Proc. Natl. Acad. Sci. USA
Vol. 74, No. 1, pp. 158–162, January 1977
Biochemistry

Relationship between the soluble glutathione-dependent Δ^5-3-ketosteroid isomerase and the glutathione *S*-transferases of the liver

(human and rat liver/ligandin/isoelectric focusing)

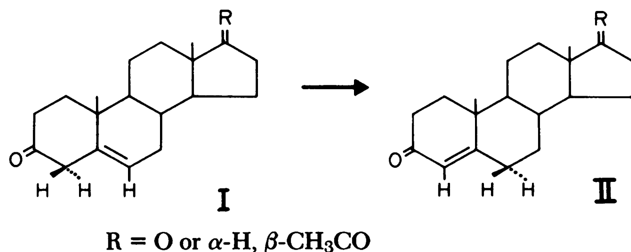
ANN M. BENSON*, PAUL TALALAY*†, JAMES H. KEEN‡, AND WILLIAM B. JAKOBY‡

* Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and ‡ Section on Enzymes and Cellular Biochemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Communicated by Eugene P. Kennedy, October 27, 1976

ABSTRACT Soluble, glutathione-stimulated Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) activity of human and rat liver resides in very basic proteins with molecular weights of about 45,000 which are present in high concentrations in these tissues. Physicochemical and immunological evidence is presented for the identity of the proteins responsible for this enzymatic activity with the glutathione *S*-transferases (RX:glutathione R-transferase, EC 2.5.1.18) that conjugate glutathione with a variety of electrophilic compounds. In the rat, the steroid isomerase is associated principally with the major transferase (B), which is also known as ligandin, and has the versatility to bind various hydrophobic compounds such as bilirubin, corticosteroids, and metabolites of a number of carcinogens. Other rat liver glutathione *S*-transferase species are far less active in the steroid isomerization reaction. The Δ^5-3-ketosteroid isomerase activity of human liver is more uniformly distributed among the five glutathione *S*-transferases that have been described. Steroid isomerization differs fundamentally from other reactions promoted by glutathione *S*-transferases in that glutathione is not consumed in the reaction. However, because the transferase enzymes promote nucleophilic attack by glutathione on a variety of largely foreign organic substrates, a similar mechanism may be involved in the isomerase reaction. Δ^5-3-Ketosteroids are among the few known naturally occurring substrates for these enzymes.

The capacity of various animal and microbial systems to promote the enzymatic conversion of Δ^5-3-ketosteroids (I) to the corresponding α,β-unsaturated Δ^4-3-ketosteroids (II) was described just over 20 years ago (1, 2). Whereas the enzyme from *Pseudomonas testosteroni*, a Δ^5-3-ketosteroid isomerase (steroid Δ-isomerase, 3-oxosteroid Δ^4-Δ^5-isomerase, EC 5.3.3.1) that does not appear to require small molecules for its activity, has been the subject of intensive study from both structural and mechanistic viewpoints (2), far less is known about the corresponding enzymes of animal origin.



R = O or α-H, β-CH_3CO

Recently, Benson and Talalay (3) reported that the cytosol fraction of the livers of a number of species contains Δ^5-3-ketosteroid isomerase activity which is markedly and specifically stimulated by (reduced) glutathione. Partial purification of the rat liver Δ^5-3-ketosteroid isomerase revealed that it was a very basic protein (pI *ca.* 10) of molecular weight 43,000–45,000 and that it appeared to constitute a substantial fraction of the total proteins of the liver cytosol.

These attributes are also characteristic of a family of liver proteins, the glutathione S-transferases which, because of their great versatility of binding and catalytic functions, have been studied in numerous laboratories in the past decade. Indeed, Litwack, Ketterer, and Arias (4) came to the conclusion that the liver proteins which had been investigated independently in each of their laboratories for the ability to bind bilirubin and bromsulfophthalein (5), to bind an azocarcinogen, at least in part covalently (6), and to bind cortisol metabolites (7), were in fact identical. The name ligandin was proposed for this protein (4). Subsequently, rat liver ligandin was shown to be identical with glutathione S-transferase B (RX:glutathione R-transferase, EC 2.5.1.18) (8), one of the enzymes that catalyze a large number of reactions in which glutathione participates as a nucleophile (9, 10). Among other reactions, these enzymes catalyze the first step in mercapturic acid formation (11), and all serve as intracellular binding proteins (12). The rat liver transferases were designated AA, A, B, C, D, and E, in reverse order of their elution from carboxymethylcellulose, and all except transferase D have been obtained in homogeneous form (13–15). Five human liver glutathione S-transferases (α, β, γ, δ, and ϵ) have also been purified to homogeneity by similar methods that include isoelectric focusing (16).

In conjunction with the exchange of enzymes, substrates, and antibody between our laboratories, we have carried out experiments that have pointed to the identity of the Δ^5-3-ketosteroid isomerase with certain glutathione S-transferases. This paper reports that the soluble Δ^5-3-ketosteroid isomerase activity of rat liver is associated principally with glutathione S-transferase B (ligandin). The Δ^5-3-ketosteroid isomerase activity of human liver is associated with transferases γ, δ, and ϵ, although transferases α and β are also active.

In the presentation of our *Results*, we shall continue to refer to "transferase" and "isomerase" preparations and activities in order to specify the assay reactions used to monitor the purification of each preparation.

EXPERIMENTAL PROCEDURES

Materials. 1-Chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene were obtained from Eastman. Δ^5-Androstene-3,17-dione was prepared as previously described (17). Ampholytes were obtained from LKB.

Assay of Enzyme Activities. Spectrophotometric determinations of initial reaction velocities were carried out according to published methods for the transferase substrates (13), 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene,

† To whom correspondence should be addressed.

and for the isomerase substrate, Δ^5-androstene-3,17-dione (3). To facilitate comparisons, we describe the precise assay procedures in Table 1. One unit of enzyme promotes the formation of 1 μmol of product per min under standard assay conditions. All velocities have been corrected, where necessary, for nonenzymatic rates. Protein was determined spectrophotometrically (18), or by the method of Lowry *et al.* (19), with crystalline bovine serum albumin as a standard.

Enzyme Preparations. Rat liver Δ^5-3-ketosteroid isomerase was purified through the CM-cellulose step as described by Benson and Talalay (3), and was then subjected to isoelectric focusing in a sucrose density gradient in the presence of carrier ampholytes (pI 9–11) in an LKB Model 810 (120 ml) column (20). After electrofocusing, the specific activities of various isomerase preparations were in the range of 1.50 to 1.84 units/mg of protein with Δ^5-androstene-3,17-dione as substrate.

The partial purification of Δ^5-3-ketosteroid isomerase from human liver was carried out on a specimen obtained from a 14 year-old girl who died of an astrocytoma, and who had received the following medications: phenobarbital, diphenylhydantoin, and dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione). The possibility that one or more of these medications had induced elevations of the liver enzyme activities should be borne in mind (21, 22). The liver was stored at the temperature of liquid nitrogen for several weeks prior to use. The purification was carried out at 0–4.° A portion of the liver (7.3 g) was homogenized in 4 volumes of water and centrifuged at 10,000 × *g* for 2 hr. The supernatant fraction was passed over a DEAE-cellulose column (1.5 × 11 cm), equilibrated and developed with 10 mM Tris-chloride at pH 8.0 (83% of the transferase activity with 1-chloro-2,4-dinitrobenzene, and 89% of the Δ^5-3-ketosteroid isomerase activity was eluted). Solid ammonium sulfate was added to the eluate to the point of saturation, and the precipitate was extracted with 10 mM potassium phosphate at pH 6.7. About 70% of the isomerase and 80% of the transferase activity (present in the DEAE-cellulose column eluate) was recovered in this fraction which was dialyzed against the same buffer and introduced into the electrofocusing column. Isoelectric focusing was carried out at 4° in a sucrose density gradient in the presence of a mixture of carrier ampholytes spanning primarily the range, pI 8–9.5; the operation was complete after 69 hr (700–900 V).

Preparations of homogeneous glutathione *S*-transferases AA (15), A (14), B (13), and C (13) were obtained from the cytosol of rat liver by described methods (13–15). Preparation of the homogeneous human transferases has also been described (16).

Immunological Procedures. Serum immunoglobulin G (IgG) was obtained from sheep immunized to homogeneous rat liver glutathione *S*-transferase B. Immunoprecipitation was carried out according to described procedures (23).

RESULTS

Superficial similarities (3, 13) between the two enzymatic reactions led us to suspect that the Δ^5-3-ketosteroid isomerase activity and the glutathione transferase activity might reside in the same molecule. Aside from the common requirement for glutathione, the two proteins had similar molecular weights, basicity, and high intracellular concentration. The best preparations of isomerase (3) proved very active as glutathione *S*-transferases when tested with glutathione and 1-chloro-2,4-dinitrobenzene as substrates.

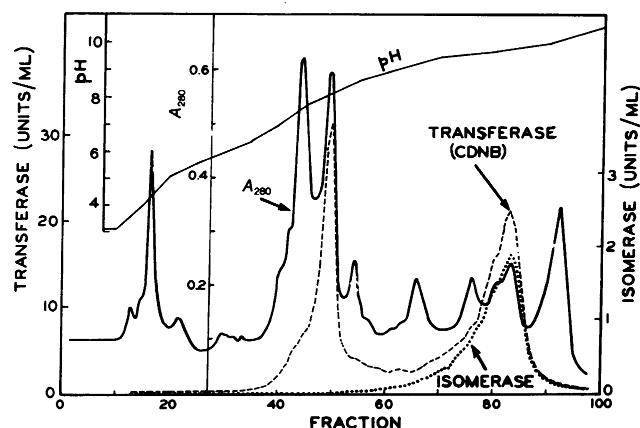


FIG. 1. Distribution of Δ^5-3-ketosteroid isomerase and glutathione *S*-transferase (1-chloro-2,4-dinitrobenzene, CDNB) activities among electrofocusing fractions of a partially purified rat liver isomerase preparation. Electrofocusing in a sucrose gradient with carrier ampholytes, primarily in the pI 9–11 range, was carried out at 4° for 88 hr. The absorbance of the column effluent at 280 nm was monitored and recorded automatically. Fractions of 1.1 ml were collected and assayed for enzyme activity.

Electrofocusing of Partially Purified Isomerase-Transferase Preparations from Rat and Human Liver. Preliminary gel filtration experiments with ultracentrifuged rat liver cytosol on Sephadex G-75 in 0.1 M potassium phosphate at pH 7.0 disclosed that the isomerase and the transferase activity were eluted in a single peak that was slightly retarded from the break-through volume of the column. The recovery of both activities in this peak was almost quantitative. Because this finding confirmed that the two activities were associated with macromolecules of similar size, more discriminatory methods were sought to probe the question of identity of the enzymes responsible.

A rat liver isomerase preparation which had been fractionated through the step of chromatography on CM-cellulose (3) was subjected to electrofocusing in a sucrose density gradient in the presence of a mixture of carrier ampholytes spanning principally the range of pI 9–11 (Fig. 1). Transferase activity with 1-chloro-2,4-dinitrobenzene was separated into two major peaks with isoelectric centers near pH 8.1 and 9.7. Whereas isomerase activity was barely detectable in the fraction with pI 8.1, it paralleled faithfully the profile of the pI 9.7 fraction, including a small shoulder on the low pH side.

A similar experiment designed to elucidate the relationship between isomerase and transferase activities in human liver is presented in Fig. 2. The human liver preparation had been subjected to only modest preliminary fractionation steps (passage through DEAE-cellulose, ammonium sulfate precipitation, and dialysis) prior to introduction into the electrofocusing system. The glutathione *S*-transferase activity focused in two regions of the gradient. No isomerase activity was associated with the acidic (pI 5.4) transferase component. Three overlapping peaks of transferase activity in the basic region (pI 9.2–9.4) contained essentially all the isomerase activity. The pattern of distribution of these two enzymatic activities is consistent with the finding (Table 1) that the two human transferases having the most basic isoelectric points (δ and ϵ) are the most effective in catalyzing the isomerization of Δ^5-androstene-3,17-dione. The variation of the ratio of these two enzymatic activities in the isoelectric focusing fractions is not unexpected since this procedure did not resolve all of the known human transferases (16) and these enzymes vary in their relative transferase and isomerase activities (Table 1).

Precipitation of Enzyme Activities by Antibody. Purified

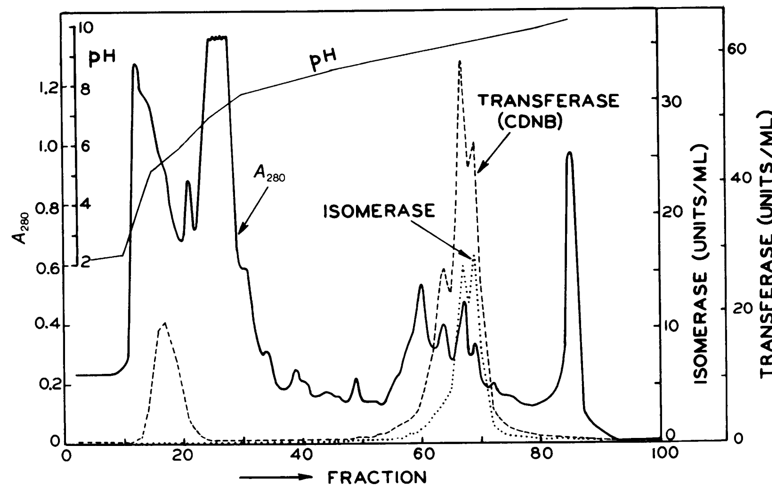


FIG. 2. Distribution of Δ^5-3-ketosteroid isomerase and glutathione S-transferase (1-chloro-2,4-dinitrobenzene, CDNB) activities among electrofocusing fractions of partially purified human liver preparation. The purification of the liver fractions was carried out as described in the *text*. Electrofocusing in a sucrose density gradient was conducted at 4° for 69 hr in the presence of a mixture of carrier ampholytes spanning principally the range of pI 8–9.5. Fractions of 1.2 ml were collected and assayed for enzyme activity.

rat liver Δ^5-3-ketosteroid isomerase was incubated with a series of concentrations of IgG obtained from the serum of a sheep injected with homogeneous rat liver glutathione S-transferase B. The glutathione S-transferase (measured with 1-chloro-2,4-dinitrobenzene) and the Δ^5-3-ketosteroid isomerase activities were removed from the supernatant fraction in a parallel manner with increasing concentrations of antibody (Fig. 3).

Table 1. Substrate specificities of purified human and rat liver glutathione S-transferase preparations

Species and transferase	Specific activity (μmol/min per mg of protein)		
		Transferase	
	Isomerase*	CDNB†	DCNB‡
Rat			
AA	0.001	14	0.008
A	0.010	62	4.3
B	1.87	11	0.003
C	0.005	10	2.0
Human			
α	0.23	19	0.049
β	0.19	16	0.065
γ	1.6	17	0.035
δ	10.2	37	0.050
ε	9.8	34	0.043

The transferase specific activities shown are those reported in earlier publications (13–16) and agreed closely with those obtained with the preparations actually used for these studies.

* All enzyme activities were determined spectrophotometrically at 25°. The Δ^5-3-ketosteroid isomerase activity was measured in the presence of 100 μM glutathione, 100 μM dithiothreitol, 25 mM Tris/potassium phosphate at pH 8.5, 68 μM Δ^5-androstene-3,17-dione in methanol [0.67% (vol/vol), final concentration]. Measurements were carried out at 248 nm (A_m = 16.3 mM^{-1} cm^{-1}). Nonenzymatic rate = 0.002–0.004 absorbance unit/min.

† Glutathione S-transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) was measured in the presence of 1 mM glutathione, 100 mM potassium phosphate at pH 6.5, 1 mM CDNB in ethanol [2.85% (vol/vol) final concentration]. Measurements were carried out at 340 nm (A_m = 9.6 mM^{-1} cm^{-1}). Nonenzymatic rate = 0.004 absorbance unit/min.

‡ Glutathione S-transferase activity with 1,2-dichloro-4-nitrobenzene (DCNB) was measured in the presence of 5 mM glutathione, 100 mM potassium phosphate at pH 7.5, 1 mM DCNB in ethanol [2.85% (vol/vol) final concentration]. Measurements were carried out at 345 nm (A_m = 8.5 mM^{-1} cm^{-1}). Nonenzymatic rate = less than 0.001 absorbance unit/min.

Substrate Specificities of Rat and Human Liver Glutathione S-Transferases. When homogeneous preparations of glutathione S-transferases AA, A, B, and C from rat liver were examined for Δ^5-3-ketosteroid isomerase activity, the results shown in Table 1 were obtained. A comparison is also made with the specific activities of these preparations for 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene. Each enzyme has its characteristic substrate preference. Thus, the

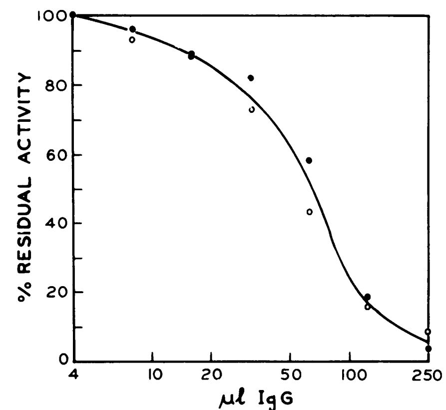


FIG. 3. Precipitation of Δ^5-3-ketosteroid isomerase (O) and glutathione S-transferase (1-chloro-2,4-dinitrobenzene) (●) activities from a purified rat liver isomerase preparation by antibody to glutathione S-transferase B. Purified rat liver Δ^5-3-ketosteroid isomerase (5 μl; 5 μg protein; specific activity 1.84) was incubated for 15 hr at 4° with 0.25 ml of 0.134 M NaCl–0.01 M potassium phosphate at pH 7.4, containing dilutions of the gamma globulin fraction of normal or anti-transferase B sheep sera. Saturated ammonium sulfate (0.125 ml; 25°; pH 5.4) was added to each sample to precipitate the antibody–antigen complex and the mixture was left at 4° for 1.5 hr. Ammonium sulfate at this concentration caused no precipitation of enzyme activity in the absence of antibody. After centrifugation, enzyme assays were performed on the supernatant fractions. Per cent residual activity is defined as the percentage of activity remaining in the supernatant fluid after treatment with anti-transferase B gamma globulins as compared to treatment with an equal quantity of normal gamma globulins, and is normalized to 100% for the highest dilution of antibody used.

specific steroid isomerase activity of the most active enzyme, transferase B, is about 200 to 2000 times higher than those of transferases AA, A, and C. The ability of these preparations to conjugate 1-chloro-2,4-dinitrobenzene with glutathione is of the same order of magnitude for all of the transferases tested. The reaction pattern with 1,2-dichloro-4-nitrobenzene is quite different: only transferases A and C promote the reaction with this substrate at a significant rate. The specific activities of the four enzymes differ by several orders of magnitude for both Δ^5-androstene-3,17-dione and 1,2-dichloro-4-nitrobenzene. It should be clear that the measured activities refer strictly to the conditions of assay employed and are not maximum velocities; selection of assay conditions is governed by substrate solubilities and by the pH-dependence of the nonenzymatic reactions of the individual substrates.

Homogeneous preparations of human liver glutathione *S*-transferases, α, β, γ, δ, and ϵ, are all effective in catalyzing the isomerization; transferases δ and ϵ are the most active of all of the transferases tested (Table 1). The human enzymes display a narrower range of specific activities with Δ^5-androstene-3,17-dione and this is also reflected with other substrates (Table 1) (9). Indeed, the human enzymes appear to have the same amino acid composition and are considered to be a single gene product; the large differences in isoelectric point among them may represent progressive deamidation of their asparagine or glutamine residues (16).

DISCUSSION

We conclude that the capacity for promoting the isomerization of Δ^5-3-ketosteroids is a catalytic activity intrinsic to some of the glutathione *S*-transferases. The argument is readily summarized: (*i*) the two enzyme activities reside in proteins of the same size. Isoelectric focusing of partially purified rat and human liver preparations results in correspondence of the isomerase activity peaks with some of the transferase peaks. Furthermore, the major isomerase activity copurifies with the major transferase (transferase B) of rat liver and has the same isoelectric pH; (*ii*) homogeneous transferase B is by far the most active in the isomerization although isomerase activity is evident with several of the other rat transferases. With some of the homogeneous human transferases, even higher isomerase specific activities are evident, and a more uniform pattern among the different enzyme species is found, in keeping with the greater similarity among these proteins; (*iii*) antibody directed against transferase B is equally effective in precipitating activity for Δ^5-androstene-3,17-dione and for 1-chloro-2,4-dinitrobenzene.

The isomerase activity of the glutathione *S*-transferases has novel aspects in relation to the previously described transferase reactions, in that glutathione is not consumed in the isomerization process[§]. There are other known glutathione-requiring reactions in which glutathione is not consumed, and some of these may proceed by mechanisms not totally dissimilar to the steroid Δ^5-3-ketosteroid isomerase reaction, e.g., glyoxalase, maleylacetoacetate isomerase, and maleylpyruvate isomerase (24, 25).

The crystalline Δ^5-3-ketosteroid isomerase from *P. testosteroni* contains no cysteine and consequently no glutathione, and its catalytic activity is not stimulated by glutathione. The bacterial isomerase exhibits no measurable glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene, even at an enzyme concentration 10^6-fold higher than that required for detection of the isomerase activity. Whether a direct intramolecular (and possibly stereospecific) transfer of a proton from C-4 to C-6 occurs with this mammalian enzyme, comparable to that catalyzed by the isomerase of *P. testosteroni*, is unknown. In the latter case, the catalytic process involves transfer of the 4β-proton to the 6β-position without exchange with the medium. There is strong evidence for the formation of an enolic intermediate, and for the participation of both proton donor and acceptor groups of the enzyme in the catalysis (26). Insofar as the possibility of a direct intramolecular proton transfer by the liver enzymes is concerned, it should be pointed out that although the sulfhydryl proton of glutathione exchanges rapidly in solution with the protons of the medium, glutathione promotes both the enzymatic and nonenzymatic isomerization of maleylpyruvate to fumarylpyruvate in a medium of D_2O, without incorporation of deuterium into the product (25).

Catalysis of the isomerization of Δ^5-3-ketosteroids by the glutathione *S*-transferases can be rationalized on the basis of the general mechanisms that have been formulated for these enzymes (9, 10). The glutathione *S*-transferases have been proposed as catalysts for a large number of reactions in which glutathione acts as a nucleophile and in which a second substrate with a sufficiently electrophilic atom is bound to the enzyme (9, 10); binding appears to require a ligand with lipophilic characteristics (12). In an extensive series of reactions that include thioether formation, the "reduction" of organic nitrate esters to nitrous acid, and the formation of cyanide from organic thiocyanates, these correlations apply. Moreover, all of the transferase reactions occur to a measurable degree in the absence of added enzyme and this is also true of the isomerization of Δ^5-3-ketosteroids. In fact, the observation was made many years ago (P. Talalay, unpublished observations) that glutathione promoted the isomerization of Δ^5-androstene-3,17-dione, although at rates many orders of magnitude lower than the isomerase of *P. testosteroni*. The isomerization of Δ^5-3-ketosteroids by glutathione *S*-transferases may be viewed as another instance in which these enzymes activate glutathione as a nucleophile, thereby initiating the isomerization.

[§] The lines of evidence supporting this assertion are: (*i*) there is no indication of a progressive decline of the reaction rate when the quantities of steroid isomerized by rat liver isomerase preparations are larger than the quantities of glutathione present; (*ii*) with purified human liver preparations, in the absence of dithiothreitol, it has been found that 10 times as much steroid product could be generated as the amount of glutathione added.

The studies at Johns Hopkins School of Medicine were supported in part by a grant from the National Institutes of Health (AM 07422).

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