH. Efforts (e.g. gel filtration in the presence of DTT) to remove tightly associated (or possibly covalently bound) GSH have not reduced the enzymatic activity to zero in the absence of added GSH. Mild treatment of the enzyme with N-ethylmaleimide reduces but does not abolish this isomerase activity, and the addition of saturating amounts of GSH restores full activity. More vigorous treatment with N-ethylmaleimide leads to progressive loss of enzyme activity and ability to respond to GSH.

The question of whether the GSH participates in an integral manner in the catalytic mechanism requires further study. GSH participates in various cis-trans isomerization reactions: the conversion of maleylacetoacetate to fumarylacetoacetate by liver (8) and by Vibrio (9) enzymes, and the isomerization of maleylpyruvate to fumarylpyruvate by a Pseudomonas enzyme (10). An interesting feature of the latter reaction is that in a medium of D_2O, the isomerization of maleylpyruvate to fumarylpyruvate promoted by the bacterial enzyme in the presence of GSH or by GSH alone, resulted in no incorporation of deuterium into the product (10). These findings led to the proposal of a mechanism for the enzymatic cis-trans isomerization involving a transient complex between GSH and the olefinic substrate (10). Experiments to establish whether the isomerization of Δ^5-androstene-3,17-dione promoted by liver

enzymes proceeds by intramolecular proton transfer, as in the cases of the crystalline enzyme of P. testosteroni (2) and the beef adrenal preparations (11) are in progress.

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