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Thiol dependent isomerization of all-*trans*-retinoic acid to 9-*cis*-retinoic acid

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Abstract The important biological effector 9-*cis*-retinoic acid can be generated by liver microsomes or by bovine serum albumin in detergent. The mechanism of this isomerization reaction is the subject of these studies. The protein mediated isomerization process is shown to be thiol- and pH-dependent. Moreover, the retinoic acids are also isomerized by 1-dodecanethiol in the presence of detergents. This isomerization process is pH-dependent as well, with isomerization rates increasing with pH. The isomerization reactions are quenched with free radical traps, such as α-tocopherol and ascorbic acid, suggesting that a thiol radical mechanism, rather than a thiolate anion-dependent mechanism, is implicated here. The pH dependence can be understood in terms of a thiol radical mechanism, because thiol radicals are produced from thiolate anions in the presence of oxygen. The facile thiol-mediated isomerization of the retinoic acids suggests that this could be a physiologically relevant mechanism for the formation of 9-*cis*-retinoic acid from all-*trans*-retinoic acid.

Key words: Isomerization; Retinoid; Thiol radical

1. Introduction

Recent studies have identified 9-*cis*-retinoic acid as a novel hormone which may be important in mammalian development [1–3]. It can bind to a subset of retinoic acid receptors known as RXR receptors, as well as binding to RAR receptors [1–3]. The biological activity of 9-*cis*-retinoic acid is presumed to be distinct from that of the well-described all-*trans*-retinoic acid [4–6].

The discovery of 9-*cis*-retinoic acid raises questions concerning the mechanism by which it is biosynthetically produced. In a simple mechanism, it could arise from all-*trans*-retinoic acid by a direct isomerization route. In previous studies it had been found that liver microsomes contain an activity capable of generating 9-*cis*-retinoic acid from added all-*trans*-retinoic acid [7]. However, the reaction is nonstereospecific, in that 13-*cis*-retinoic acid is generated, and the reaction is also not saturable with substrate at physiologically relevant concentrations [7]. This means that there is a chemical mechanism readily available for the isomerization of retinoic acids in cells which either masks or, indeed, is the mechanism of the physiological synthesis of 9-*cis*-retinoic acid.

The isomerization of retinoic acid is mediated by thiols. This point is clearly made here by showing that both microsomal and BSA/SDS mediated catalysis are strongly inhibited by thiol blocking reagents. Moreover, the isomerization of retinoic acid is also catalyzed by thiols, such as 1-dodecanethiol, in SDS. It is further shown that the isomerization of retinoic acid is probably thiol radical-mediated since isomerization can be blocked by free radical trapping agents.

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Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CMC, critical micelle concentration; DT, 1-dodecanethiol; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; NEM, *N*-ethylmaleimide; PHMB, *para*-hydroxymercuribenzoate; RA, all-*trans*-retinoic acid; SDS, sodium dodecyl sulfate.

2. Experimental

2.1. Materials

Retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinal, α-tocopherol and β-carotene were purchased from Sigma Chemical Co. All-*trans*-retinol was purchased from Fluka Chemical Company. Trolox was purchased from Aldrich. Ascorbic acid was purchased from Fisher and recrystallized from isopropanol/chloroform. Tritium labeled retinoids, 11,12-[^{3}H]retinoic acid and 11,12-[^{3}H]retinol were purchased from NEN. 9-*cis*-Retinoic acid was prepared from 9-*cis*-retinal or 9-*cis*-retinol by NaCN catalyzed MnO_2 oxidation in methanol followed by hydrolysis of the resulting methyl ester [8,9]. 9,13-*dicis*-Retinoic acid was prepared by HPLC purification from a photoisomerate of all-*trans*-retinoic acid. Its identity was established by NMR spectroscopy, and by comparing it with known spectra [10]. Fresh bovine liver was purchased from Research 87 (Revere, MA).

2.2. Preparation of bovine liver microsomal fraction

Bovine liver microsomes were prepared as previously described [7]. A Bio-Rad D_C Protein assay kit was used on protein samples prepared by deoxycholate/TCA precipitation [11].

2.3. Extraction of retinoic acid isomers from reaction mixtures

Retinoic acid isomers were extracted from microsomally catalyzed isomerization mixtures (500 μl) by addition of 850 μl 7:3:1 $CHCl_3$/CH_3OH/HCOOH containing 0.1% BHT and a nonradioactive isomerate of retinoic acid. The mixtures were then shaken vigorously, and centrifuged to separate the layers. Extracts were concentrated in vacuo. The residue was dissolved in 5 μl $CHCl_3$, then 200 μl heptane containing 0.1% BHT was added. The resulting heptane solutions were centrifuged to precipitate solids, and then were analyzed by HPLC. All incubations, extractions, and manipulations of the retinoic acids were performed in the dark in tubes wrapped in aluminum foil to prevent any artifactual photoisomerization reactions from occurring.

For reactions catalyzed by 1-dodecanethiol, extraction proceeded as follows. To the reaction (~100 μl) was added simultaneously 20 μl HCOOH and 250 μl heptane. The mixtures were shaken vigorously, then 20 μl saturated NaCl was added (in the case of Triton X-100 micelles), and the reactions were centrifuged to separate the layers. To the heptane layers was added 1 μl of a nonradioactive isomerate of retinoic acid. The heptane layers were analyzed by HPLC.

2.4. HPLC analysis of isomerization reaction mixtures

Extracts from isomerization assay reactions were analyzed by HPLC on two LiChrosorb Si-60 (5 μm particle size) columns connected in series as previously described [7]. The mixtures were eluted in a solvent

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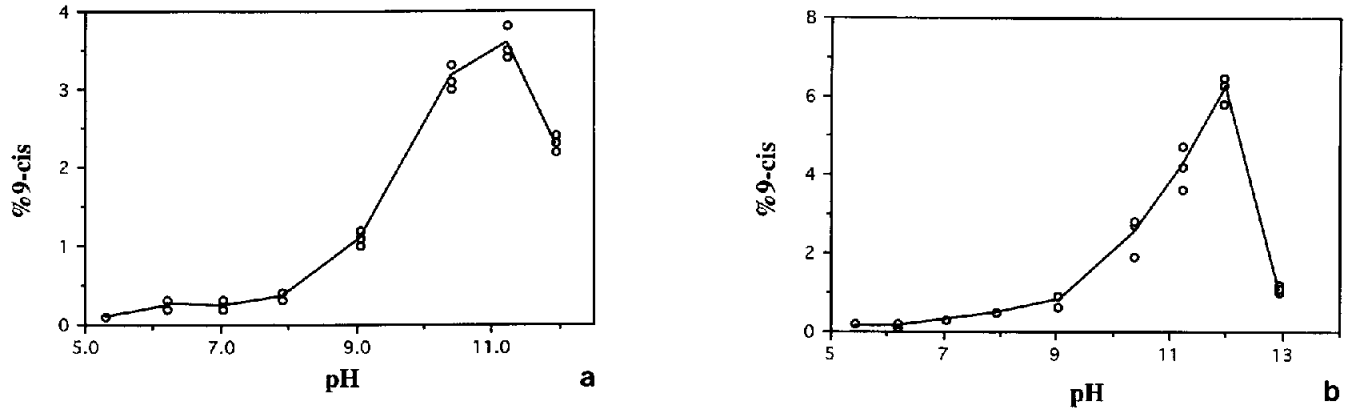

Fig. 1. Effects of pH on the isomerization of retinoic acid in detergents. (a) A preincubated, vortexed solution of 1μCi all-*trans*-retinoic acid in 50 μl 1% Triton X-100 was added to a preincubated solution of 50 μl 100 μM DT in 1% Triton X-100 with 400 mM Na-phosphate buffers of various pH values. The mixture was immediately vortexed and incubated 5 min at 37°C. Reactions were quenched by simultaneous addition of 20 μl HCOOH and 250 μl heptane containing 0.1% BHT. Extraction and HPLC analysis were conducted as described in section 2. (b) A preincubated, vortexed solution of 1μCi all-*trans*-retinoic acid in 50 μl 1% SDS was added to a preincubated solution of 50 μl 100 μM 1-dodecanethiol in 1% SDS with 400 mM Na-phosphate buffer. The mixture was immediately vortexed and incubated 10 min at 37°C. Reactions were quenched by simultaneous addition of 20 μl HCOOH and 250 μl heptane containing 0.1% BHT. Extraction and HPLC analysis were conducted as described in section 2.

containing 1.4% octanol in hexane with 10 mM TFA, run at a flow rate of 1.5 ml/min. Nonradioactive retinoic acid isomerates for use as standards in HPLC analysis were prepared by irradiation of heptane solutions of retinoic acid containing 0.1 mg I_2 with fluorescent lights for 15–30 min. This produced all the major isomers present at thermal equilibrium, including all-*trans*, 9-*cis*, 13-*cis*, and 9,13-*dicis*-retinoic acids. These isomers had been previously identified by comparison of retention times of the isomerate with those of authentic samples of the isomers. Under the described HPLC conditions, the retention times of the retinoic acid isomers were as follows: 13-*cis*, 10.1 min; 9,13-*dicis*, 12.3 min; 9-*cis*, 13.5 min; and all-*trans*, 15.3 min.

3. Results

In order to elucidate the contribution of thiols to the microsomally and BSA/SDS catalyzed isomerization of retinoic acid, the effect of various thiol reagents on retinoic acid isomerization was studied, both in microsomes and in BSA/SDS (Table 1). In both systems, *N*-ethylmaleimide (NEM) and *para*-hydroxymercuribenzoate (PHMB) were effective in greatly reducing microsomal and BSA-catalyzed isomerization. Therefore it seems likely that in the cases so far investigated the isomerization reaction is thiol-mediated.

This is directly demonstrated by showing that isomerization is strongly catalyzed by 1-dodecanethiol (DT) in detergents (Fig. 1). Since thiols are ionizable, it was of interest to determine the effects of pH on the catalyzed isomerization of all-*trans*-retinoic acid. The effects of pH on the isomerization of all-*trans*-retinoic acid in liver microsomes, in SDS and in Triton X-100 were determined and are shown in Figs. 1 and 2. Isomerization only occurs in the presence of thiol (in this case DT) when detergents are used. Isomerization in microsomes occurs in the absence of added thiol. As can be seen in Fig. 1, the rates of isomerization in the detergents increase for pH up to 1–12, and then decrease. The rates of isomerization of retinoic acid were also enhanced at higher pHs in microsomes (Fig. 2), but here a maximum was not reached.

The fact that the isomerization rates are still increasing at pH ~11 and beyond cannot easily be reconciled with a thiolate-catalyzed mechanism, given that the protonated retinoic acid would of necessity be the Michael acceptor. Since the pK_a of retinoic acid in solution is approximately 4 [12], the interaction of retinoic acid with micelles would have to shift its pK by approximately +7 pK units for a thiolate-mediated mechanism to reasonably be supposed to occur. This remote possibility, however, can be tested directly by determining the pK_a values for retinoic acid, as well as those for thiol, in micelles.

The pK_a of DT was readily measured by following the absorption at 240 nm as a function of pH. The pK_a was measured to be approximately 11.5 in water and between 13 and 14 in detergents (data not shown). Thus, the detergents had a substantial effect on the acidity of DT, raising it by at least 2 pK units.

The pK_a values of retinoic acid in the detergents were readily measured by the published procedure [13]. The pK_a was measured to be approximately 7 in Triton X-100, and 8 in SDS (data not shown). The effect of detergent on the acidity of retinoic acid was approximately 3–4 pK units, and was somewhat more

Table 1
Effects of sulfhydryl blocking reagents on the BSA and microsome-mediated isomerization of retinoic acid

Catalyst mixture	Thiol reagent	% 9-*cis*-isomer in product	% decrease in isomerization vs. controls
BSA/SDS	None	6.7 ± 1.2	–
BSA/SDS	NEM	0.6 ± 0.1	91%
BSA/SDS	PHMB	4.2 ± 0.3	39%
Microsomes	None	2.1 ± 0.1	–
Microsomes	NEM	0.4 ± 0.1	81%
Microsomes	PHMB	0.5 ± 0.3	76%

Solutions containing 50 mM (NEM) and 50 mM PHMB were prepared. The PHMB solution was cloudy and was vortexed prior to use. To 1250 μl aliquots of liver microsomes containing 16 mg/ml protein in 50 mM Na-borate pH 9 was added 312 μl of distilled water (as a control), 50 mM NEM, or 50 mM PHMB. Likewise, to 1250 μl aliquots of 1% BSA/1%SDS was added 312 μl of thiol reagent or distilled water. The mixtures were incubated for 150 min on ice. To solutions containing 1 μCi [^{3}H]-all-*trans*-retinoic acid in 100 μl 100 mM Na-borate, pH 8, was added 500 μl treated microsome suspension or BSA/SDS solution. Reactions were incubated 1h at 23°C before extraction with 7:3:1 $CHCl_3$/CH_3OH/HCOOH, 0.1% BHT. Extracts were then concentrated and analyzed by HPLC as described in section 2.

pronounced than in the case of DT. However, even though the pK_a values of retinoic acid were substantially positively shifted in the detergents, they were not shifted enough to explain the pH dependence on isomerization if a thiolate mechanism were to be operative. Therefore, a free radical basis for the reaction was sought.

A variety of free radical trapping agents, such as α-tocopherol, β-carotene, Trolox and ascorbate, were tested as inhibitors of the isomerization process catalyzed by microsomes or by DT in detergents. As shown in Table 2, some of these radical traps had profound effects on the isomerization process. In the case of DT in SDS, Trolox virtually abolished isomerization and both α-tocopherol and ascorbic acid markedly decreased it. In microsomes the effects were less profound, although both ascorbic acid and Trolox did decrease the amount of isomerization. It is concluded from these experiments that the isomerization mechanism is likely to be free radical in nature, both in micelles and in microsomes.

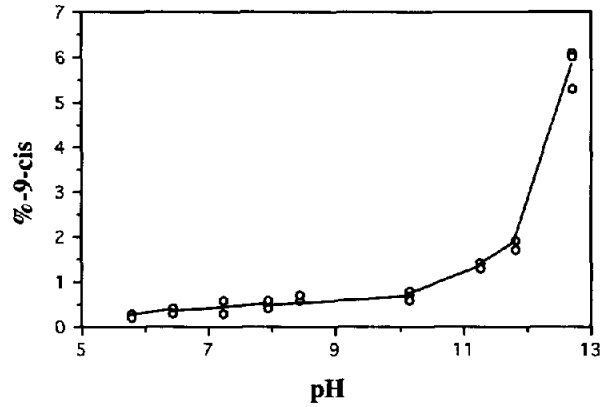

Fig. 2. Effects of pH on the isomerization of retinoic acid by liver microsomes. To 400 μl of a microsomal suspension (3.6 mg protein) was added 100 μl 500 mM Na-phosphate buffers at a variety of pH values. The combined, preincubated microsome mixtures were added to 5 μl solutions containing 1 μCi all-*trans*-retinoic acid in ethanol. Reactions were incubated with shaking at 23°C for 5 min, then 850 μl 7:3:1 $CHCl_3/CH_3OH/HCOOH$ containing a retinoic acid isomerate was added and the mixture was vortexed. Extraction and analysis proceeded as described in section 2.

4. Discussion

Retinoic acid receptors are among the superfamily of steroid hormone receptors and are found in two classes: the RAR and the RXR receptors [1,2]. The RAR receptors bind both all-*trans*-retinoic and 9-*cis*-retinoic acids with equal affinities, while the RXR receptors favor binding of the 9-*cis* stereoisomer by at least a factor of 40 [1,2]. The retinoic acids themselves are readily isomerized by thiols in the presence of detergents or by microsomal membranes. Isomerization does not occur in aqueous buffer in the absence of detergents or membranes, and is enhanced at higher pHs. The facile isomerization of the retinoic acids is not shared by the retinals(ols) and provides a mechanism through which developmental mediator 9-*cis*-retinoic acid can be synthesized in cells from all-*trans*-retinoic acid.

It is interesting to note though that generation of 9-*cis*-retinoic acid from all-*trans*-retinoic acid added to cells in culture shares many of the characteristics of the chemical isomerization route described here [1,2]. The reported cellular synthesis is

Table 2
Effects of free radical traps on the isomerization of retinoic acid

Incubation conditions	Antioxidant added	% 9-*cis* formed	% reduction in 9-*cis* formation	% 9,13-*dicis* formed	% reduction in 9,13-*dicis* formation
DT/SDS; pH 11	None	3.7 ± 0.37	–	1.6 ± 0.082	–
DT/SDS; pH 11	α-Tocopherol (0.1 mg/ml)	1.6 ± 0.082	60%	0.73 ± 0.12	73%
DT/SDS; pH 11	Ascorbic acid (1 mM)	1.0 ± 0.05	77%	0.73 ± 0.26	73%
DT/SDS; pH 11	β-Carotene (10 mM)	3.7 ± 0.33	0%	1.7 ± 0.09	0%
DT/SDS; pH 11	Trolox (1 mM)	0.33 ± 0.05	96%	0.4 ± 0.08	100%
SDS; pH 11	None	0.2	–	0.4 ± 0.082	–
Microsomes; pH 9	None	5.1 ± 0.47		2.3 ± 0.25	
Microsomes; pH 9	Ascorbic acid (5 mM)	3.0 ± 0.14	51%	1.2 ± 0.047	56%
Microsomes; pH 9	Trolox (1 mM)	4.5 ± 0.26	15%	2.6 ± 0.57	0%
pH 9	None	1.0 ± 0.14	–	0.37 ± 0.094	–

Bovine liver microsomes (3.6 mg) in 0.5 ml 100 mM sodium phosphate buffer pH 9.0 were mixed with 1 μCi freshly purified [^{3}H]-all-*trans*-retinoic acid in 5 μl ethanol and the reactions were incubated 3 h at 23°C. Where indicated, 5 μl of a radical inhibitor solution was added before addition of microsomes. Radical inhibitor solutions used were 0.1 M Trolox in 0.15 M NaOH or 0.5 M sodium ascorbate. As a control, 100 mM sodium phosphate buffer was added to 1 μCi [^{3}H]-all-*trans*-retinoic acid in 5 μl ethanol and the reaction was incubated 3 h at 23°C. Extractions and HPLC analyses of the reactions were conducted as described in section 2 for microsome catalyzed reactions. Experiments were performed in triplicate. For DT-catalyzed reactions, stock solutions were prepared containing 0.4 M sodium phosphate pH 11, 1% SDS, 100 μM DT (from 10 mM DT in ethanol). To 200 μl aliquots of this solution was added either 4 uL distilled water, 4 μl 10 mg/ml α-tocopherol in ethanol, 4 μl 1 mM β-carotene in DMSO, 4 μl 0.1 M Trolox in 0.15 M NaOH (pH 11.5), or 4 μl 0.1 M ascorbic acid in 0.15 M NaOH (pH 11.4). To solutions of 1 μCi [^{3}H]-all-*trans*-retinoic acid in 50 μl 1% SDS preincubated at 37°C was added 50 μl of the solutions described above preincubated at 37°C and vortexed before use. Mixtures were vortexed and incubated 5 min at 37°C before extraction and HPLC analysis as described in the methods section. As a control, 50 μl 0.4 M sodium phosphate pH 11 with 1%SDS was added to 1 μCi [^{3}H]-all-*trans*-retinoic acid in 50 μl 1%SDS and incubated in identical fashion to the reactions containing DT.

nonstereospecific, with 13-*cis*-retinoic acid being formed, which suggests a non-enzymatic process [1,2]. Since 9-*cis*-retinoic acid arises spontaneously from all-*trans*-retinoic acid, and since 9-*cis*-retinoic acid can activate both forms of retinoic acid receptors, it is difficult to dissect out the actual biological role(s) of all-*trans*-retinoic acid. The addition of all-*trans*-retinoic acid to cultures would always give rise to some 9-*cis*-retinoic acid, so that any effect observed could not immediately be ascribed to an all-*trans*-retinoic acid-mediated effect.

Although an extensive study on the mechanism of the thiol catalyzed isomerization has not been performed, it is likely to be thiol radical-mediated since free radical trapping agents block the reaction. A thiolate anion-mediated mechanism seems a remote possibility first because it should not be inhibitible by free radical trapping agents. Secondly, assuming that catalysis occurs by a Michael addition of the thiolate anion to the uncharged retinoic acid, then it would appear that the two relevant pK_a values are too far apart for catalysis to occur. This is because, by the time the thiol was ionized, only one part in approximately 10^6 of the retinoic acid would be in the conjugate acid form, and hence susceptible to nucleophilic attack. A thiol radical addition mechanism would explain how retinoate could be the actual substrate for isomerization. The pH dependence of the isomerization process.could result from the well-known base catalyzed formation of thiol radicals from thiolate anions in the presence of oxygen [14].

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