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Thiol-Catalyzed cis-trans Isomerization of Oleic Acid

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Various thiols were found to catalyze the geometrical isomerization of oleic acid to *trans*-Δ^9-octadecenoic acid. The reaction proceeds in neutral aqueous solution at mild temperatures and at relatively low thiol concentration, 5–20 meq/liter. Hydrogen from the medium was not incorporated into the product, and no trace of Δ^8 or Δ^{10} octadecenoic acid could be detected among the products. The reaction is proposed to involve the formation of a mixed micelle of fatty acid and thiol, nucleophilic attack of the double bond by the thiol, rotation about the former double bond, and elimination of the thiol to produce the thermodynamically more stable *trans* isomer. The cationic reagent, 2-mercaptoethylamine, was the most efficient catalyst tested. This system should prove to be useful for the preparation of labeled *trans* unsaturated fatty acids.

INTRODUCTION

Enzymatic *cis–trans* isomerizations of double bonds fall into two general classes, those which occur with bond migration, and those which occur without bond migration (*1*). Only three enzymes have been purified which catalyze this second class of reaction. Maleate isomerase from *Pseudomonas fluorescens* (*2*) has an absolute requirement for an exogenous mercaptan, such as glutathione or cysteine. Maleate isomerase from *Alcaligenes faecalis* (*3*) does not require a sulfhydryl cofactor, but is inhibited by sulfhydryl reagents, suggesting a catalytically essential cysteine residue at the active center. Maleylacetone isomerase from Vibrio 01 (*4*) specifically requires glutathione as a cofactor. All these enzymes involve a substrate containing an α–β unsaturated carbonyl group.

Chemical isomerization of double bonds without bond migration may be catalyzed by a number of reagents, including thiyl or phosphinyl radicals (*5*). These reactions are not very analogous to the enzymatic isomerization, however, as they are carried out in organic solvents and involve a free radical mechanism. We have studied the *cis–trans* isomerization of an unsaturated fatty acid catalyzed by thiols in neutral aqueous solution as a possible model for biological isomerization.

MATERIALS AND METHODS

Oleic acid was obtained from Supelco, Inc. [1-^{14}C]oleic acid was obtained from the New England Nuclear Co. and was purified by argentation thin-layer chromatography

of the methyl ester. The purified product was >99% *cis*-octadec-9-enoic acid. Dithiothreitol, 2-mercaptoethanol, 2-mercaptoethylamine, 2,3-dimercaptopropanol, and bovine serum albumin, type F (fatty-acid free) were obtained from Sigma. The 2-mercaptoethanol was redistilled before use. Lubrol WX was a gift of ICI America, Inc. Solutions of Lubrol WX were filtered through 0.22-μm Millipore filters immediately before use. Deuterium oxide, 99.86 atoms % excess ^2H, was obtained from BioRad. All organic solvents were redistilled before use. Thiol concentrations were determined with Ellman's reagent (*6*). Double-bond positions were determined by the method of Niehaus and Ryhage (*7*). These analyses, and those for the incorporation of deuterium, were performed on an LKB 9000 gas chromatograph–mass spectrometer using a column of 4% SE-30 on Supelcoport.

Incubation Conditions

Ten microliters of [1-^{14}C]oleic acid (10^5 dpm/μmole, 50 m*M* in methanol) was added to 1 ml of 0.05 *M* phosphate buffer, pH 7.0, and preincubated for 10–30 min. The turbidity of the mixture was then measured using an American Instruments Co. SPF 125 spectrofluorometer, with excitation and emission at 400 nm. The slit widths were 0.5 mm and the observed relative intensity ranged from 0 to 6.5. The values were reproducible to $\pm$0.2. The highest relative intensity in each experiment ($\sim$6.5) was arbitrarily assigned a relative turbidity of 100%. The thiol compound, dissolved in 50 μl of buffer was added, the tubes were closed with corks, and incubated in the absence of direct light. Preliminary experiments showed no differences in isomerization rate between samples incubated in this way and samples incubated in the dark under a nitrogen atmosphere. After the incubation period, samples were acidified with HCl, fatty acids were extracted with ether, the ether was shaken with saturated aqueous $AgNO_3$ to remove all thiols which had been extracted, and the ether was dried over $MgSO_4$. The fatty acids were esterified with diazomethane, and *cis* and *trans* isomers were separated by low-temperature argentation thin-layer chromatography [silica gel G impregnated with 3% $AgNO_3$; pentane:ether (96:4)]. Chromatographic standards of methyl oleate and methyl elaidate were used, and were visualized by spraying with 2′7′-dichlorofluorescein and viewing under ultraviolet light. The spots were marked, scraped into scintillation vials, and counted in a Beckman LS200B liquid scintillation spectrometer using a fluid containing 0.45% diphenyloxazole in toluene:Triton × 100:H_2O (60:30:10). The incubations containing bovine serum albumin were acidified with perchloric acid instead of HCl to precipitate the protein and allow quantitative extraction of the fatty acids.

RESULTS

Type and Concentration of Thiol Catalyst

Several different thiol compounds were surveyed for their ability to effect the *cis–trans* isomerization of oleic acid. Results were expressed as the percentage of the original oleic acid which was converted to the *trans* isomer during the incubation period. The effect of thiol concentration on isomerization by 2,3-dimercaptopropanol, 2-mercaptoethanol, dithiothreitol, and 2-mercaptoethylamine is shown in Fig. 1. These incubations were carried out at pH 7 and 37°C for 30 min. The production of the *trans* isomer was

linear with time over this incubation period for all samples except for the two highest concentrations of 2-mercaptoethylamine, in which the *cis–trans* mixture is approaching the equilibrium ratio. The data presented represent the average of three incubations. Values are reproducible to ±10%. Incubations carried out for longer periods produced up to 80–85% of the *trans* isomer which is in good agreement with the equilibrium ratio achieved when selenium or nitrous acid (*8*) or thiyl radicals (*5*) are used as catalyst.

Under these same conditions, cysteine, glutathione, and thioglycolic acid were ineffective as isomerization catalysts. Incubation of oleic acid with these thiols at 20 meq/liter for 1 hr resulted in less than 3% conversion to the *trans* isomer.

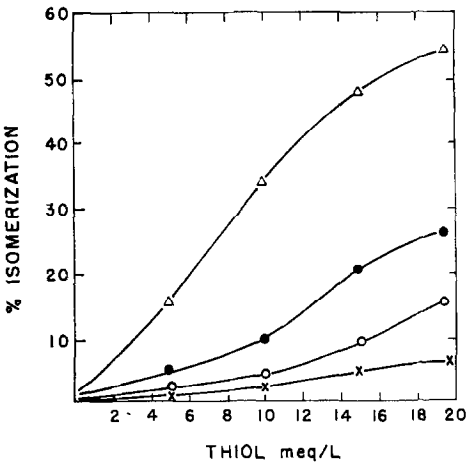

FIG. 1. Isomerization of oleic acid as a function of thiol concentration. Incubation conditions: oleic acid, 0.5 m*M*; pH 7.0; 37°C; 30 min. Percentage isomerization represents the percentage of the original oleic acid converted to the *trans* isomer. ×, 2,3-dimercaptopropanol; ○, 2-mercaptoethanol; ●, dithiothreitol; △, 2-mercaptoethylamine.

The relative ineffectiveness of 2,3-dimercaptopropanol may be due to its sparing solubility in water. At the highest concentration tested (20 meq/liter) the sample became turbid upon standing at 37°C for 2 hr. No turbidity was noted during the course of the 30-min incubation, however. The partition of the various thiols between equal volumes of phosphate buffer and ether was estimated. The percentage partitioning into the aqueous phase was: 2,3-dimercaptopropanol 20%; 2-mercaptoethanol 60%; dithiothreitol 60%; 2-mercaptoethylamine 45%; thioglycolic acid 30%. Cysteine and glutathione remain totally in the aqueous phase under these conditions. It, therefore, appears that to be an effective isomerization catalyst, a thiol must possess a significant degree of both aqueous and organic solubility.

Although 2-mercaptoethylamine was the most efficient catalyst tested, most of the experiments used dithiothreitol as catalyst, to avoid possible ambiguities due to the ionized amine group.

Effect of pH

The pH profile of isomerization was determined using dithiothreitol (15 meq/liter) at 37°C for 30 min. Buffers were 0.05 *M* phosphate (pH 6.0, 7.0, 8.0) and 0.05 *M* Tris

(pH 8.0, 9.0). The percentage isomerization to *trans* octadecenoic acid at the various pH values was as follows: pH 6.0, 3%; pH 7.0, 12%; pH 8.0, 17%; pH 9.0, 6%. No isomerization occurred in 0.05 *M* NaOH.

The isomerization rate was higher at pH 8 than at pH 7 whether dithiothreitol or 2-mercaptoethanol was used to catalyze the isomerization. The apparent pH optimum was independent of the buffer employed (Tris, phosphate, glycyl glycine, or histidine), but the absolute rate of isomerization was somewhat dependent on the buffer species. Phosphate consistently gave the highest rates and was used in most of the experiments. The rates in Tris and glycyl glycine were 10–15% lower, and the rates in histidine buffer were about 50% lower. We have no explanation for these observations at the present time.

Effect of Temperature

The effect of temperature on isomerization was investigated using dithiothreitol (20 meq/liter) in 0.05 *M* phosphate buffer, pH 7.0. Since the reactions were linear for only a short time at the higher temperatures, results are expressed as percentage of the oleic acid converted to the *trans* isomer per minute: 20°C, 0.3%; 30°C, 0.4%; 40°C, 0.8%; 50°C, 1.0%; 60°C, 2.0%. When analyzed by the Arrhenius equation, this corresponds to an apparent activation energy of 9 kcal/mole. The samples at 60°C reached thermodynamic equilibrium (80° *trans*) after about 60 min.

Effect of Solubilizing Agents

At the concentration used in these experiments (0.5 m*M*) oleic acid does not exist in true aqueous solution, but in a turbid micellar suspension. The suspension is stable for at least 6 hr, as evidenced by absence of change in the turbidity. The turbidity does not

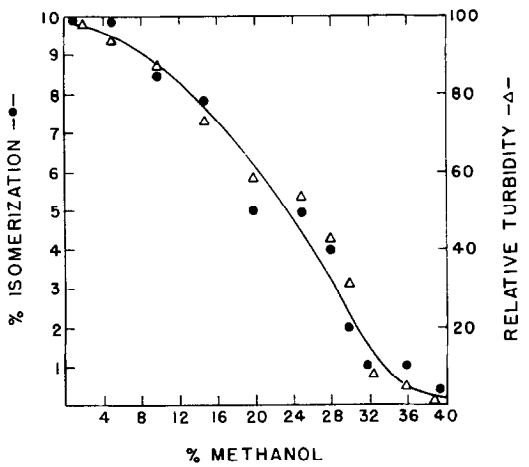

FIG. 2. The effect of methanol on the isomerization of oleic acid by dithiothreitol. Incubation conditions: oleic acid, 0.5 m*M*; pH 7.0; 37°C; 30 min; 20 meq/liter thiol.

change upon isomerization of up to 50% of the *cis* fatty acid and to the *trans* isomer. Various agents which alter this micellar state of the fatty acids were tested for their effect on the thiol-catalyzed *cis–trans* isomerization. The addition of methanol to con-

centrations above 40% results in an optically clear system, in which oleic acid is presumably present in true monomolecular solution. As shown in Fig. 2, with an increase in the concentration of methanol, the rate of isomerization of oleic acid by dithiothreitol decreases uniformly as does the turbidity of the oleic acid suspension. No isomerization is seen in anhydrous methanol using either dithiothreitol or 2-mercaptoethanol at 25 m*M* for 60 min.

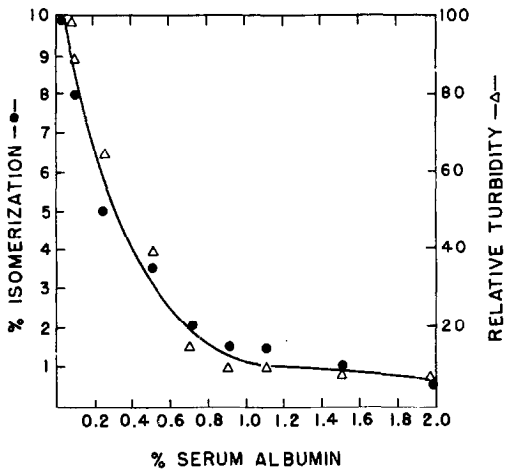

FIG. 3. The effect of bovine serum albumin on the isomerization of oleic acid by dithiothreitol. Incubation conditions: oleic acid, 0.5 m*M*; pH 7.0; 37°C; 30 min; 20 meq/liter thiol.

The addition of serum albumin changes the micellar state of the oleic acid suspension by adsorption of the fatty acid molecules to the protein. Again, as shown in Fig. 3, upon addition of increasing amounts of albumin, the thiol-catalyzed isomerization decreases in parallel with the turbidity.

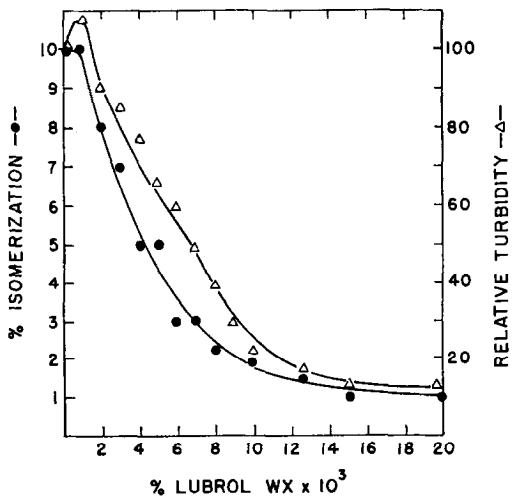

FIG. 4. The effect of the nonionic detergent, Lubrol WX, on the isomerization of oleic acid by dithiothreitol. Incubation conditions: oleic acid, 0.5 m*M*; pH 7.0; 37°C; 30 min; 20 meq/liter thiol.

The results achieved with the nonionic detergent, Lubrol WX, are only slightly less straightforward (Fig. 4). With an increase in the concentration of detergent, the rate of thiol-catalyzed isomerization falls somewhat more rapidly than does the turbidity. The turbidity measurements may be somewhat high, however, as at very low concentrations (0.5–1.0 mg/100 ml) the detergent actually causes an increase in the turbidity of the oleic acid suspension. This increased turbidity was more marked with another non-ionic detergent, Triton X 100, which, overall, had qualitatively similar effects on isomerization as did Lubrol WX.

Absence of Incorporation of ^2H from ^2H_2O

To assay for the incorporation of hydrogen from the medium during the isomerization, oleic acid was isomerized in a medium very highly enriched in ^2H_2O. Buffer was prepared by evaporating the water from the 0.05 *M* phosphate buffer (pH 7.0) and redissolving the residue in ^2H_2O. To this was added 2-mercaptoethylamine (10 m*M*) and the mixture was preincubated at 37°C for 10 min, whereupon oleic acid was added (0.5 m*M*). The overall enrichment of ^2H was greater than 95%. Samples were extracted at 30 min (20% *trans*), 60 min (36% *trans*), and 120 min (49% *trans*). The *cis* and *trans* fatty acid methyl esters were isolated by preparative argentation thin-layer chromatography and analyzed for deuterium content by gas chromatography–mass spectrometry. The molecular ion peak at *m/e* 296 and the peak at 297 (*M* + 1) were used to calculate the extent of deuterium incorporation. In no case could more than 5% of the molecules of *trans*-octadecenoic acid have contained deuterium, which is within the experimental error of the analytical procedure.

Lack of Double Bond Migration

A sample of oleic acid was incubated with 50 m*M* 2-mercaptoethanol at 37°C for 24 hr. The *trans* isomer produced (85%) was isolated, oxidized with alkaline $KMnO_4$ to the dihydroxy fatty acid, methylated with methyl iodide to yield the dimethoxy fatty acid methyl ester, and analyzed by gas chromatography–mass spectrometry (*7*). The major fragmentation, occurring between the vicinal methoxyl groups, would yield fragments of *m/e* 201 and 157 for a derivative of a Δ^9 fatty acid. The corresponding fragments are at *m/e* 215 and *m/e* 143 for a Δ^{10} fatty acid, and *m/e* 187 and 171 for a Δ^8 fatty acid. Comparison of the intensities of these peaks shows that the sample contains less than 0.5% Δ^{10} and less than 1% Δ^8 fatty acid. Hence, the isomerization occurred without any observable bond migration.

Lack of a Detectable Free Radical

A cursory examination for free radical signals by electron spin resonance spectroscopy at room temperature revealed the complete absence of any detectable radical buildup during the course of isomerization reaction. The samples contained 0.5 m*M* oleic acid in phosphate buffer, pH 7.0 which had been incubated from 0 to 4 hr with 50 m*M* dithiothreitol or mercaptoethanol.

DISCUSSION

The *cis–trans* isomerization of double bonds without bond migration can be effected by thermal or photochemical stimulus, or can be catalyzed by univalent atoms,

molecules with odd electrons, free radicals, mineral acids, and paramagnetic molecules or atoms (*9*). The proposed mechanisms, whether ionic or free radical, invoke the formation of an addition complex between the unsaturated substrate and the catalyst, free rotation about the axis of the former double bond, and the net formation of the thermodynamically more stable *trans* isomer. For example, maleylacetoacetate and maleylacetone undergo a facile conversion to the corresponding *trans* isomers in the presence of low concentrations of Ag^+ at moderate temperature and neutral pH. The β diketone grouping is essential, as maleic acid is not converted to fumaric acid under the same conditions (*11*). The Ag^+-catalyzed isomerization has been shown to proceed by a nonradical path, and does not involve the exchange of vinyl protons. The methyl ester of maleylacetone is isomerized more rapidly than is maleylacetone. It is suggested that the reaction involves production of a π complex of Ag^+ with the monoanion of maleylacetone, loss of the enol proton, and rotation about the former double bond (*10*). Maleylacetoacetate is also isomerized slowly by glutathione at neutral pH. This reaction was proposed to occur by a radical mechanism, but no radical initiator was included in the reaction mixture (*12*). Trace metals and/or dissolved oxygen may have served to generate small concentrations of thiyl radicals. Earlier reports also implied the role of thiols in the *cis–trans* isomerization of maleic acid and maleylacetoacetic acid (*13*). Recently, dithiols, such as dithiothreitol, reduced DL-α-lipoate, and 2,3-dimercaptopropanol have been shown to effect the isomerization of all-*trans*-retinal in neutral aqueous solution (*14*). This isomerization was much more efficiently catalyzed by dihydroflavins, however. The isolated double bond of an unsaturated fatty acid has been shown to undergo *cis–trans* isomerization under fairly vigorous conditions, e.g., treatment with selenium or nitrous acid (*8*). Sgoutas and Kummerow (*5*) have reported the isomerization of monoenoic and dienoic fatty acid methyl esters catalyzed by thiols and diphenylphosphine in the presence of the radical initiator azobisisobutylnitrile. These reactions took place in organic solvent at 65°–75°C over 6–8 hr. Earlier reports had documented the isomerization of simple olefins (*15–17*) and of *cis, trans, trans* cyclodecatriene (*18*) by thiophenol, thioglycolic acid, or thiolacetic acid. These reactions involve thiyl free radicals generated by light or by radical initiators such as benzoyl peroxide or azobisisobutylnitrile.

Enzyme-catalyzed *cis–trans* isomerizations, on the other hand, are unlikely to occur via a free radical mechanism. Those isomerizations which occur with bond migration (*19–22*) involve the incorporation of hydrogen from water, and are proposed to involve a carbonium ion (*19–21*) or carbanion (*22*) intermediate. The isomerases which catalyze interconversion of *cis* and *trans* isomers without double-bond migration require one or more sulfhydryl groups for activity. The sulfhydryl may be an integral part of the enzyme protein, as in maleate isomerase from *Pseudomonas fluorescens* (*3*) or may be furnished by a cofactor, such as glutathione, as in maleylacetone isomerase from Vibrio 01 (*4*). The mechanism of isomerization is postulated to involve addition of glutathione to the carbon–carbon double bond with enolization of the diketone system. It is suggested that the enzyme may form a Schiff base with the substrate which enhances nucleophilic attack by glutathione (*4*). It is obvious that the formation of an enzyme–substrate–glutathione complex, with or without Schiff base formation, produces a situation in which the SH group is bound in close proximity to the reactive double bond of the substrate, thus facilitating the nucleophilic attack.

The isomerization of oleic acid by dithiothreitol, 2-mercaptoethanol, and, especially, 2-mercaptoethylamine may be considered to be analogous to the enzymatic systems which isomerize maleic acid and maleylacetone. No enzymes have been described which catalyze *cis–trans* isomerization of fatty acids without bond migration, however. Due to the presence of *trans* monoenoic fatty acids in nature, one may perhaps postulate that such enzymes do exist. An effort is underway to demonstrate this *cis-trans* isomerization of monoenoic fatty acids in bacterial extracts.

The reaction is proposed to involve nucleophilic attack of the double bond by –SH, facilitated by the formation of a mixed micelle of oleic acid and thiol. Thus the effective concentration of –SH at the double bond would be much higher than the overall –SH concentration of the system. This interpretation is supported by the following findings:

1. The effectiveness of a thiol as an isomerization catalyst is related to its relative solubilities in aqueous and organic systems, and thus to its tendency to dissolve in the oleic acid micelles. Cysteine and glutathione, which do not dissolve in organic solvents, are totally ineffective as catalysts. 2,3-Dimercaptopropanol, which is very slightly soluble in water, and tends to undergo phase separation from the aqueous system, is a rather poor catalyst. 2-Mercaptoethanol and dithiothreitol, which are readily soluble in both aqueous and organic solvents, are quite effective isomerization catalysts.

2. The effectiveness of a thiol as an isomerization catalyst is related to its ionic nature, and thus its ability to interact with the negatively charged oleic acid micelles. Thus, thioglycolic acid is totally ineffective as an ionization catalyst, presumably due to electrostatic repulsion from the negatively charged oleic acid micelle, and 2-mercaptoethylamine is by far the most efficient catalyst tested, presumably due to electrostatic attraction to the negatively charged oleic acid micelle.

3. The rate of isomerization of oleic acid by dithiothreitol is decreased in parallel with the disruption of the micellar system by agents such as methanol, serum albumin, and nonionic detergents. Presumably, as the fatty acid system becomes less turbid, its micellar nature is lost, and there is no longer the possibility of forming mixed micelles in which thiol is present in very high local concentration.

4. There is no evidence for the existence of free radical intermediates in the isomerization reaction, although we cannot rigorously exclude the transient presence of very low concentrations of radicals.

The thiol compounds have been referred to as catalysts even though it is recognized that their concentration greatly exceeds the concentration of substrate in these experiments. The position of equilibrium between *cis* and *trans* isomers appears to be independent of the thiol type or concentration employed. However, prolonged incubations (10–24 hr) result in the production of substantial quantities of polar products which have not yet been identified. Under all the incubation conditions described in this manuscript, more than 95% of the fatty acid could be accounted for as *cis*- and *trans*-octadecenoic acid.

The finding that various thiols can effect the *cis–trans* isomerization of fatty acids under very mild conditions must serve as a warning to investigators who work with lipid systems. Thiols such as mercaptoethanol and dithiothreitol are frequently added to incubation mixtures at concentrations which could cause isomerization of unsaturated fatty acids, and perhaps could cause isomerization of unsaturated fatty acids of

membrane phospholipids. It should be noted that methyl oleate is isomerized at about 20% of the rate observed with oleic acid. If the addition of thiols is essential for enzyme stability in a system containing unsaturated fatty acid, glutathione or cysteine appear to be the reagents of choice, since they do not cause isomerization of the double bond.

Finally, treatment of aqueous suspensions of unsaturated fatty acids with 2-mercaptoethylamine appears to be the method of choice for the preparation of *trans* unsaturated fatty acids from the *cis* isomers. The method employs extremely mild reaction conditions, does not involve free radical initiators, produces equilibrium mixtures of *cis* and *trans* isomers in a matter of 1–2 hr, and is totally free of double-bond migration. In addition, the 2-mercaptoethylamine hydrochloride is not extracted into ether from acid solution, and, therefore, the extracted fatty acids need no intermediate purification prior to methylation and separation by argentation chromatography. The method is particularly applicable to small-scale preparation of radioactive fatty acids and, due to the extremely mild conditions, may be well suited to use with polyunsaturated fatty acids.

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