NONENZYMATIC ISOMERIZATION OF 9-CIS-RETINOIC ACID CATALYZED BY SULFHYDRLY COMPOUNDS

TZU-WEN SHIH, TSU-HAN LIN, Y. FULMER SHEALY, AND DONALD L. HILL

Southern Research Institute

(Received November 6, 1995; accepted September 26, 1996)

ABSTRACT:

Certain thiol-containing compounds catalyze, in a chemical reaction, the isomerization of 9-cis-retinoic acid to a mixture of all-trans-retinoic acid, 9-cis-retinoic acid, 13-cis-retinoic acid, and 9,13-dicis-retinoic acid. In the presence of such catalysts, all-trans-retinoic acid gives rise to the same mixture. Reactions approaching equilibrium contain more all-trans-retinoic acid than either of the other isomers. Small molecules effective as catalysts are mercaptoethanol, l-cysteine methyl ester, glutathione, and N-acetyl-l-cysteine. Apoferritin (a thiol-containing protein), native microsomes, and, to a lesser extent, boiled microsomes catalyze the reaction. In intact cells, these interconversions also occur in a process inhibited by a sulfhydryl-specific reagent. The thiol-catalyzed isomerization of 9-cis-retinoic acid may be relevant in the biological activity of this compound.

In the absence of other forms of vitamin A, all-trans-RA, a naturally occurring compound, supports growth of animals and allows epithelial differentiation (1, 2). In animal models, RA and similar compounds with a side chain that possesses, or is readily convertible to, a free carboxylic acid group (retinoid acids) prevent and/or inhibit the development of preneoplastic and neoplastic lesions, especially in the skin and bladder (see ref. 3). Although the presence of such a group seems to be necessary for chemopreventive activity in the skin, such structures are generally associated with greater toxicity (4, 5) and teratogenic activity (6, 7). In clinical trials, RA is useful in the treatment of acute promyelocytic leukemia (8) and bladder papillomas (9), and 13-cis-RA has notable effects for the treatment of leukoplakia (10) and for the prevention of head and neck cancer in patients who have been successfully treated for the first occurrence of this disease (11).

Many biological effects of retinoid acids are mediated through the RARs (12, 13) and RXRs, which may function as homodimers or as RAR/RXR heterodimers (14, 15). RA is a ligand for the RARs, and 9-cis-RA is a ligand for both the RARs and RXRs (16, 17). Ligand binding activates the capacity of these receptors to alter gene transcription. As the only known physiologic ligand for RXRs, 9-cis-RA may play an important role in tumor differentiation (18).

Retinoids readily undergo isomerization. After pharmacological doses of RAs, 13-cis-RA appears in tissues of intact animals (19, 20), and RA appears after doses of 13-cis-RA (21–24). Furthermore, RA administered to rats gives rise to the biliary metabolite, 13-cis-retinyl-β-glucuronide, formed by isomerization of the free acid before glucuronidation (25, 26). As produced by liver microsomes from hamsters, 13-cis-4-oxo-RA is a metabolite common to both RA and 13-cis-RA (27).

This study was supported by Grant PO1 CA34968 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

Send reprint requests to: Dr. Donald L. Hill, Southern Research Institute, P.O. Box 55305, Birmingham, AL 35255-5305.
were grown to a density of $3 \times 10^6$ cells/ml. To exclude effects of components of the medium, cells were harvested and resuspended in an isotonic buffer containing glucose (41). To portions (2 ml) of these cells, either RA or 9-cis-RA was added at a final concentration of 30 $\mu$M. To some cultures, PHMB, a compound known to penetrate cells (42), was added at a final concentration of 1 mM 2 hr before addition of the retinoid. Cultures were incubated at 37°C. After 4 hr, the cells were harvested. To those preparations not previously exposed to PHMB, this compound was added at a concentration of 1 mM. Cells were harvested, suspended in 1 ml of water, and frozen before analysis. To 0.1 ml of each cell suspension, 9 volumes of acetonitrile were added, and preparations were centrifuged. Supernatants were evaporated to dryness and reconstituted in the HPLC mobile phase before analysis.

The chemical reaction mixtures, without solvent extraction, were transferred to amber vials and analyzed by reversed-phase HPLC. For this purpose, a Beckman liquid chromatograph system composed of a solvent delivery module 110B, analog interface module 406, programmable detector module 166 set at 340 nm, and an autosampler 507 (Beckman Instruments, Inc., San Ramon, CA) was used. Samples (40 $\mu$l) were injected onto a 4.6 mm i.d. 3.250 mm Spherisorb ODS 5-$\mu$m column (Chromanetics, Williamston, NJ) and were eluted at a flow rate of 1 ml/min, with a solvent of acetonitrile:1% acetic acid (7:3, v/v). The column was fitted with a Spherisorb ODS guard column.

With this HPLC system, the 9-cis-, 13-cis-, 9,13-dicis-, and all-trans-isomers of RA were separated with retention times of 13.2, 11.5, 12.2, and 14.6 min, respectively. Standard curves for the retinoids were determined to be linear over the range of 4–200 pmol/injection. All procedures involving manipulation of retinoids were performed under subdued yellow light. With the standard conditions of analysis, no measurable degradation of retinoids was observed.

Results

In a buffered system, mercaptoethanol catalyzed the conversion of 9-cis-RA to RA, 13-cis-RA, and 9,13-cis-RA. These retinoids were detected and measured by use of the HPLC method described previously (fig. 1). With concentrations of 20, 80, and 320 $\mu$M mercaptoethanol, the initial rates for formation of the three products increased with increasing concentration. In the presence of 80 or 320 $\mu$M mercaptoethanol, the rates of isomerization of 9-cis-RA to RA were essentially linear with time for $\sim$4 hr (fig. 2A). At a concentration of 320 $\mu$M mercaptoethanol, the formation of 13-cis-RA and 9,13-cis-RA from 9-cis-RA was also linear, but only for 2–3 hr (fig. 2, B

![Figure 1](https://example.com/fig1.png)

**Fig. 1. Chromatographic separation of the isomers of RA.**

In this example, the starting material was 9-cis-RA. Peaks are identified as follows: **A**, 13-cis-RA; **B**, 9,13-cis-RA; **C**, 9-cis-RA; and **D**, RA.

![Figure 2](https://example.com/fig2.png)

**Fig. 2. Relationship of time and mercaptoethanol concentration on the isomerization of 9-cis-RA.**

Products are (**A**) RA, (**B**) 13-cis-RA, and (**C**) 9,13-cis-RA. Standard conditions of analysis were used, except that the time of incubation and concentration of mercaptoethanol were varied as indicated. CRA, cis-RA; O, 20 $\mu$M; ▲, 80 $\mu$M; ▲, 320 $\mu$M.

![Figure 3](https://example.com/fig3.png)

**Fig. 3. Relationship between the concentration of 9-cis-RA and its rate of isomerization.**

Standard conditions of analysis were used, except that the concentration of 9-cis-RA was varied as indicated. ▲, RA; O, 13-cis-RA; ▲, 9,13-dicis-RA. and C; at this concentration of mercaptoethanol, formation of products approached a maximum at 4–8 hr. At a concentration of 640 $\mu$M (not shown), however, the rate of isomerization was not as fast as that...
for 320 μM. After 24 hr of incubation, the reactions approached equilibrium. At this time, there was 72% present as RA, 12% as 9-cis-RA, 11% as 13-cis-RA, and 5% as 9,13-cis-RA.

With 9-cis-RA as starting material, the rate of formation of RA increased linearly with increasing 9-cis-RA concentrations up to 20 μM; at 60 μM; maximum isomerization was approached (fig. 3). Formation of 13-cis-RA was linear for concentrations up to 40 μM, and formation of 9,13-cis-RA was linear for concentrations up to 60 μM.

Of various thio and other compounds tested as catalysts for the isomerization of 9-cis-RA, mercaptoethanol and L-cysteine methyl ester were most active (table 1). Apoferritin, GSH, N-acetyl-L-cysteine, and native microsomes had moderate activity; boiled microsomes, oxidized GSH, and bovine serum albumin demonstrated minimal activity; L-cystine, L-cysteine, thiocyanate, thiourea, and ethanolamine were without detectable activity. In the presence of native microsomes, the relative amount of 9,13-dicis-RA was higher than for other catalysts.

With increasing concentrations of catalyst, the maximum rate of isomerization of 9-cis-RA to RA (~1 nmol/hr) occurred with 160 μM mercaptoethanol (fig. 4A); higher concentrations of catalyst were inhibitory. Concentrations of L-cysteine methyl ester in the range of 160–640 μM were maximal; no inhibition at high concentrations was noted (fig. 4B). With apoferritin, a sulfhydryl-containing protein, as

### TABLE 1

**Effect of various compounds as catalysts for the isomerization of 9-cis-RA**

<table>
<thead>
<tr>
<th>Compound (Concentration)</th>
<th>RA 13-cis-RA 9,13-cis-RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercaptoethanol (0.1 mM)</td>
<td>0.975 0.170 0.113</td>
</tr>
<tr>
<td>L-Cysteine methyl ester (0.1 mM)</td>
<td>0.523 0.091 0.072</td>
</tr>
<tr>
<td>Apoferritin (1 mg/ml)</td>
<td>0.315 0.069 0.049</td>
</tr>
<tr>
<td>GSH (0.1 mM)</td>
<td>0.301 0.057 0.047</td>
</tr>
<tr>
<td>N-Acetyl-L-cysteine (0.1 mM)</td>
<td>0.244 0.040 0.021</td>
</tr>
<tr>
<td>Microsomes (1 mg/ml)</td>
<td>0.154 0.032 0.063</td>
</tr>
<tr>
<td>Boiled microsomes (1 mg/ml)</td>
<td>0.019 &lt;0.002 &lt;0.002</td>
</tr>
<tr>
<td>GSSG&lt;sup&gt;b&lt;/sup&gt; (0.1 mM)</td>
<td>0.010 &lt;0.002 &lt;0.002</td>
</tr>
<tr>
<td>Bovine serum albumin (1 mg/ml)</td>
<td>0.008 &lt;0.002 &lt;0.002</td>
</tr>
<tr>
<td>L-Cystine (0.1 mM)</td>
<td>&lt;0.002 &lt;0.002 &lt;0.002</td>
</tr>
<tr>
<td>L-Cysteine (0.1 mM)</td>
<td>&lt;0.002 &lt;0.002 &lt;0.002</td>
</tr>
<tr>
<td>Potassium thiocyanate (0.1 mM)</td>
<td>&lt;0.002 &lt;0.002 &lt;0.002</td>
</tr>
<tr>
<td>Thiourea (0.1 mM)</td>
<td>&lt;0.002 &lt;0.002 &lt;0.002</td>
</tr>
<tr>
<td>Ethanolamine (0.1 mM)</td>
<td>&lt;0.002 &lt;0.002 &lt;0.002</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard conditions of analysis were used, except that the catalyst was varied as indicated.

<sup>b</sup> GSSG, oxidized glutathione.

---

**FIG. 4. Relationship between catalyst concentration and the rate of isomerization of 9-cis-RA.**

Catalysts are (A) mercaptoethanol, (B) L-cysteine methyl ester, (C) apoferritin, and (D) native microsomes. Standard conditions of analysis were used, except that the catalyst and its concentration were varied as indicated. ▲, RA; O, 13-cis-RA; ×, 9,13-dicis-RA.
catalyst, the rate of formation of RA increased with increasing concentration of protein (fig. 4C). Up to a concentration of ~3 mg of microsomal protein/ml, the rate of isomerization of 9-cis-RA to RA increased with increasing concentrations of microsomes (fig. 4D). The relative amount of 9,13-dicis-RA formed was greater than that for other catalysts.

The rate of isomerization of 9-cis-RA to RA increased with increasing temperature (fig. 5). For mercaptoethanol, l-cysteine methyl ester, apoferritin, and microsomes, the reaction rate increased more rapidly than for microsomes. Derived from data in this figure, the energy of activation for the reaction in the presence of catalysts was as follows: mercaptoethanol, 18,968; L-cysteine methyl ester, 15,938; apoferritin, 20,756; and microsomes, 6,469 cal/mol.

Mercaptoethanol was also effective as a catalyst in the conversion of RA to 9-cis-RA, 13-cis-RA, and 9,13-cis-RA (fig. 6, A–C). In the range of 20–320 μM, the initial rates for formation of the three products increased with increasing concentration of mercaptoethanol. In the presence of 320 μM mercaptoethanol, the rate of isomerization of RA to 13-cis-RA was essentially linear with time for ~4 hr. In the reactions resulting in formation of 13-cis-RA and 9,13-cis-RA from RA, no linearity was noted. After 24 hr of incubation, the reactions approached equilibrium. At this time, there was 78% present as RA, 8% as 9-cis-RA, 9% as 13-cis-RA, and 5% as 9,13-cis-RA. At a concentration of 640 μM mercaptoethanol (not shown), however, the rates were generally not as fast as that for 320 μM.

Combinations of mercaptoethanol (30 μM) and microsomes (1 mg/ml) and combinations of mercaptoethanol (30 μM) and GSH (30 μM) catalyzed isomerization of 9-cis-RA, but to an extent less than additive for the individual components (not shown).

In cultured Hep #2 cells exposed to RA or 9-cis-RA, the concentration of the added retinoid decreased over a period of 2 hr; concentrations of the other isomers increased proportionally (table 2). Formation of other isomers was greatly reduced in the presence of PHMB.

**Discussion**

As catalyzed by nucleophiles, geometrical isomerization is most efficient for α,β-unsaturated compounds (see ref. 43). A sulphydryl group that may be either an integral part of an enzyme or part of a coenzyme is involved in cis-trans-isomerases not accomplishing bond migration. Although an enzyme exists to catalyze the isomerization of maleylacetone, GSH alone accomplishes the reaction (44). Also, in chemical reactions, thiocyanate catalyzes the isomerization of maleic acid (45) and cis-β-acetylaceylacrylic acid (46); and various thiol compounds catalyze the isomerization of oleic acid (47).

The rates of isomerization of 9-cis-RA to RA and of RA to 9-cis-RA in the presence of mercaptoethanol are similar to those previously observed for the interconversion of 13-cis-RA and RA in the presence of GSH (29). Although, in the previous effort, we did not
detect 9,13-cis-RAl, we now note that the chromatographic method used at that time does not allow separation of 9,13-cis-RAl from 13-cis-RAl. In the previous experiment involving the isomerization of RA to 13-cis-RAl, the values reported for 13-cis-RAl could be ~1.5 times the actual values. If so, there would, nevertheless, be no change in interpretation of the results.

Of the geometric isomers of RA, RA itself is most stable thermodynamically; in all of the systems we have tested, its concentration at equilibrium is greatest. The ratio of products is similar when either RA or 9-cis-RAl is used as the starting material. Reported values for RA and its isomers at equilibrium in a system containing 1% bovine serum albumin and 1% sodium dodecyl sulfate are as follows: RA, 61.4%; 13-cis-RAl, 17.2%; 9-cis-RAl, 14.8%; and 9,13-cis-RAl, 6.7% (32). Values reported herein are similar. 9-cis-RAl (33). Values reported herein are similar. 9-cis-RAl and 13-cis-RAl have equivalent thermodynamic properties; and 9,13-dicis-RAl is the least preferred form. The main difference is that we found, at equilibrium, a greater percentage of RA. The formation of relatively large amounts of 9,13-dicis-RAl in the presence of microsomes could be due to stabilization of this compound by some protein component of the micromolar preparation.

As in the isomerization of 13-cis-RAl to RA catalyzed by mercaptoethanol and especially by GSH (29), high concentrations of mercaptoethanol result in a slower rate of isomerization of 9-cis-RAl. At such concentrations, the sulfhydrol groups may interact with other molecules of mercaptoethanol and thus be unavailable for catalysis.

Although washed microsomes and some individual proteins catalyze the interconversion of RA, 13-cis-RAl, 9-cis-RAl, and 9,13-cis-RAl, it is unlikely that the reaction in intact cells is predominantly enzymatic for the following reasons: the reaction is not stereospecific; small, thio-containing molecules catalyze the reaction; boiled microsomes retain a portion of their original activity; and, over a wide concentration range, including amounts where micelle formation would be expected (48), the isomerization of 9-cis-RAl to RA, 13-cis-RAl, and 9,13-cis-RAl is directly proportional to its concentration. It remains possible that specific enzymes could also accomplish some of the isomerizations, but their presence would not be necessary for the reactions to proceed.

The basis for the activity of l-cysteine methyl ester and N-acetyl-l-cysteine as catalysts for the isomerization reaction, whereas l-cysteine itself is ineffective, may be due to the lack of penetration of the zwitterionic l-cysteine molecule into the micelles of retinoid and sodium dodecyl sulfate, which would presumably exist under these conditions (49).

Boiled microsomes are apparently less active than native microsomes due to oxidation of most of the free sulfhydryl groups during the heating process, such is known to occur during the boiling of proteins (50). Apoferritin, which has 72 sulfhydryl groups/molecule (51), readily catalyzes the isomerization process; bovine serum albumin, which has only one reactive sulfhydryl group/molecule (52), has lower catalytic activity. In intact cells, the chemical isomerization of retinoids may be accomplished, in part, by GSH, which is present in all tissues, mostly in the cytoplasm (53). Sulfhydryl groups of proteins would probably contribute to the reaction. For mercaptoethanol and microsomes, relative values for the calculated energy of activation for formation of RA from 9-cis-RAl are in the same order (mercaptoethanol > microsomes) as those for the same catalysts for the conversion of 13-cis-RAl to RA (29).

The isomerization of 9-cis-RAl in intact cells demonstrates that this process occurs in biological systems, as has been demonstrated for RA and 13-cis-RAl (19–24). Inhibition by PHMB indicates that the isomerization process is accomplished with the involvement of sulfhydryl groups.

These results indicate that isomerization of retinoids in intact cells can be accomplished by GSH and/or by sulfhydryl groups on proteins. In mouse tissues, the GSH content ranges from 1 to 11 μmol/g (~1–11 mM) (54); the total Ellman-reactive sulfhydryl content is ~20 mM, as calculated from the sulfhydryl content of individual HeLa cells (55). As demonstrated by the in vitro reactions reported herein, these concentrations are sufficiently high to allow isomerization.

We previously noted that the isomerization of 13-cis-RAl and RA is much faster with l-cysteine methyl ester than with l-cysteine, and suggested that a nucleophilic attack by a sulfhydryl group occurs on the unsaturated retinoid system and that isomerization results from an addition-elimination mechanism (29). The present results are consistent with this suggestion.

References