Glutathione S-transferases act as isomerases in isomerization of 13-*cis*-retinoic acid to all-*trans*-retinoic acid *in vitro*

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A discovery that rapid enzymic isomerization of 13-*cis*-retinoic acid (13-cRA) to all-*trans*-retinoic acid (t-RA) can be catalysed by purified hepatic glutathione S-transferases (GSTs; EC 2.5.1.18) from rat is now reported. Rates of *cis*–*trans* isomerization were determined quantitatively by HPLC. GST-catalysed reactions reached equilibrium rapidly, in marked contrast with uncatalysed or GSH-catalysed isomerizations. The GST-catalysed reaction exhibited substrate saturation kinetics with a K_m of approx. 8 μ M. The maximal velocity of the reaction and the catalytic efficiency of GSTs were determined. The initial rate of the reaction increased linearly as a function of enzyme concentration. Catalysis by GSTs was independent of the presence of GSH, indicating that GSTs act as GSH-independent isomerases as well as transferases. Incubation with guanidine (7–8 M) or heat-inactivation of GSTs (100 °C for 3 min)

decreased isomerase activities by approx. 50% and 75% respectively. The same heat treatment did not significantly inhibit isomerization catalysed by GSH and apoferritin, indicating that the observed decrease in isomerase activity by heat inactivation was not primarily due to oxidation of protein thiol groups in the GSTs. The specific activity of GSTs was approx. 23- and 340-fold those of GSH and apoferritin respectively when comparisons were made on the basis of free thiol concentrations, indicating that free thiol in GSTs cannot account for the majority of observed isomerase activities and suggesting that specific conformations of GSTs are important for such activities. Complete inhibition of the reaction by low concentrations of *N*-ethylmaleimide (10 μ M) demonstrated that intact protein thiols are required for the isomerase activities of GSTs.

INTRODUCTION

Steric isomerization of 13-*cis*-retinoic acid (isotretinoin; 13-cRA) to all-*trans*-retinoic acid (t-RA) is of great importance to nuclear retinoid receptor-mediated biological activities. As is well documented both *in vitro* and *in vivo*, t-RA binds with high affinity and activates retinoic acid receptors. Ligand binding triggers a cascade of events in various signal transduction pathways [1–4]. Research indicates that 13-cRA binds nuclear retinoid receptors with extremely low affinities and is unlikely to trigger the same signal transduction pathways directly [4–6]. *Trans* isomerization of 13-cRA seems to be responsible for its potent therapeutic activities in the treatment of skin disorders and certain types of cancer [4].

The steric isomerization of 13-cRA to t-RA has long been observed both *in vitro* and *in vivo* [7–9]. The mechanisms regulating and controlling rates of the reaction, however, are not fully understood. *Cis*–*trans* isomerization of retinoic acids can be non-enzymic and a variety of thiol compounds have exhibited catalytic activities for the reactions [7]. Whether the rate of this reaction is under enzymic control remains unknown, although enzymic catalysis has been suggested [10–13].

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a multifunctional group of enzymes that catalyse the metabolic detoxification of electrophilic compounds via conjugation [14]. GSTs also participate in the intracellular binding and transport of a variety of xenobiotic compounds [15] and catalyse key steps in the synthesis of prostaglandins and leukotrienes [16].

Interestingly, several GST isoforms can catalyse the *cis*–*trans* isomerization of maleoylactone to fumaroylacetone, and maleoylacetoacetic acid to fumaroylacetoacetic acid [14]. Other GST isoenzymes possess ketosteroid isomerase activity and catalyse the conversion of Δ^5 -3-ketosteroids to Δ^4 -3-ketosteroids [17]. Potential isomerase activities of GSTs in *cis*–*trans* conversions of retinoids seem not to have been reported.

We recently observed evidence for a retinoid isomerase activity in rat embryonic tissues [13]. This observation prompted us to perform a thorough study of whether GSTs can act as isomerases in the *cis*–*trans* isomerization of retinoic acids. We initiated an investigation of the enzymic catalysis of isomerization of 13-cRA to t-RA by using purified hepatic GSTs from rat as an enzyme source. There were several reasons for the choice of purified GSTs. First, complete amino acid and cDNA sequences for GST isoenzymes have been elucidated [14]. The sequence information is of great value in understanding the potential roles of GSTs in catalysis of retinoic acid isomerization. Secondly, two families of GST isoenzymes (Alpha and Mu) have been identified in rat hepatic tissues [18]. The known polymorphism of GSTs will facilitate future investigations of each individual GST in the catalysis of isomerization of retinoic acids as they become accessible. Finally, affinity chromatography-purified GSTs are now commercially available; thus significant efforts are spared in terms of purification and stabilization of the enzymes.

This study focused on investigations of the GST-catalysed isomerization of 13-cRA to t-RA, in which 13-cRA can be converted to a high-affinity ligand of RARs. HPLC was used for

Abbreviations used: GST, rat hepatic glutathione S-transferases; GSH, reduced glutathione; GSSG, oxidized glutathione; t-RA, all-*trans*-retinoic acid; 13-cRA, 13-*cis*-retinoic acid; 9-cRA, 9-*cis*-retinoic acid; NEM, *N*-ethylmaleimide; DCNB, 1,2-dichloro-4-nitrobenzene; *p*-NPEB, *p*-nitrophenethyl bromide; *p*-NBC, *p*-nitrobenzyl chloride.

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assessing t-RA generated from 13-cRA under catalysis by GSTs and other biological thiol compounds. The kinetic parameters of GST-catalysed reactions were determined and the effects of enzyme inhibitors were investigated. The objectives of this study were: (1) to compare the catalytic activity of GSTs with a variety of thiol catalysts in the isomerization of 13-cRA to t-RA; (2) to demonstrate that the GST-catalysed reaction is an enzymic reaction; and (3) to characterize the enzymic conversions of 13-cRA to t-RA in terms of kinetic parameters.

MATERIALS AND METHODS

Chemicals

Rat hepatic GSTs, horse spleen apoferritin, BSA, L-cysteine, reduced glutathione (GSH), oxidized glutathione (GSSG), *N*-ethylmaleimide (NEM), guanidine, t-RA, 13-cRA and 9-cRA were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Benzoylated dialysis tubing (molecular mass cut less than 1200 Da) was also purchased from Sigma. 1,2-Dichloro-4-nitrobenzene (DCNB), *p*-nitrophenethyl bromide (*p*-NPEB), and *p*-nitrobenzyl chloride (*p*-NBC) were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All other chemicals and reagents utilized were of the highest purity commercially available.

Preparations of GST and other chemical solutions

GST was weighed and dissolved in sodium phosphate buffer (0.1 M, pH 7.5). Protein concentrations of the enzyme solutions were determined by the method of Lowry et al. [19]. BSA was used as a standard protein for the quantification. GSH, GSSG, L-cysteine, apoferritin or BSA were also weighed and dissolved in the same buffer for experimental purposes.

Dialysis of GST

To eliminate the possibility of contamination of GST with low-molecular-mass thiol compounds during enzyme purification and stabilization, GST solutions were dialysed against sodium phosphate buffer (1:1000, v/v) at 4 °C for 24 h. After 12 h the dialysis solutions were changed with fresh buffer and dialysis was continued for an additional 12 h. The protein contents of dialysed GST solutions were also determined by the method of Lowry et al. [19]. Specific isomerase activities of non-dialysed GST were compared with those of dialysed GST and the comparisons showed no statistically significant differences.

Catalysis of thioether formation by GST

GST-catalysed thioether formation reactions were performed by testing the transferase activities of GST. Procedures for the assays are described by Habig and Jakoby [20]. Briefly, DCNB (1 mM), *p*-NPEB (0.1 mM) or *p*-NBC (1 mM) was incubated with GSH (5 mM) plus GST in potassium phosphate buffer (0.1 M) at 25 °C. For conjugation of DCNB, the pH of the buffer was adjusted to 7.5 and the absorbance wavelength was 340 nm. For conjugation of *p*-NPEB and *p*-NBC, the pH of the buffer was adjusted to 6.5 and the absorbance wavelength was 310 nm. Complete assay mixtures without GST served as controls. A Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA, U.S.A.) was used for measurements of changes in absorbance. $\Delta\epsilon$ (nm⁻¹ cm⁻¹) for the DCNB-dependent reaction was 8.5, and for *p*-NPEB and *p*-NBC was 1.2 and 1.9 respectively. Data obtained are expressed as means $\pm$ S.D. for three or four measurements with standard deviations.

Conversion of 13-cRA to t-RA

For measurements of uncatalysed isomerization, 13-cRA was incubated in sodium phosphate buffer (0.1 M, pH 7.5) at 37 °C in a water bath with continuous shaking for various times in total darkness. For GST and various chemical-catalysed reactions, 13-cRA was incubated with GST or chemicals under the same conditions as those utilized for measurements of uncatalysed isomerization. To investigate the effects of enzyme inhibitor on GST-catalysed reactions, GST was preincubated with NEM for 20 min before the addition of 13-cRA to start the reactions. At the end of the incubations, an equal volume of ice-cold propan-2-ol was added to the incubation mixtures, which were then vortex-mixed for 1 min and centrifuged for 30 min at 16000 *g* at 4 °C in the dark. The supernatant was saved and stored at -80 °C for subsequent HPLC analyses.

Identification and quantification of retinoic acids by HPLC

The solvent delivery system for HPLC consisted of two model 100 A dual-piston Beckman pumps linked together for activation of a binary gradient. The system was interfaced with a Shimadzu SPD-10A UV-VIS detector (set at 354 nm) and a Shimadzu C-R5A Chromatopac data processor. The HPLC was equipped with a Beckman mixing chamber and manual injector. The identification and quantification of retinoids for conversion of 13-cRA to t-RA were conducted with a Zorbax octadecylsilane column (0.46 cm $\times$ 15 cm) (MacMod Analytical, Inc., Chadds Ford, PA, U.S.A.) and by the method described by Kim et al. [21], with modifications. The analytical eluents consisted of solvent A [acetonitrile/H₂O/acetic acid (49.75:49.75:0.5, by vol.)] and solvent B [acetonitrile/H₂O/acetic acid (90:10:0.4, by vol.)], both containing 10 mM ammonium acetate. The HPLC conditions were as follows: 80% solvent B plus 20% solvent A with a flow rate of 0.6 ml/min. A mixture (100 μ l) of authentic 13-cRA, 9-cRA and t-RA or supernatant fraction of incubation mixture were loaded on the HPLC column and the elution time of each individual standard retinoic acid was used to identify the peaks eluting from the HPLC column. The detection limit of the HPLC system for concentrations of retinoic acids was 1 ng/100 μ l. Concentrations of retinoic acids below 1 ng/100 μ l were designated as not detected.

Statistical analyses

All experimental data were expressed as means $\pm$ S.D. for three or four experimental measurements. Student's *t* tests were conducted with a Microexcell statistics package (Microsoft, Redmond, WA, U.S.A.) for the statistical significance of differences between mean values.

RESULTS

Figure 1 shows a typical separation of authentic 13-cRA, 9-cRA and t-RA with an HPLC system using a Zorbax octadecylsilane column. Retinoic acids were identified by comparing elution times with those of purified authentic standard compounds. Concentrations of retinoic acids were determined quantitatively by a calculation program installed in a Shimadzu C-R5A Chromatopac data processor.

GST-catalysed thioether formation reactions were assessed to examine the transferase activities of the GST preparations utilized (Figure 2). Purified GST effectively catalysed GSH-dependent conjugation reactions with *p*-NBC, DCNB and *p*-NPEB as substrates. With catalysis by GST, reactions proceeded extremely rapidly, indicating that the GST preparations exhibited excellent transferase activities.

Figure 1 A typical HPLC chromatogram showing the separation of authentic 13-cRA, 9-cRA and t-RA

Elution times for 13-cRA, 9-cRA and t-RA were 13.0, 14.5 and 16.0 min respectively. For chromatographic conditions see the Materials and methods section.

Figure 2 Catalysis of GSH conjugation by GST

p-NBC (○, 1 mM), DCNB (●, 1 mM) or *p*-NPEB (□, 0.1 mM) was incubated with GSH (5 mM) plus GST (0.11 mg) in potassium phosphate buffer (0.1 M, pH 7.5 or 6.5) at 25 °C. Reactions were initiated by adding substrate. Complete assay mixtures without GST served as controls. The final volumes of incubation mixtures were 1 ml. For further details see the Materials and methods section.

Typical progress curves of GST-catalysed and uncatalysed 13-cRA isomerizations are shown in Figure 3. GST-catalysed reactions showed an estimated initial reaction velocity of 133 pmol/min. As expected, the velocity diminished as the

Figure 3 Steric conversions of 13-cRA (3.3 μ M) to t-RA in sodium phosphate buffer, pH 7.5, at 37 °C in the dark

Symbols: ○, GST-catalysed reaction; ●, uncatalysed reaction. The final volumes of incubation mixtures were 1.0 ml and the quantity of GST was 0.056 nmol. No GSH was present in the reaction mixtures. For further details see the Materials and methods section.

reactions approached equilibrium. In sharp contrast, uncatalysed isomerization of 13-cRA proceeded extremely slowly with an initial reaction velocity of only 5 pmol/min.

Figure 4 is a double-reciprocal (Lineweaver–Burk) plot of initial velocity against concentrations of substrate with GST as an isomerization catalyst. The maximal velocity ($V_{\max}$) was approx. 154 nmol/min per mg of protein. The Michaelis constant (K_m) was approximated as 9 μ M and k_{cat} ($V_{\max}/[E_0]$) of GST was approx. 2406 nmol of t-RA/min per nmol of GST (molar concentrations of GST were calculated on the basis of an average molecular mass of approx. 52 kDa).

Table 1 shows percentages of retinoic acids at various times when 13-cRA (10 μ M) was incubated with or without GST at 37 °C in the dark. In GST-catalysed reaction mixtures, t-RA

Figure 4 Double-reciprocal (Lineweaver–Burk) plot of initial velocity against concentrations of substrate

Slope of the plot ($K_m/V_{\max}$) was 0.059 and r^2 was 0.97. Incubations were conducted at 37 °C in the dark for 15 min. The concentrations of 13-cRA used were 10, 13.4, 20 and 27 μ M. The total quantity of GST was 0.064 nmol in all incubations. For further details see the Materials and methods section.

Table 1 Distributions of 13-cRA, 9-cRA and t-RA after incubations of 13-cRA (10 μ M) at 37 °C in the dark with or without GST

Incubations of 13-cRA (10 μ M) with or without GST (650 nM) in 0.1 M sodium phosphate buffer, pH 7.5, were conducted at 37 °C in the dark. The final volumes of reaction mixtures were 1 ml. Abbreviation: n.d., not detected (less than 1 ng/100 μ l).

Time (min)	GST-catalysed reaction			Uncatalysed reaction		
	13-cRA (%)	9-cRA (%)	t-RA (%)	13-cRA (%)	9-cRA (%)	t-RA (%)
0	100	n.d.	n.d.	100	n.d.	n.d.
15	67 $\pm$ 2.6	n.d.	33 $\pm$ 3.0	95 $\pm$ 2.0	n.d.	5 $\pm$ 1.0
60	40 $\pm$ 1.0	n.d.	60 $\pm$ 0.5	94 $\pm$ 1.0	n.d.	6 $\pm$ 0.6
180	28 $\pm$ 2.0	4 $\pm$ 0.5	68 $\pm$ 1.5	83 $\pm$ 1.0	n.d.	17 $\pm$ 0.8

Table 2 Total and specific activities of GST and other proteins/chemicals in the catalysis of isomerization of 13-cRA to t-RA

Incubations of 13-cRA (3.3 μ M) with GST or other compounds were performed in 0.1 M sodium phosphate buffer, pH 7.5 at 37 °C in the dark. GST-catalysed and GSH-catalysed reactions were conducted for 5 min, whereas reactions catalysed by other chemicals were conducted for 30 min. The final volumes of reaction mixtures were 1 ml. Abbreviation: n.d., not detected (less than 1 ng/100 μ l).

Compounds	Concentration (μ M)	Total activity (pmol/min per ml)	Specific activity (pmol/min per nmol)
GST	0.30	366 $\pm$ 28	1144 $\pm$ 88
GST	1.6	759 $\pm$ 12	474 $\pm$ 7
Apo ferritin	0.44	15 $\pm$ 2	34 $\pm$ 3
BSA	0.30	2 $\pm$ 0.2	7 $\pm$ 0.5
GSH	3.0	150 $\pm$ 14	50 $\pm$ 6
L-Cysteine	3.0	12 $\pm$ 0.5	0.04 $\pm$ 0.003
GSSG	3.0	n.d.	n.d.

accounted for approx. 30 % and 60 % of total retinoic acids after 15 and 60 min. After 1 h, isomerization seemed to reach equilibrium; there was no significant increase in t-RA generation as incubation times were increased from 1 to 3 h. The reaction mixtures contained only approx. 4 % of total retinoids as 9-cRA after 3 h of incubation. For uncatalysed reactions, in contrast, t-RA was far below its equilibrium percentage even after 3 h of incubation and 9-cRA was not detectable within that period.

Catalytic activities of GST and various thiol-containing compounds with reference to 13-cRA isomerization were compared (Table 2). The observed total isomerase activity of GST (0.3 μ M) was 2.5-fold and 31-fold the activities exhibited by GSH (3 μ M) and L-cysteine (3 μ M) respectively. There is an estimated average of 10 cysteine residues per molecule of GST according to Hayes and Pulford [14]. When comparisons were made at equimolar thiol concentrations, the specific activity of GST, which assesses the catalytic efficiency of the enzymes, was far greater than those of GSH and L-cysteine (23-fold and 28600-fold greater respectively). The total activity of GST was approx. 24-fold and 183-fold greater than those of apoferritin and BSA. Apoferritin reportedly contains 72 cysteine residues per molecule [7]. At the same molar holoprotein concentrations, the thiol concentration of apoferritin should be approx. 7-fold that of GST. Interestingly, the specific activity of apoferritin was approx. 1/34 that of GST. With increases in concentrations of GST, total activity increased significantly but the specific activity declined as expected. GSSG did not exhibit any detectable catalytic activity.

GSH (0.003–10 mM) was tested for total and specific activities

Table 3 Total and specific activities of GSH in catalysis of isomerization of 13-cRA to t-RA

GSH was incubated with 13-cRA (3.3 μ M) in 0.1 M sodium phosphate buffer (pH 7.5) at 37 °C for 15 min in the dark. The final volumes of incubation mixtures were 1 ml.

Concentration (mM)	Total activity (pmol/min per ml)	Specific activity (pmol/min per nmol)
0.003	150 $\pm$ 14	50 $\pm$ 6
0.1	384 $\pm$ 81	4.0 $\pm$ 0.8
5	42 $\pm$ 8	8.4 $\times 10^{-3}$
7	20 $\pm$ 3	2.7 $\times 10^{-3}$
10	14 $\pm$ 5	1.4 $\times 10^{-3}$

Figure 5 Direct comparison of catalytic activities of GST (0.2 mM) and GSH (10 mM) in conversions of 13-cRA to t-RA

Symbols: ○, GST; ●, GSH. GST or GSH was incubated with 13-cRA (3.3 μ M) in sodium phosphate buffer (0.1 M, pH 7.5) at 37 °C in the dark. The final volumes of incubation mixtures were 1 ml.

in the catalysis of *cis*–*trans* isomerization of retinoic acids (Table 3). The optimal concentrations for catalysing the reaction seemed to be near 0.1 mM. Very importantly, at physiological concentrations (5–10 mM), both total and specific activities of GSH were drastically decreased. At 10 mM, for example, the total and specific activities of GSH were decreased to approx. 1/27 and 1/35714 respectively from the optimal activities described above.

Direct comparisons at physiological concentrations of the catalytic activities of GST with GSH in conversions of 13-cRA to t-RA are presented in Figure 5. The physiological concentration of GST is approx. 0.2 mM, whereas physiological concentrations of GSH are approx. 10 mM [22]. The results showed definitively that GST was a much more effective catalyst than GSH in catalysing 13-cRA isomerization under conditions approximating those *in vivo*.

Heating GST at 100 °C for 3 min sharply decreased its isomerase activity by approx. 70 %. In contrast, the same heat treatment decreased the isomerization activities of GSH and apoferritin by only approx. 10 % and 20 % respectively and these changes were not statistically significant. Guanidine also inhibited the GST-catalysed isomerization reactions and the inhibition was concentration-dependent. At relatively low

Figure 6 Effects of variations in pH on GST-catalysed 13-cRA isomerization

Substrate (3.3 μ M) was incubated with GST in 0.1 M sodium phosphate buffer at various pH values at 37 °C in the dark for 15 min. Measurements obtained from uncatalysed reactions were subtracted. For further details see the Materials and methods section.

Figure 7 Effect of NEM on GST-catalysed 13-cRA isomerization

GST (0.048 nmol) was preincubated with various concentrations of NEM in 0.1 M sodium phosphate buffer, pH 7.5, at 37 °C in the dark for 20 min. The reactions were initiated by adding 13-cRA to the reaction vessels and the final concentration of substrate was 3.3 μ M. The reaction mixtures were incubated for a further 15 min under the same conditions. Incubation of 13-cRA with NEM alone did not generate detectable t-RA. The specific activity of the control was 1209 ± 5 pmol/min per nmol.

concentrations (0.5 M), guanidine showed virtually no effect. GST activity was decreased by less than 20 % when the concentration of guanidine was increased to 2 M. Increases in guanidine concentrations did not result in a further decline until its concentration was raised to 6 M. Activities then decreased abruptly with concentrations between 7 and 8 M. At each of the highest concentrations, GST's isomerase activities were decreased by approx. 60 %.

The catalysis of isomerization by GST was also pH-sensitive and the effects of variations in pH on GST-dependent activity are presented in Figure 6. When the pH was between 6.6 and 7.0 no significant changes in GST's activity could be detected. When the pH was increased to 7.6, however, the isomerase activity increased linearly. From pH 7.8 to 8.0, the activity tended to decrease. The

optimal pH for GST-catalysed reaction was estimated as approx. 7.8.

Figure 7 presents the effects of NEM on GST-catalysed 13-cRA isomerization. At concentrations below 0.1 μ M, NEM did not affect GST's isomerase activities detectably. From 0.1 to 10 μ M, however, additions of NEM resulted in a sharp decline in GST's activities. For example, at final concentrations of 1 and 10 μ M, the isomerase activities were decreased by approx. 60 % and 90 % respectively.

DISCUSSION

Our results showed that purified GST exhibited exceptional catalytic activities for the isomerization of 13-cRA to t-RA. The enzymic nature of the reaction was demonstrated by several important observations and experiments. First, at equilibrium, t-RA accounts for 60–70 % of total retinoic acids depending on conditions utilized [7,23]. GST-catalysed reactions proceeded extremely rapidly compared with the uncatalysed or GSH-catalysed reactions, and the GST-catalysed reactions approached equilibrium very quickly. Secondly, the initial reaction velocity was a function of GST concentrations (0–240 μ M) and was clearly saturable. Thirdly, the reaction was highly sensitive to protein-denaturing treatments such as heat and guanidine, and the reaction exhibited a pH optimum. Lastly, complete inhibition by NEM of the observed isomerase activities indicated that intact thiol groups are required for GST-catalysed *cis-trans* isomerization of retinoic acids. This is consistent with the concept that all isomerases so far identified contain intact thiol groups [24].

The GST-catalysed isomerization of 13-cRA to t-RA can be complicated by the concomitant non-enzymic reaction catalysed by its own protein thiol groups. Reportedly, there is an estimated average of 10 cysteine residues in individual GST molecules [14]. These cysteine thiol residues are likely to catalyse conversions of 13-cRA to t-RA in a pattern of thiol-dependent, non-enzymic catalysis. Isomerase activities remaining after inactivation by heat and guanidine (approximately 25 % and 35 % respectively) were apparently due to non-enzymic reactions. However, evidence strongly suggested that the rapid *cis-trans* isomerization of 13-cRA to t-RA catalysed by GST is due primarily to its enzymic catalysis rather than to non-enzymic catalysis. First, catalysis of GST seemed to be specific for the conversion of 13-cRA to t-RA and seemed not to catalyse other steric conversions. For example, at equilibrium, 9-cRA should account for roughly 15 % of a mixture of *cis-trans* retinoic acid isomers [23]. Concentrations of 9-cRA were still far below the equilibrium percentage when t-RA reached equilibrium concentration. Secondly, if non-enzymic catalysis were to account for the majority of the observed GST activities, GST should exhibit roughly the same activities as other thiol compounds at the same free thiol concentrations. However, GST (0.3 μ M) exhibited approx. 23-fold, 34-fold, 163-fold and 28 600-fold higher specific activity than GSH, apoferritin (a protein containing 72 free thiol groups per molecule), BSA and L-cysteine. The exceptional catalytic activity of GST is evidently due to a specific protein conformation rather than to its free thiol groups. Thirdly, inactivation of GST by heat (100 °C for 3 min) sharply decreased its activity by more than 70 %. In contrast, the same heat treatment decreased the catalytic activity of GSH by only approx. 10 %. Thiol groups can ultimately be oxidized through heat treatment but much longer times are required (more than 30 min) [25]. The sensitivity of GST to 3 min of heat treatment strongly indicated that enzymic catalysis accounts for the majority of the observed isomerase activities in a GST-catalysed reaction.

For enzymic *cis-trans* isomerization, isomerases have exhibited a requirement for one or more thiol groups for enzymic activity [24]. The necessity of thiol groups for isomerase activity was demonstrated with a virtually complete inhibition of maleate isomerase activity by thiol-blocking agents in purified isomerase systems [24]. In contrast with maleate isomerase, GST seemed to act as a thioltransferase rather than strictly as an isomerase in GST-mediated *cis-trans* isomerization of maleylacetone and maleylacetoacetate [17,24]. In *cis-trans* isomerization of 13-cRA to t-RA, GSTs seemed to act more strictly as isomerases rather than as thioltransferases or glutathione transferases. First, the GST-catalysed isomerization was completely independent of free GSH. Secondly, saturation of the reaction with substrate suggests that binding between 13-cRA and enzymes is important. Thirdly, GST's activities were inhibited by NEM at very low concentrations.

It also seems probable that GST has an important role in regulating the rates of interconversions of retinoic acids *in vivo*. First, the enzymes are extremely efficient for the isomerization of 13-cRA to t-RA at expected physiological concentrations. *In vivo*, when retinoic acids were orally administered to rats, equilibrium concentrations of 13-cRA, 9-cRA and t-RA in serum were observed within 30–60 min [9]. Highly effective enzymic catalysis is apparently required for such rapid interconversions. As shown in this study, 13-cRA was quickly converted to t-RA and equilibrium was achieved within 60 min under catalysis by GST. Secondly, specific catalytic activities of GSTs are much higher than endogenous thiol compounds such as GSH. Thirdly, at physiological concentrations, GSTs showed much higher catalytic activities than GSH in catalysing 13-cRA isomerization. Fourthly, GST accounts for approx. 10% of cytosolic proteins in the liver and is widely distributed in various tissues [26]. The total isomerase activities are expected to be high because of the abundance of the enzymes. Finally, because the expressions of GSTs are regulated by multiple genes, the same regulating mechanisms might also participate in the regulations of isomerization of 13-cRA to t-RA *in vivo*.

In summary, we have demonstrated that GST can effectively catalyse *cis-trans* isomerization of 13-cRA to t-RA *in vitro* and probably acts as a retinoic acid isomerase(s) *in vivo*. In rat hepatic tissues, two classes of cytosolic GST isoforms have been identified [18]. Alpha and Mu families account for approx. 37% and 63% respectively of total GST; however, which individual isoenzymes and to what extent they can catalyse the *cis-trans* isomerization of retinoic acids are not clear at present and are currently under investigation. It certainly will be of great importance to further

investigate the *cis-trans* isomerization of retinoic acids catalysed by individual GST isoenzymes.

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