Recombinant human glutathione S-transferases catalyse enzymic isomerization of 13-*cis*-retinoic acid to all-*trans*-retinoic acid *in vitro*

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The steric conversion of 13-*cis*-retinoic acid (13-cRA) to all-*trans*-retinoic acid (t-RA) has been proposed as an activation mechanism for the observed therapeutic and teratogenic activities of 13-cRA. Here we have investigated the catalysis of isomerization of 13-cRA to t-RA by recombinant human glutathione S-transferases (GSTs). Substrate was incubated with GST in 0.1 M sodium phosphate buffer, pH 7.5, at 37 °C in total darkness. The t-RA generated was measured quantitatively by HPLC. Under the reaction conditions used, GSTP1–1 was far more effective than human GSTM1–1 or human GSTA1–1 in catalysing the isomerization reaction. The reaction catalysed by GSTP1–1 showed substrate saturation and the K_m and V_{max} values for the reaction were approx. 7 μM and 650 pmol/min per nmol respectively. The reaction rate increased linearly with increasing enzyme concentration. The reaction was inhibited both by heat treatment and by *S*-decylglutathione (a potent inhibitor of transferase activity associated with GST). Additions of polyclonal rabbit antiserum for human GSTP1–1 to the reaction resulted in a significant decrease in generation of t-RA (70–80 %). In addition, ethacrynic acid, a selective substrate for Pi isoforms of GST, also inhibited the isomerization of 13-cRA to t-RA catalysed by GSTP1–1. Under the same reaction conditions, GSTP1–1 was much less effective in catalysing the steric conversion of 9-*cis*-retinoic acid to t-RA, indicating that the enzyme was stereospecific for the conversion of 13-cRA to t-RA. These observations suggest that enzymic catalysis was the primary mechanism for the GSTP1–1-dependent conversion of 13-cRA to t-RA. Reactions catalysed by a purified rat hepatic GST Pi isoenzyme proceeded more slowly than reactions catalysed by human GSTP1–1. Comparative studies also showed that there were marked species differences in catalytic activities between various purified mammalian hepatic GST mixtures.

INTRODUCTION

In addition to the catalysis of glutathione conjugation reactions, certain isoforms of glutathione S-transferases (GSTs) can catalyse *cis–trans* isomerizations of maleylacetone to fumarylacetone, maleylacetoacetic acid to fumarylacetoacetic acid, and Δ^5-3-ketosteroids to Δ^4-3-ketosteroids [1,2]. Studies also have indicated that GSTs possess prostaglandin isomerase activities [3,4].

It was demonstrated recently [5] that purified rat hepatic GSTs could also act as retinoid isomerases, with effective catalysis of the steric isomerization of 13-*cis*-retinoic acid (13-cRA) to all-*trans*-retinoic acid (t-RA). Because the conversion of 13-cRA to t-RA has been proposed as a prerequisite step for observed potent therapeutic as well as teratogenic effects of 13-cRA [6], the GST-catalysed isomerization of 13-cRA to t-RA might have a significant role in regulating the biological activities of 13-cRA *in vivo*.

Currently at least 20 cytosolic GST isoenzymes have been identified in human tissues; they belong to four classes: Alpha, Mu, Pi and Theta [1]. However, it is not known whether, to what extent or which of these individual isoforms can catalyse the steric conversion of 13-cRA to t-RA.

The overall purpose of this study was to investigate the potential catalysis by human GST of the isomerization of 13-cRA to t-RA. For this study, three commercially available recombinant human GST isoforms (GSTA1–1, GSTM1–1 and GSTP1–1) were investigated for their retinoic acid isomerase activities. Investigations of the Theta isoforms were not performed owing to commercial unavailability. HPLC was employed to separate and identify the steric isomers of retinoic acid. Antisera against human GST, *S*-decylglutathione (a potent inhibitor of the transferase activity associated with GSTs) and ethacrynic acid (a selective substrate of Pi class GST) were used for investigating the nature of human GST-catalysed reactions. For purposes of comparison, purified GSTs from various mammalian hepatic tissues also were characterized for their capacities to catalyse the same reactions. Isomerase activities were compared between human GSTs and other mammalian GSTs.

MATERIALS AND METHODS

Chemicals

Purified (purity more than 95 %), certified GSH-free, recombinant human GST Pi, Alpha and Mu isoforms (GSTP1–1, GSTA1–1 and GSTM1–1) were purchased from PanVera Corp. (Madison, WI, U.S.A.). Specific transferase activities were measured by the spectrophotometric determination of 1-chloro-2,4-dinitrobenzene conjugation with GSH (PanVera Corp.). Purified rat hepatic GST (Pi isoform) and polyclonal rabbit antiserum against human GSTP1–1 were purchased from Oxford Biochemical Research (Oxford, MI, U.S.A.). Specific transferase activities of these enzymes also were measured by spectrophotometric determinations of 1-chloro-2,4-dinitrobenzene conjugation with GSH (Oxford Biochemical Research). Affinity-chromatography-purified rat hepatic GST, bovine hepatic GST, rabbit hepatic GST, pig hepatic GST and horse hepatic GST were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Abbreviations used: 9-cRA, 9-*cis*-retinoic acid; 13-cRA, 13-*cis*-retinoic acid; GST, glutathione S-transferase; t-RA, all-*trans*-retinoic acid.

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Retinoic acids, GSH, *N*-ethylmaleimide, ethacrynic acid and *S*-decylglutathione were also purchased from Sigma. All other chemicals and reagents utilized were of the highest purity commercially available.

Conversion of 13-cRA to t-RA catalysed by GSTs

The isomerization substrate (13-cRA) was incubated with GST in 0.1 M sodium phosphate buffer, pH 7.5, at 37 °C in a water bath with continuous shaking in total darkness. Incubations without additions of GST or other catalysts served as controls. The final volume of incubation mixtures was 1 ml. Reactions were terminated by the addition of an equal volume of ice-cold propan-2-ol and 1 μmol of *N*-ethylmaleimide, which is a potent inhibitor of thiol-dependent reactions [5]. For incubation durations of 0–30 min the generation of products increased linearly. Incubations were completed within 15 or 30 min so that rates of reactions could be determined and compared under zero-order rate conditions. The incubation mixtures 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 for a maximum of 30 days at −80 °C for subsequent analyses by HPLC.

Inactivation by heat of human GST-catalysed conversions of 13-cRA to t-RA

Substrate was incubated with freshly prepared GST solutions or heat-inactivated GST solutions (100 °C for 3 min). Incubations were conducted under the same conditions as described above. The effects of inactivation by heat were determined by assessing the differences between the generation of t-RA in incubations with freshly prepared GST and that in incubations with heat-inactivated GST.

Inhibition by *S*-decylglutathione or ethacrynic acid of GST-catalysed conversion of 13-cRA to t-RA

Substrate, human GSTP1–1 and inhibitor (*S*-decylglutathione or ethacrynic acid) were incubated under the same conditions as described above. Incubations of substrate alone, substrate plus GST, or substrate plus inhibitor served as controls. The inhibitory effects of *S*-decylglutathione or ethacrynic acid were determined by assessing the differences between the generation of t-RA in incubations of GST plus *S*-decylglutathione or ethacrynic acid and from that in incubations of *S*-decylglutathione or ethacrynic acid alone. The effect of *S*-decylglutathione on the GSH-catalysed iosmerization of 13-cRA was also investigated. Instead of GSTP1–1, GSH (0.1 mM or 100 nmol/ml) was added to the incubation vessels by following the procedure described above. The reason for using 0.1 mM GSH was that the rate of GSH-catalysed isomerization of 13-cRA was maximal at this concentration [7].

Inhibition by antiserum against GST of GST-catalysed conversion of 13-cRA to t-RA

Antiserum against GSTP1–1 (70 μg/ml) was preincubated with GSTP1–1 (1.25 μg/ml) in 0.1 M sodium phosphate buffer, pH 7.5, at 37 °C for 20 min. The reaction was initiated by the addition of 13-cRA (3.3 μM) to the reaction vessels; incubations were performed for 30, 60 and 120 min. Incubations of 13-cRA with GSTP1–1 or with polyclonal antiserum alone served as controls. The inhibitory effects of polyclonal antiserum for human GSTP1–1 were determined by assessing the differences between the generation of t-RA in incubations of GST plus the antiserum and that in incubations of the antiserum alone.

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 the preparation 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 system was equipped with a Beckman mixing chamber and manual injector. The identification and quantification of retinoic acids were conducted with a Zorbax octadecylsilane column (0.46 cm × 25 cm) (MacMod Analytical, Chadds Ford, PA, U.S.A.) and by the method described by Kim et al. [8], with modifications. The analytical eluents consisted of solvent A [acetonitrile:water:acetic acid (49.75/49.75/0.5, by vol.)] and solvent B [acetonitrile:water:acetic acid (90/10/0.04, 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 0.7 ml/min. A 100 μl sample of a mixture of authentic 13-cRA, 9-*cis*-retinoic acid (9-cRA) and t-RA or the supernatant fraction of the incubation mixture was loaded on the HPLC column; the elution times of the standard retinoic acids were used to identify the peaks eluted from the HPLC column. The detection limit of the HPLC system for concentrations of retinoic acids was 1 ng/100 μl; concentrations of retinoic acids below 1 ng/100 μl were designated non-detectable.

Protein determination

The method of Lowry et al. [9] was used for the quantitative determination of the concentrations of proteins for rat hepatic GST, bovine hepatic GST, rabbit hepatic GST, pig hepatic GST and horse hepatic GST. BSA was used as a standard protein for the quantification.

Statistical analyses

All experimental data were expressed as the means ± S.D for three or four experimental measurements. A Microsoft Excel statistics package (version 5.0, Microsoft, Redmond, WA, U.S.A.) was used for all statistical analyses. For conversions of 13-cRA to t-RA catalysed by human GST, one-way and two-way analyses of variance (ANOVAs) with replication were used for determining whether GST-catalysed reactions were time-dependent and whether there was a statistically significant interaction between the effects of incubation times and the effects of individual isoforms of human GST for observed isomerization reactions. For inhibition by ethacrynic acid or antiserum of human GSTP1–1-catalysed isomerization reactions, both one-way and two-way ANOVAs with replication were used for determining whether the reactions were time-dependent and whether there was a statistically significant interaction between the effects of incubation times and the effects of doses of ethacrynic acid and antiserum for observed inhibition. Group-to-group comparisons with *t*-tests also were conducted (*post hoc*) to test further for statistical differences between mean values.

RESULTS

Figure 1 shows conversions of 13-cRA to t-RA catalysed by human GSTA1–1, GSTM1–1 or GSTP1–1. Two-way ANOVAs clearly indicated that there were significant differences in isomerase activities between the individual isoforms of GST ($P < 0.05$). The Pi class GST was by far the best catalyst when compared with the Mu and Alpha classes for the reactions investigated. The rates of the reactions catalysed by the Pi isoform were approx. 6-fold and 7-fold greater than those of the

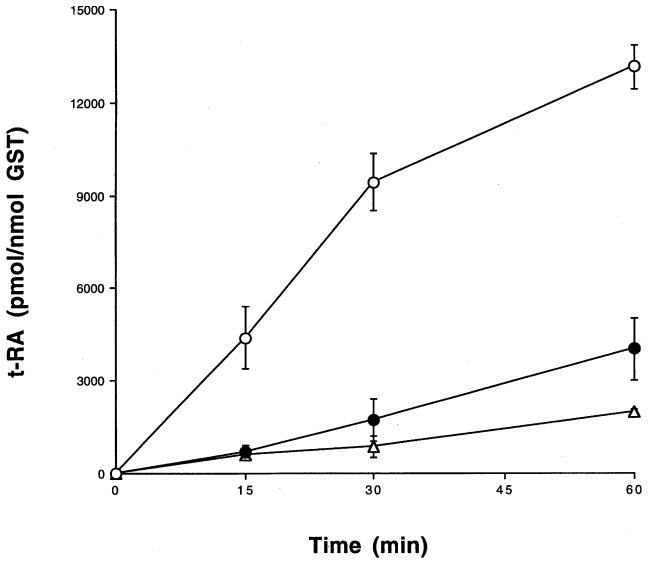

Figure 1 Direct comparisons of isomerase activities between isoforms of human GST for catalysis of conversions of 13-cRA to t-RA: ○, GSTP1–1; ●, GSTM1–1; △, GSTA1–1

Substrate (3.3 μM) was incubated with GST (0.24 nmol/ml) in 0.1 M sodium phosphate buffer, pH 7.5, at 37 °C in the dark. For further details see the Materials and methods section.

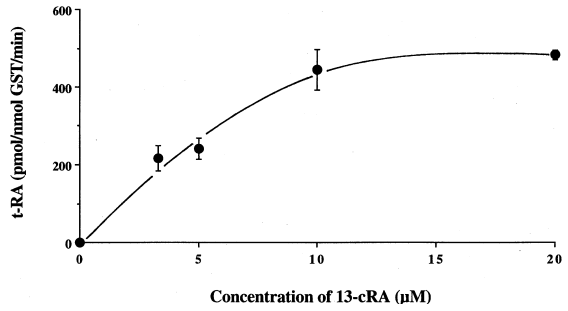

Figure 2 Effects of varying the concentration of substrate on initial rates of isomerization of 13-cRA to t-RA catalysed by human GSTP1–1

Substrate was incubated with GSTP1–1 (0.24 nmol/ml) in 0.1 M sodium phosphate buffer, pH 7.5, at 37 °C in the dark for 15 min. For further details see the Materials and methods section.

Table 1 Comparison of isomerase activities between human GSTP1-1, rat GSTP1-1 and various mammalian hepatic GSTs in catalysis of conversions of 13-cRA to t-RA

Substrate (16 μM) was incubated with GST in 0.1 M sodium phosphate buffer, pH 7.5, at 37 °C in the dark for 15 or 30 min. For further details see the Materials and methods section.

Enzyme	Catalytic activity (pmol/min per μg of protein)
Human GSTP1-1	21 ± 1.3
Rat GSTP1-1	9 ± 1.5
Rabbit hepatic GST	7 ± 1.0
Horse hepatic GST	4 ± 0.8
Rat hepatic GST	3 ± 0.4
Pig hepatic GST	3 ± 0.7
Bovine hepatic GST	0.7 ± 0.06

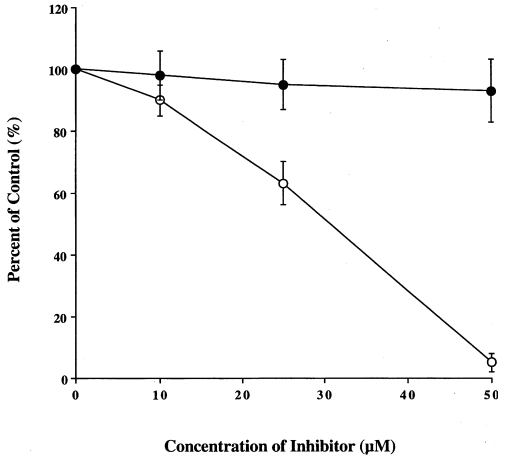

Figure 3 Effect of *S*-decylglutathione on isomerization of 13-cRA to t-RA catalysed by human GSTP1–1 (○) or GSH (●)

Substrate (16 μM) was incubated with GSTP1–1 (0.024 nmol/ml) or GSH (100 nmol/ml) plus *S*-decylglutathione at various concentrations (0–50 μM) in 0.1 M sodium phosphate buffer, pH 7.5, at 37 °C in the dark for 30 min. Control rates of GST and GSH-catalysed reactions (no inhibitor added) were 1942 ± 46 and 3.3 ± 0.7 pmol/min per nmol respectively. For further details see the Materials and methods section.

reactions catalysed by the Mu and Alpha isoenzymes respectively. Interestingly, human GSTP1–1 did not detectably catalyse the conversion of 9-cRA to t-RA unless a 10-fold greater quantity of enzyme was incorporated into the incubation mixtures. Even under such conditions, human GSTP1–1 was much less efficient for the catalysis of 9-cRA isomerization (approx. 50 % of the observed activity for catalysing the conversion of 13-cRA to t-RA). It was observed that the rates of reactions catalysed by human GSTP1–1 increased linearly with the quantity of enzyme (0–2.5 μg/ml). The human GST-catalysed conversion of 13-cRA to t-RA was also heat-sensitive. After heat treatment at 100 °C for 3 min, the enzyme lost approx. 70 % of its observed isomerase activity.

Figure 2 indicates the relationship between initial rates of reactions catalysed by human GSTP1–1 and concentrations of substrate. As concentrations of substrate increased, the reaction rates were also increased. Beyond 10 μM, however, the initial rates of the reactions did not increase significantly and there was clearly a substrate saturation. The Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) for the reaction were determined by using a double-reciprocal Lineweaver–Burk plot. The K_m and V_{max} values for the reaction were approx. 7 μM and 650 pmol/min per nmol respectively.

Table 1 presents direct comparisons for isomerase activities of human GSTP1–1, rat GSTP1–1 and various mammalian hepatic GSTs. Human GSTP1–1 exhibited the highest catalytic activity for the conversion of 13-cRA to t-RA.

Figure 3 presents the effect of *S*-decylglutathione on the human GSTP1–1- or GSH-catalysed conversion of 13-cRA to t-RA. *S*-Decylglutathione was a potent inhibitor of the GSTP1–1-catalysed reaction. The addition of 25 or 50 μM *S*-decylglutathione to reaction vessels resulted in a 35 % or 95 % decrease in the generation of t-RA respectively. In contrast, *S*-decylglutathione did not show a statistically significant inhibition of GSH-catalysed reactions at the same concentrations.

Ethacrynic acid, a selective substrate for Pi GST isoforms, showed inhibitory effects on the conversion of 13-cRA to t-RA catalysed by human GSTP1–1. At 0.5 or 2 mM, ethacrynic acid inhibited the reaction by approx. 50 % or 90 % respectively. Two-way ANOVAs indicated that the inhibitory effects of ethacrynic acid were concentration-dependent.

The addition of polyclonal rabbit antiserum against GSTP1–1 to the reaction resulted in a 70–80 % decrease in the generation of t-RA. Two-way ANOVAs showed that the inhibitory effects were statistically significant ($P < 0.05$).

DISCUSSION

Human classes Alpha, Mu and Pi GST homodimers were all shown to possess catalytic activity for the conversion of 13-cRA to t-RA, although class Pi was much more effective than the others. Unlike GST-catalysed *cis–trans* isomerizations of maleylacetone and maleylacetoacetate [2,10], the GST-catalysed *cis–trans* isomerization of retinoic acids seemed to be GSH-independent and thus GST seems to act more strictly as an isomerase enzyme than as a non-enzymic, thiol-dependent catalyst.

Several lines of evidence indicated that the observed human GST-catalysed 13-cRA isomerization was due mainly to enzymic catalysis. First, 13-cRA is a small hydrophobic compound; it can therefore bind readily to the hydrophobic substrate site of GST. In reactions catalysed by GSTP1–1, conversions of 13-cRA to t-RA were inhibited by ethacrynic acid, a selective substrate for transferase reaction catalysed by Pi class GST. This tended to suggest that 13-cRA and ethacrynic acid could share the same binding sites of GST. Secondly, the rates of human GST-catalysed isomerization reactions increased linearly as a function of enzyme concentration and the reaction exhibited substrate saturation kinetics. Thirdly, the GST-catalysed reaction was heat-sensitive: isomerase activity decreased sharply with heat treatment. In addition, antiserum against human GST showed a statistically significant inhibitory effect on the reaction. Lastly, the preference for 13-cRA over 9-cRA as a substrate indicated that the human GST-catalysed isomerization of retinoic acids was stereospecific. Considered together, the results strongly indicate that enzymic catalysis was the primary mechanism for the GST-dependent isomerization of 13-cRA to t-RA.

The steric isomerization of retinoic acids can also be catalysed by small thiol molecules such as GSH; that reaction is usually referred to as a thiol-dependent isomerization reaction [7,11–13]. The free thiol groups associated with human GST could therefore catalyse 13-cRA isomerization reactions via a non-enzymic mechanism. Therefore it was necessary to determine whether a non-enzymic catalysis might have contributed significantly to human GST-catalysed isomerization reactions. In the present study, *S*-decylglutathione was used for distinguishing non-enzymic catalysis from enzymic catalysis for two reasons. First, *S*-decylglutathione is a potent inhibitor of prostaglandin isomerase activity associated with GST [4]. Secondly, as shown in the results, *S*-decylglutathione had virtually no effect on GSH-catalysed 13-cRA isomerization, a free thiol-dependent catalysis of the isomerization reaction. Consequently any catalytic activity decreased by incubation with *S*-decylglutathione could be attributed to enzymic catalysis. Our results showed that *S*-decylglutathione inhibited GST-catalysed isomerization reactions almost completely at a concentration of 50 μM, suggesting that the non-enzymic catalysis was virtually insignificant for human GST-dependent 13-cRA isomerization.

In the present study, the catalytic activities of other purified mammalian hepatic GSTs were also investigated for the conversion of 13-cRA to t-RA. Rabbit hepatic GST exhibited the highest isomerase activity (7 pmol/min per μg of GST), whereas the activity of bovine hepatic GST was much lower (0.7 pmol/min per μg of GST). The observed differences in catalytic activity could be due to the compositions of individual GST isoenzymes in the tissues. As shown in the present study, GST classes Pi and Mu exhibited higher isomerase activities than class Alpha. Therefore tissues containing high concentrations of Pi or Mu isoenzymes might be expected to show higher catalytic activities in catalysing 13-cRA isomerization.

Class Pi GST isoenzymes might have a significant role in embryotoxicity induced by 13-cRA. As shown in this study, both human and rat Pi GST isoenzymes can effectively catalyse the conversion of 13-cRA to t-RA, which is much more embryotoxic than 13-cRA [6]. Studies have shown that GST class Pi is expressed in human and rat embryonic tissues during organogenesis [14,15]. Therefore the expression and regulation of GST class Pi might be an important determinant for the susceptibility of embryos to the insult of excessive 13-cRA. The extent to which tissues/cellular distribution of these enzymes is important will be of particular interest in future studies. Because 13-cRA is an established human teratogen, it will also be of great interest to investigate the expression and regulation of class Pi GST isoenzyme in human embryonic tissues during organogenesis. Such studies will provide valuable information for understanding the mechanisms by which 13-cRA produces profound teratogenic effects on the human fetus.

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