Evidence for Catalysis by a Conceptual Isomerase

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ABSTRACT:

The purpose of this study was to investigate whether and to what extent the steric isomerization of retinoic acids in conceptual tissues can be attributed to enzymatic catalysis in addition to thiol-dependent, nonenzymatic catalysis. Conversions of 13-cis-retinoic acid and 9-cis-retinoic acid to all-trans-retinoic acid catalyzed by cell-free preparations of conceptual rat tissues (gestational day 12.5) were investigated. Substrates and rat conceptual homogenates (RCH) were incubated in sodium phosphate buffer (0.1 M, pH 7.5) at 37°C in the dark. Incubation mixtures were quantitatively analyzed by HPLC. In RCH-catalyzed reactions, conversions of 13-cis-retinoic acid or 9-cis-retinoic acid to all-trans-retinoic acid were very rapid, in comparison with uncatalyzed isomerization reactions (incubations without RCH). Comparisons of the rates of reactions catalyzed by freshly prepared vs. freshly prepared/dialyzed RCH showed no significant differences, indicating that small, sulfhydryl-containing molecules such as reduced glutathione did not significantly contribute to the RCH-catalyzed reactions. Furthermore, at physiological concentrations (2.5–10 mM), reduced glutathione exhibited very low specific catalytic activities, indicating that nonenzymatic, sulfhydryl-dependent catalysis was not a major mechanism in catalyzing interconversions of retinoic acids in vivo. Enzymatic catalysis by RCH of the conversion of 13-cis-retinoic acid to all-trans-retinoic acid was further characterized by showing 1) substrate saturation kinetics, 2) reaction rates that increased proportionally with protein concentrations, and 3) much greater sensitivity of the reactions to heat inactivation and denaturation by urea, compared with nonenzymatic, glutathione-catalyzed reactions. Thus, isomerization of retinoids in conceptual tissues appeared to be under enzymatic control.

It is generally agreed that the embryotoxicity of retinoids is positively correlated with the binding affinities of retinoids for RARs (Levin, 1995), and an abundance of experimental data appears to support this concept (Kochhar and Penner, 1987; Creech Kraft and Juchau, 1992, 1993a,b; Creech Kraft et al., 1994). Interestingly, isotretinoin (13-cRA) is regarded as a potent human teratogen, but it binds to RARs very weakly (Levin, 1995). Evidence indicates that biotransformation of 13-cRA to t-RA (a high-affinity ligand for RARs) is probably a prerequisite process for the teratogenic effects of 13-cRA (Levin, 1995; Kim et al., 1994; Repa et al., 1993).

Studies have demonstrated that 9-cRA is a high-affinity ligand for retinoid X receptors (Levin et al., 1992; Heyman et al., 1992) and is a potent, directly acting dysmorphismogen (Creech Kraft and Juchau, 1993b; Creech Kraft et al., 1994). Although 9-cRA can be generated from its carotenoid precursors, isomerization of t-RA to 9-cRA has been suggested as an alternative pathway for the biogenesis of 9-cRA (Napoli, 1996). Therefore, interconversions between 9-cRA and t-RA may contribute significantly to the regulation of endogenous levels of both ligands in embryonic tissues.

Steric interconversions of retinoic acids have long been observed both in vitro and in vivo (Zile et al., 1967, 1982; Kalin et al., 1981; Sundaresan and Bhat, 1982; Kojima et al., 1994). At equilibrium, t-RA is the dominant isomer, accounting for approximately 60–70% of total retinoic acids, depending on the conditions used (Shih et al., 1986, 1994a). The reactions can be catalyzed by a variety of low-molecular weight sulfhydryl compounds in vitro and thus are often referred to as thiol-dependent, nonenzymatic, isomerization reactions (Shih et al., 1986, 1997; Urbach and Rando, 1994a). However, the physiological significance of such nonenzymatic catalysis can be questionable. Firstly, not all thiol compounds are capable of effectively catalyzing the reactions. For example, L-cysteine shows virtually no catalytic activity for conversions of 13-cRA/9-cRA to t-RA, although it contains a free thiol group (Shih et al., 1986, 1997). This raises the question of whether the nonenzymatic reaction is solely sulfhydryl dependent. Secondly, some sulfhydryl compounds that have shown high catalytic activities, such as mercaptopoethanol, apparently do not exist endogenously in biological tissues (Shih et al., 1986, 1997). Thirdly, GSH, a very important endogenous sulfhydryl compound, is effective in catalyzing isomerizations of retinoic acids but only at very low (nonphysiological) concentrations (0.05–0.15 mM) (Shih et al., 1986, 1997). At concentrations above this range, rates of GSH-catalyzed retinoid isomerizations are very low (Shih et al., 1986, 1997). Finally, it is difficult to...
imagine that interconversions of important retinoid acid ligands would be completely regulated by nonenzymatic processes (Napoli, 1996).

The existence of all-trans/11-cis-retinol isomerase, the key enzyme of the vertebrate visual cycle, was demonstrated 1 decade ago (Bernstein et al., 1987a,b; Fulton and Rando, 1987). Although that isomerase has not yet been completely purified, the following important biochemical properties of the isomerase have been established: 1) its activity is eliminated by heat denaturation of proteins, 2) its activity is sensitive to inactivation by many protein-denaturing detergents, and 3) its activity is membrane-associated (Bernstein and Rando, 1990). Whether a similar isomerase system exists in embryonic tissues and catalyzes the very important isomeric interconversions of retinoic acids has not been investigated, although the possibility has been raised (Napoli, 1996; Mangelsdorf et al., 1994; Tang and Russell, 1990).

We previously observed that t-RA was the predominant product of oxidative conversions of 13-cis- and 9-cis-retinal catalyzed by conceptal cytosol and microsomes (Chen and Juchau, 1997). One possible explanation for those observations is that 13-cRA and 9-cRA, generated from their parent aldehydes (13-cis- and 9-cis-retinal, respectively), were rapidly converted to the thermodynamically more stable t-RA. To examine this hypothesis, 13-cRA was incubated with conceptal cytosol under the same conditions used for the oxidative conversions of retinals to retinoic acids. As expected, 13-cRA was quickly converted to t-RA and the rapid reaction presumably was catalyzed, at least in part, by an embryonic isomerase (Chen and Juchau, 1997).

In the present investigations, our interest was to study steric conversions of retinoic acids catalyzed by potential embryonic isomerases during organogenesis (gestational days 9–13). It is now generally appreciated that embryos are very sensitive to retinoids during the stage of organogenesis. We focused on the conversions of 13-cRA and 9-cRA to t-RA for three primary reasons. Firstly, conversions to t-RA are thermodynamically favorable, relative to the corresponding reverse reactions (i.e. conversions of t-RA to 13-cRA and 9-cRA), and studies of the former are thus much more practical for such investigations. Secondly, enzymatic catalysis of the conversion of 13-cRA to t-RA represents an important bioactivation mechanism for retinoid receptor-mediated signal transduction pathways. Thirdly, isomerases catalyzing the conversion of 9-cRA to t-RA would also catalyze the reverse reaction (t-RA to 9-cRA), which could be one of the pathways for biogenesis of 9-cRA.

In these investigations, conversions of 13-cRA and 9-cRA to t-RA catalyzed by cell-free RCH were characterized and compared with corresponding uncatalyzed and GSH-catalyzed reactions. To focus on nonenzymatic reactions catalyzed by small sulfhydryl compounds in the tissue homogenates, RCH were dialyzed and the reactions catalyzed by dialyzed RCH and freshly prepared RCH were compared. To determine whether enzymatic catalysis was primarily responsible for the observed rapid conversions of retinoic acids catalyzed by RCH, GSH-catalyzed reactions were characterized under the same conditions and compared. To further examine the enzymatic nature of RCH-catalyzed reactions, effects of heat inactivation and urea (protein denaturants) on RCH- or GSH-catalyzed reactions were investigated. It was expected that enzymatic reactions would be much more sensitive to either heat inactivation or inhibition by urea.

**Materials and Methods**

**Chemicals.** t-RA, 13-cRA, 9-cRA, GSH, and urea were all purchased from Sigma Chemical Co., (St. Louis, MO). The purity of all retinoic acids was >98% (by HPLC). Benzoylated dialysis tubing (molecular weight cut-off, <1200) was also purchased from Sigma Chemical Co. All other chemicals and reagents used were of the highest purity commercially available.

**Preparation of RCH.** Time-mated pregnant rats (Sprague-Dawley, Wistar-derived) were obtained from Tyler Laboratories (Bellevue, WA) on day 12.5 of gestation. Preparation of RCH was as previously described (Chen et al., 1995).

**Dialysis of RCH.** To exclude cis-trans isomerization catalyzed by low-molecular weight materials in embryonic tissues, freshly prepared RCH were dialyzed against sodium phosphate buffer (1:1000, v/v) at 4°C. The buffer was changed after 7 hr of dialysis, and dialysis was then continued overnight (12 hr). Specific isomerase activities of fresh nondialyzed RCH were compared with those of dialyzed RCH.

**Uncatalyzed Conversions of 13-cRA or 9-cRA to t-RA.** For uncatalyzed isomerization reactions, 13-cRA or 9-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. Incubations were conducted in a darkened room with yellow lights, to prevent both photosomerization and photooxidation of retinoids. At the end of the incubations, an equal volume of ice-cold isopropanol was added to the incubation mixtures. The mixtures were then vortex-mixed for 1 min and centrifuged for 30 min at 16,000g at 4°C in the dark. The extraction procedure was equally effective for 13-cRA, 9-cRA, and t-RA, and extraction efficiencies for individual retinoid acids were all >95%. The supernatant was saved and stored at −80°C for subsequent HPLC analysis.

**RCH- or GSH-Catalyzed Conversions of 13-cRA or 9-cRA to t-RA.** For RCH- or GSH-catalyzed isomerization reactions, 13-cRA or 9-cRA was incubated with RCH or GSH under the same conditions as those used for uncatalyzed isomerization. Briefly, RCH or GSH was added to sodium phosphate buffer (0.1 M, pH 7.5) and mixed well. The reactions were initiated by the addition of substrate (13-cRA or 9-cRA) and were carried out for various times (0–120 min) at 37°C in the dark. Preliminary experiments indicated that short incubation times (<60 min) greatly reduced the contributions of uncatalyzed isomerization to t-RA or GSH-catalyzed reactions. Therefore, most of incubations were completed within 60 min or less. Extraction of retinoids followed the procedure described previously (Chen et al., 1995). Samples were also saved and stored at −80°C for subsequent HPLC analysis.

**Effects of Heat Inactivation on RCH- or GSH-Catalyzed 13-cRA Isomerization.** RCH or GSH was boiled for various times (3–10 min at 100°C). RCH, heat-treated RCH, GSH, or heat-treated GSH was added to the buffer. The reactions were initiated by addition of 13-cRA and were carried out for 20 min at 37°C in the dark. Generations of t-RA from freshly prepared RCH- or GSH-catalyzed reactions served as controls.

**Effects of Urea on RCH- or GSH-Catalyzed 13-cRA Isomerization.** RCH alone, urea (7 M) alone, or RCH plus urea (7 M) was added to sodium phosphate buffer (0.1 M, pH 7.5). Incubations were initiated by addition of 13-cRA, and the reactions were carried out for 20, 40, 60, or 120 min at 37°C in the dark. Longer incubation times (60 and 120 min) were used in this experiment, to ensure that the denaturation of isomerases by urea could be observed at several time points. The effects of urea denaturation were determined by assessing the differences between the t-RA generation from incubations with RCH plus urea and the t-RA generation from incubations with urea alone. Following the same procedures, GSH alone, urea alone, or GSH plus urea was incubated with 13-cRA, and the effects of urea on GSH-catalyzed 13-cRA isomerizations were determined.

**Identification and Quantitation of Retinoic Acids by HPLC.** The solvent delivery system for HPLC consisted of two model 100A dual-piston Beckman pumps linked together for activation of a binary gradient. The system was interfaced with a Shimadzu SPD-10A UV/visible 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. Identification and quantitation of isomers of retinoic acid in incubation mixtures were conducted by using a Zorbax octadecylsilane column (0.46 × 15 cm; MAC- MOD Analytical, Inc., Chadds Ford, PA) and the method described by Kim et al. (1992), with modifications. The analytical eluents consisted of solvent A (acetonitrile/H₂O/acetic acid, 49.75:49.75:0.5, v/v/v) and solvent B (acetonitrile/H₂O/acetic acid, 90:10:0.04, v/v/v), both containing 10 mM ammonium acetate. The HPLC conditions included 80% solvent B and 20% solvent A, with a flow rate of 0.6 ml/min. One hundred microliters of a mixture of authentic 13-cRA, 9-cRA, and t-RA of the supernatant fraction from an
incubation mixture were loaded onto the HPLC column, and the elution time of each standard retinoic acid was used to identify the peaks eluting from the HPLC column. The detection limit of the HPLC system for retinoic acids was 1 ng/100 μl. Detected concentrations of retinoic acids below 1 ng/100 μl were designated as not detected. The concentrations of individual retinoic acids were determined by a quantitative calculation program installed in the CRS01 Chromatopac data processor, using known amounts of standard retinoic acids for calibration.

**Protein Determination.** The method of Lowry et al. (1951) was used to quantitatively determine the concentration of protein in RCH. Bovine serum albumin was used as a standard protein for the quantitation.

**Statistical Analysis.** All experimental data were expressed as the means ± SD of three or four experimental measurements. A Microsoft Excel statistics package (version 5.0; Microsoft, Redmond, WA) was used for all statistical analyses. Both one-way and two-way ANOVAs, with replication, were used for determining statistical significance and potential interactions of the experimental data (for greater than two-way comparisons). Post hoc group-to-group comparisons with t tests were also performed to test statistical differences between mean values involving two-way comparisons.

**Results**

Fig. 1A presents a typical HPLC chromatogram of a mixture of authentic retinoic acids. Individual retinoic acids were well separated under the conditions used and thus were accurately quantified. Fig. 1, B and C, shows HPLC chromatograms of 13-cRA and of 13-cRA after incubation with RCH for 15 min.

Results from uncatalyzed isomerization reactions of 13-cRA and 9-cRA are presented in table 1. Absolute amounts (nanograms) and percentage concentrations of individual retinoic acids in the incubation mixtures are given. Uncatalyzed conversions of 13-cRA and 9-cRA to t-RA were extremely slow. For example, after 30 min of incubation, no t-RA was detected in the mixtures from incubations with 13-cRA or 9-cRA. Even after 4 hr of incubation, t-RA accounted for only approximately 18–19% in the mixtures from incubations with 13-cRA or 9-cRA. It was also noted that approximately 30–35% of the retinoic acids were lost by the end of 4-hr incubations with 13-cRA or 9-cRA. Those losses of retinoic acids could be the result of slow nonenzymatic autoxidation and catabolic decomposition of the retinoic acids.

Catalysis by components of RCH greatly accelerated conversions of 13-cRA/9-cRA to t-RA (table 2). After 60 min of incubation, t-RA accounted for approximately 63% of the total retinoic acids in RCH-catalyzed 13-cRA isomerization reactions. For RCH-catalyzed 9-cRA isomerization reactions, t-RA accounted for approximately 44% of the total retinoic acids after 60 min of incubation.

Fig. 2 shows direct comparisons of isomerase activities of freshly prepared RCH and dialyzed RCH in catalyzing conversions of 13-cRA/9-cRA to t-RA (table 2). After 60 min of incubation, t-RA accounted for approximately 63% of the total retinoic acids in RCH-catalyzed 13-cRA isomerization reactions. For RCH-catalyzed 9-cRA isomerization reactions, t-RA accounted for approximately 44% of the total retinoic acids after 60 min of incubation.

Fig. 3 shows linear increases in the conversion of 13-cRA to t-RA as the concentrations of RCH proteins were increased. When concentrations of proteins were increased from 0.014 mg to 0.03 mg, generation of t-RA increased from 200 pmol/ml to approximately 350 pmol/ml. For values greater than 0.03 mg, increases of protein concentrations resulted in slow increases in the conversion of 13-cRA to t-RA.

Initial reaction rates for RCH-catalyzed 13-cRA/9-cRA isomerization reactions were determined by drawing tangents to initial parts of the progress curves shown in fig. 4. The initial rates (slopes of the tangents) for RCH-catalyzed conversions of 13-cRA and 9-cRA to t-RA were approximately 950 and 620 pmol/min/mg protein, respectively. Regression analyses were performed to determine whether conversions to t-RA from 13-cRA/9-cRA were increased linearly as incubation times were increased. There were statistically significant linear relationships between incubation times (0–30 min) and generation of t-RA from 13-cRA (r² = 0.95) and from 9-cRA (r² = 0.97).

Specific catalytic activities of RCH (2.5–10 mM) were directly compared, and the results are provided in table 3. Overall, RCH were far more effective than GSH in catalyzing conversions of both 13-cRA and 9-cRA to t-RA. The catalytic activities of GSH were substantially decreased as its concentrations were increased.

Fig. 5 shows the effects of varying substrate concentrations on the rates of conversion of 13-cRA to t-RA catalyzed by RCH or GSH. For RCH-catalyzed reactions, saturation by substrate was observed as the concentration of 13-cRA was increased from 2.0 to 3.5 μM. In contrast, no saturation by substrate was observed for GSH (1 and 4 mM)-catalyzed reactions. Instead, linear regression analyses indicated that there were linear relationships between the concentrations of substrate and the initial rates for GSH-catalyzed reactions (r² > 0.95 in both cases). Fig. 5 clearly shows that the rates of reactions catalyzed by RCH were much greater than the rates of those catalyzed by GSH (1 and 4 mM) with the same concentrations of substrate. The Kₘ of...
and $V_{\text{max}}$ for RCH-catalyzed conversion of 13-cRA to t-RA were estimated as 3.0 $\mu$M and 3000 pmol/mg protein/min, respectively, from a double-reciprocal, Lineweaver-Burke plot.

The effects of heat inactivation on specific activities of RCH and GSH (0.1 mM) for catalysis of the conversion of 13-cRA to t-RA are shown in Fig. 6. Treatment of RCH at 100°C for 3 and 5 min reduced isomerase activities by approximately 35 and 40%, respectively. In contrast, the same treatments with GSH as catalyst caused only approximately 5 and 20% reductions, respectively, in catalytic activ-

### TABLE 1

Distributions of 13-cRA, 9-cRA, and t-RA after incubations of 13-cRA or 9-cRA (uncatalyzed isomerization reactions)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>13-cRA as Substrate</th>
<th>9-cRA as Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13-cRA</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>50 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>40 ± 1</td>
<td>100</td>
</tr>
<tr>
<td>60</td>
<td>34 ± 2</td>
<td>94</td>
</tr>
<tr>
<td>120</td>
<td>30 ± 1</td>
<td>88</td>
</tr>
<tr>
<td>240</td>
<td>28 ± 3</td>
<td>82</td>
</tr>
</tbody>
</table>

Incubations of 13-cRA or 9-cRA (3.3 $\mu$M) in 0.1 M sodium phosphate buffer (pH 7.5) were conducted at 37°C in the dark. Final volumes of the reaction mixtures were 1 ml. Values are expressed as means ± SD of four experimental measurements. For additional details, see Materials and Methods.

### TABLE 2

Distributions of 13-cRA, 9-cRA, and t-RA after incubations of 13-cRA or 9-cRA with RCH

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>13-cRA as Substrate</th>
<th>9-cRA as Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13-cRA</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>50 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>41 ± 1</td>
<td>89</td>
</tr>
<tr>
<td>15</td>
<td>27 ± 2</td>
<td>68</td>
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<tr>
<td>30</td>
<td>23 ± 1</td>
<td>58</td>
</tr>
<tr>
<td>60</td>
<td>11 ± 2</td>
<td>31</td>
</tr>
</tbody>
</table>

Incubations of 13-cRA or 9-cRA (3.3 $\mu$M) were with RCH (1.1 mg protein/ml) in 0.1 M sodium phosphate buffer (pH 7.5) and were conducted at 37°C in the dark. Final volumes of the reaction mixtures were 1 ml. Values are expressed as means ± SD of four experimental measurements. For additional details, see Materials and Methods.

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**Fig. 2.** Direct comparisons of isomerase activities of freshly prepared RCH (□) and dialyzed RCH (●) in catalyzing conversions of 13-cRA and 9-cRA to t-RA.

RCH or dialyzed RCH (0.35 mg protein/ml) was incubated with 13-cRA or 9-cRA (both at 3.3 $\mu$M) in sodium phosphate buffer (0.1 M, pH 7.5) for 30 min at 37°C in the dark. Final volumes of the incubation mixtures were 1 ml. For additional details, see Materials and Methods.

**Fig. 3.** Effects of varying concentrations of RCH protein on the isomerization of 13-cRA to t-RA.

RCH (0–0.014 mg protein/ml) was incubated with 13-cRA (0.2 $\mu$M) in sodium phosphate buffer (0.1 M, pH 7.5) at 37°C in the dark for 60 min. Final volumes of the incubation mixtures were 1 ml. For additional details, see Materials and Methods.
As heat inactivation was extended to 7 and 10 min, catalytic activities of both RCH and GSH were reduced sharply. Two-way ANOVA indicated that there was a strong interaction between heat-treated RCH and heat-treated GSH in catalyzing the conversion of 13-cRA to t-RA ($p < 0.001$), indicating that RCH-catalyzed reactions were significantly more sensitive to heat treatment than were GSH-catalyzed reactions.

Fig. 7 shows the effects of urea on RCH- and GSH-catalyzed conversions of 13-cRA to t-RA. RCH-catalyzed reactions were very sensitive to urea. After only 20 min of incubation with urea (7 M), catalytic activities of RCH were reduced sharply (by approximately 50%). After 40 min of incubation, the activities were further reduced (by 75%). In contrast, GSH-catalyzed reactions appeared to be far less sensitive to treatment with urea. Even after 40 min of incubation with urea, there was no statistically significant decrease in GSH-dependent catalytic activity. Catalytic activities of GSH were reduced by approximately 20 and 55% at longer incubation times of 1 and 2 hr, respectively. Two-way ANOVA indicated that there was a strong interaction between RCH- and GSH-catalyzed reactions when RCH and GSH were incubated with urea ($p < 0.001$), indicating that RCH-catalyzed reactions were significantly more sensitive to treatment with urea than were GSH-catalyzed reactions.

**Discussion**

In this study, we demonstrated that cell-free embryonic tissue preparations were very effective in catalyzing the isomerization of retinoic acids, compared with uncatalyzed reactions. RCH-catalyzed isomerizations were chemically characterized and directly compared with nonenzymatic, GSH-catalyzed, isomerization reactions. Two lines of evidence strongly supported the concept that enzymatic catalysis accounts for most of the bioactivation of 13-cRA to t-RA via isomerization in embryonic tissues. Firstly, the majority of the observed isomerase activity of RCH was not due to low-molecular weight materials such as GSH. Embryonic concentrations of GSH were estimated to be approximately 25 nmol/mg protein (Harris et al., 1986; Hernandez and Lijinsky, 1989). In our studies, the final concentrations of GSH in the incubation vessels were 0.001–0.02 mM. At
incubation with 13-cRA. Under the conditions used, an approximately 10^6-fold dilution of GSH was achieved after dialysis (assuming that equilibrium had been approached during dialysis). At these extremely low concentrations, GSH showed virtually no catalytic activities (Shih et al., 1986). Indeed, comparisons between reactions catalyzed by freshly prepared RCH and by dialyzed RCH showed no statistically significant differences in catalysis of the conversion of 13-cRA to t-RA. This combined evidence strongly suggested that small sulfhydryl-containing molecules, such as GSH, were not the main source of the catalytic activities exhibited in embryonic tissues. Secondly, rates of RCH-catalyzed conversion of 13-cRA to t-RA were proportional to concentrations of conceptal proteins and the reactions showed saturation kinetics. Substrate saturation suggested that binding between the substrate and the enzyme was required for the reactions. In comparison, nonenzymatic, GSH-catalyzed reactions showed a nearly linear relationship ($r^2 = 0.95$) between reaction rates and concentrations of substrate.

RCH-catalyzed 13-cRA isomerization reactions were also very sensitive to heat inactivation. Observed isomerase activities of RCH were reduced by >35 and >40% after 3 and 5 min, respectively, of heating at 100°C. In contrast, GSH-catalyzed reactions appeared to be far less sensitive to heat treatment. Only 5 and 25% reductions in the catalytic activities of GSH were observed after GSH was boiled for 3 and 5 min, respectively. The difference in the reductions of catalytic activities between RCH- and GSH-catalyzed reactions was likely the result of denaturation of enzymes. Interestingly, both RCH and GSH retained approximately 40% of their original catalytic activities after 7 and 10 min of heat inactivation. The retained activities were likely due to nonenzymatic catalysis. It was reported that sulfhydryl groups could be oxidized through heat treatment but that much longer heating times (>30 min) were required (Hamm and Hofmann, 1965). To observe complete inhibition of catalytic activities of RCH and GSH, longer heat inactivation treatment times appeared to be necessary.

RCH-catalyzed conversions of 13-cRA to t-RA were also far more sensitive to urea (a potent protein denaturant) than were GSH-catalyzed reactions. Isomerase activities of RCH declined by approximately 50 and 75% after incubation with urea for 20 and 40 min, respectively, at 37°C. The sharp decreases in the catalytic activities of RCH were likely the result of denaturation of the conceptal isomerase by urea, rather than the effects of urea on nonenzymatic catalysis. This was supported by the observation that urea showed no effects on GSH-catalyzed reactions at the same incubation times (20 and 40 min). Although urea could eventually affect nonenzymatic, GSH-catalyzed reactions, much longer incubation times (1 and 2 hr) were required to observe significant inhibition. Comparison of the sensitivities to urea provided additional support for the concept that enzymatic catalysis was the major mechanism for RCH-catalyzed conversion of 13-cRA to t-RA.

The putative embryonic isomerase may have important physiological significance. Firstly, the enzymatic catalysis of the conversion of 13-cRA to t-RA appeared to be very efficient. Equilibrium concentrations of retinoic acids in serum were observed within 30–60 min when retinoic acids were administered orally to rats (9), and the efficiency of the isomerase was in agreement with the rapid isomerizations observed in vivo. Secondly, the putative isomerase showed a relatively low $K_m$ (approximately 3.0 μM) for conversion of 13-cRA to t-RA. Endogenous levels of retinoic acids in rodent embryos during organogenesis were estimated to be in the low nanomolar range (Creech Kraft and Juchau, 1992). After treatment with retinoids, the serum and embryonic levels of retinoic acids are expected to increase drastically. Under such circumstances, the isomerase should be readily saturated and thus the $V_{max}$ of the reaction could be achieved.

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**Fig. 6. Effects of heat inactivation on RCH-catalyzed (○) or GSH-catalyzed (●) 13-cRA isomerization.**

RCH or GSH was boiled for 0, 3, 5, 7, or 10 min and cooled to room temperature. RCH (0.028 mg protein/ml) or GSH (0.1 mM) was incubated with 13-cRA (3.3 μM) in sodium phosphate buffer (0.1 M, pH 7.5) at 37°C in the dark for 20 min. Final volumes of the incubation mixtures were 1 ml. Specific activities for freshly prepared RCH and GSH were 2639 ± 67 pmol/min/mg protein and 2702 ± 111 pmol/min/mg GSH, respectively. For additional details, see Materials and Methods.

**Fig. 7. Effects of urea on RCH-catalyzed (○) or GSH-catalyzed (●) 13-cRA isomerization reactions.**

Substrate (3.3 μM) was incubated with RCH (0.028 mg protein/ml) or GSH (0.1 mM), with or without the addition of urea (7 M), in sodium phosphate buffer (0.1 M, pH 7.5) at 37°C in the dark for 20 min. Final volumes of the incubation mixtures were 1 ml. Specific activities for freshly prepared RCH and GSH were 2639 ± 67 pmol/min/mg protein and 2702 ± 111 pmol/min/mg GSH, respectively. For additional details, see Materials and Methods.
Finally, at physiological GSH concentrations (2–10 mM), GSH-dependent catalysis was so ineffective that it required a much longer time to bring 13-cRA and t-RA into equilibrium, in contrast to RCH-catalyzed reactions. In vivo, therefore, enzymatic catalysis seems to be crucial for bioactivation of 13-cRA to t-RA.

Although nonenzymatic, GSH-dependent catalysis was much less effective for isomerization of 13-cRA to t-RA with physiological GSH concentrations, it might be an important complementary mechanism to enzymatic catalysis under various conditions. Firstly, the distribution and localization of the embryonic isomerase are not presently known. It is possible that the isomerase may be expressed only at specific sites of specific cells in embryos during organogenesis. GSH, on the other hand, is present more ubiquitously in all known tissues and cells and thus could substitute for the isomerase and catalyze isomerizations of retinoic acids. Secondly, overall concentrations of GSH in rodent embryonic tissues were estimated to be 1–2 mM, levels significantly lower than hepatic concentrations (approximately 10 mM) (Harris et al., 1986; Hernandez and Lijinsky, 1989). In addition, embryonic GSH concentrations may vary, and levels of GSH in certain types of cells may be lower than 1 mM. As demonstrated in this investigation and other studies (Shih et al., 1986, 1997), GSH can be very efficient in catalyzing conversions of 13-cRA and 9-cRA to t-RA at lower concentrations, such as 0.1 mM. Therefore, for certain types of cells at specific developmental stages, GSH may be important in regulating levels of retinoid receptor ligands.

Although evidence has suggested that an isomerase may play an important role in conceptual isomerization of 13-cRA to t-RA, other sulfhydryl-containing proteins may also contribute to the observed RCH isomerase activities. This is because dialysis cannot separate those proteins from the potential isomerase, and heat inactivation may also denature those proteins. Complete elucidation of enzymatic catalysis would rely on future isolation and purification of the isomerase enzyme(s).

In summary, we have presented evidence that an embryonic isomerase may play an important role in steric conversions of 13-cRA and 9-cRA to t-RA, in contrast to thiol-dependent, nonenzymatic catalysis, at concentrations similar to those observed in vivo. The existence of an isomerase has been demonstrated by high catalytic efficiencies, saturation kinetics, and the inactivation of catalytic activities by heat and urea. Additional studies are needed to further identify the enzyme and to completely elucidate the physiological significance of a retinoid isomerase in retinoid-induced cytotoxicity.

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References


