Microsomal Enzymes of Cholesterol Biosynthesis from Lanosterol

SOLUBILIZATION AND PURIFICATION OF STEROID 8-ISOMERASE*

(Received for publication, September 30, 1985)

Young-Ki Paik, Jeffrey T. Billheimer, Ronald L. Magolda, and James L. Gaylor

From the Central Research & Development Department, E. I. du Pont de Nemours & Company, Inc., Experimental Station, Wilmington, Delaware 19888

Steroid-8-ene isomerase that catalyzes isomerization of Δ7- to Δ8-sterols has been solubilized from rat liver microsomes with a mixture of two detergents, octylglycoside and sodium taurodeoxycholic acid. During a 40-fold enrichment of the solubilized enzyme, other enzymes of cholesterol biosynthesis, endogenous lipids, and electron carriers are removed.

A comparison of properties of the solubilized and partially purified isomerase with the membrane-bound enzyme shows they are essentially identical with respect to pH profile, effect of inhibitors and cofactors, substrate specificity, and Km values. Addition of phospholipid to the partially purified enzyme stimulates activity as much as 1.8-fold over control rates. Although the relative rate of isomerization of cholesta-8,24-dien-3β-ol is six times that observed with cholest-8-en-3β-ol, the Δ8 to Δ7 ratio at equilibrium is approximately equal. The reversibility of the reaction has been demonstrated by the direct conversion of cholest-7-en-3β-ol to cholest-8-en-3β-ol; at equilibrium the Δ7-isomer is predominant (19/1). The purified enzyme does not catalyze isomerization of cholesta-8,14-dien-3β-ol and cholest-8(14)-en-3β-ol under conditions that result in equilibrium mixtures of isomers from cholest-8(9)-en-3β-ol. These results are consistent with the earlier suggestion that Δ9,14-sterols are neither formed nor metabolized by the same microsomal enzymes that catalyze transformation of lanosterol to cholesterol.

In the later stages of cholesterol biosynthesis from lanosterol,1 isomerization of the Δ5- to Δ7-double bond of sterol biosynthetic intermediates appears to be a prerequisite to oxidative introduction of the Δ5-double bond (1). Rat liver microsomes contain a steroid 8-ene isomerase that catalyzes anaerobic conversion of sterol-8-ene to the sterol-7-ene isomer (2). The membrane-bound enzyme requires neither oxygen nor added cofactors for catalytic activity (2). Several other properties of the microsomal enzyme have been reported since this early investigation, including stereochemical elucidation, isotopic reversibility, and substrate specificity (2-11). Although solubilization with Triton WR-1339 was achieved by this laboratory several years ago (12), use of Triton WR-1339 and other higher molecular weight nonionic detergents has not allowed further enrichment and resolution of the solubilized enzyme from other enzymes of cholesterol synthesis. We now describe a novel solubilization procedure, from which enrichment of 8-isomerase has been achieved, as well as resolution of the 8-isomerase from other enzymes of hepatic microsomal cholesterol biosynthesis.

The enriched enzyme appears to be the same as the microsomal-bound enzyme that catalyzes the obligatory isomerization of Δ7- to Δ8-sterol intermediates. Furthermore, our preparation is free of contaminating enzymes that catalyze metabolism of various sterol biosynthetic intermediates. We have shown that the enzyme is stimulated by phospholipids and can be incorporated into artificial liposomal particles. Thus, the resolved enzyme appears suitable for reconstitution experiments.

As Bloch and co-workers (13) pointed out in 1969 there are no "standard procedures" for the solubilization and purification of microsomal enzymes of cholesterol biosynthesis. Indeed, to date, each membrane-bound enzyme has been investigated independently, and optimal conditions for solubilization and purification have varied widely between enzymes and laboratories. In this report, we describe the development of procedures for solubilization, enrichment, and resolution of 8-isomerase. We have used similar protocols to solubilize and enrich nine microsomal enzymes and electron carriers of cholesterol biosynthesis. Therefore, this protocol appears to satisfy some criteria for at least a generalized approach. Accordingly, rationale for various choices of procedures as well as more detailed descriptions of methods used are reported here to assist others who may wish to solubilize and purify microsomal enzymes.

EXPERIMENTAL PROCEDURES*

* This is Publication 3869 from the Central Research and Development Department, E. I. du Pont de Nemours & Company, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations and steroid common names used are: lanosterol, 4,4,14a-trimethyl-5α-cholesta-8,24-dien-3β-ol; zymosterol, 5α-cholesta-8,24-dien-3β-ol; desmosterol, cholest-8-en-3β-ol; lathosterol, cholest-7-en-3β-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol. In this report the Δ7-steroid to Δ8-steroid isomerase is referred to as 8-isomerase. The NADPH-dependent reductases that catalyze reductions of the 14-double bond of Δ4,14-steroid dienes, the 24-double bond of Δ5,24-steroid dienes, the 7-double bond of Δ8-steroid dienes, and the 3-ketones to 3-hydroxy steroids are referred to as 14-, 24-, 7-reductase, and 3-ketosteroid reductase, respectively. The 5-desaturase is the cytochrome b5-dependent oxidative enzyme that catalyzes the introduction of the 5-double bond into Δ7-sterol substrates. STDC, sodium taurodeoxycholate; OG, n-octylglycoside; PEG, polyethylene glycol 3000; TDCA, taurodeoxycholic acid; PMSP, phenylmethylsulfonyl fluoride; HPLC, high pressure liquid chromatography; GLC, gas-liquid chromatography; GC/MS, gas chromatography-mass spectrometry.

2 Portions of this paper ("Experimental Procedures," Fig. 1, and Footnotes 3 and 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry. Vol. 261, No. 14, Issue of May 15, pp. 4470-4477, 1986 Printed in U.S.A.
RESULTS AND DISCUSSION

Solubilization Procedure—After the development of an assay and characterization of the membrane-bound 8-isomerase (2, 12) in this laboratory, considerable effort was devoted to attempted solubilization of the enzyme. Various ionic and nonionic detergents that have been used successfully for solubilization of other membrane-bound enzymes were investigated singly and in combination. In general, when microsomes were treated under solubilizing conditions with several different detergents, stable 8-isomerase could be detected in the supernatant fraction (S-1). However, as in the solubilization of 8-isomerase with Triton WR-1339 and other detergents as reported earlier (12), the liberated 8-isomerase was unstable or refractory to further enrichment. It is possible that this is due to the high concentrations of nonionic detergents needed to liberate activity into the S-1 during routine solubilization attempts. Furthermore, the initial step in enrichment generally has been either filtration on Sephadex or precipitation with PEG. Both gel filtration and protein precipitation have been difficult procedures to conduct in the presence of nondialyzable, nonionic detergents. Accordingly, an alternate procedure has now been developed with the use of OG, a dialyzable, nonionic detergent.

Although OG has been reported by others as a solubilizing detergent for other membrane-bound proteins (21–24) in the absence of a second detergent, complete solubilization of 8-isomerase activity could not be obtained with the use of 0.5 to 2.6 mg of detergent/mg of microsomal protein (Table I). However, activity was consistently released and found to be quite stable for several hours of storage in the presence of combined detergents including OG and STDC (8:1, w/w, 2 mg of detergent/mg of protein in solution). Therefore, a combination of the nonionic OG and various ionic detergents was used to solubilize, in general, each of these enzymes has been very difficult to release from microsomes using various detergent solubilization protocols. However, with the development of the OG plus TDCA procedure, only modest adjustments of detergent concentrations and exposure duration have been necessary to yield a solubilized preparation. In all cases, the 3:1 ratio of OG to TDCA acid (e.g. Table I) has been nearly optimal. It is suggested that initial solubilization attempts of membrane-bound enzymes should start with these conditions. With a fixed detergent ratio of 3:1, OG-TDCA (w/w) ratios of detergent to microsomal protein from 0.5/1 to 4/1 were examined. At ratios in excess of 1.5 mg of detergent/mg of microsomal protein, quantitative release of protein into the supernatant fraction was observed. Maximal recovery of 8-isomerase activity (80% of microsomal activity) was obtained at a ratio of detergent to protein of two. Higher concentrations of detergent resulted in a decrease in the stability of 8-isomerase. For the assay of S-1 fractions, dilution to <0.2% of detergent was found to be inhibitory to the assay of both total protein and enzymic activity. With a fixed detergent ratio of 3:1, OG-TDCA acid afforded almost quantitative solubilization of protein (>90%) with release of most activity (~90%) (Table I). Simultaneous release of protein and 8-isomerase could not be enhanced further by the additions of a third detergent such as Triton WR-1339.

A similar mixture of OG and TDCA has now been used to solubilize Δ2-sterol 5-desaturase (1), the two microsomal electron carriers of the 5-desaturase, Δ14-sterol 14-reductase (15), the cytochrome P-450 of 14a-methyl group oxidation of lanosterol plus accompanying cytochrome P-450 reductase (25), Δ5-sterol 7-reductase, 3-ketosteroid reductase, and Δ3-sterol 24-reductase. In general, each of these enzymes has been very difficult to release from microsomes using various detergent solubilization protocols. However, with the development of the OG plus TDCA procedure, only modest adjustments of detergent concentrations and exposure duration have been necessary to yield a solubilized preparation. In all cases, the 3:1 ratio of OG to TDCA acid (e.g. Table I) has been nearly optimal. It is suggested that initial solubilization attempts of

<table>
<thead>
<tr>
<th>Solubilizing conditions</th>
<th>Recovery in S-1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octylglucoside</td>
<td></td>
</tr>
<tr>
<td>Taurodeoxycholic acid</td>
<td></td>
</tr>
<tr>
<td>Triton WR-1339</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Activity</td>
</tr>
<tr>
<td>mg detergent/mg microsome protein</td>
<td>%</td>
</tr>
<tr>
<td>0.5</td>
<td>54.6 ± 4.3</td>
</tr>
<tr>
<td>1.0</td>
<td>68.5 ± 2.9</td>
</tr>
<tr>
<td>1.5</td>
<td>81.0 ± 1.7</td>
</tr>
<tr>
<td>2.0</td>
<td>94.3 ± 3.4</td>
</tr>
<tr>
<td>0.25</td>
<td>36.2 ± 2.2</td>
</tr>
<tr>
<td>0.5</td>
<td>55.5 ± 3.5</td>
</tr>
<tr>
<td>1.0</td>
<td>75.0 ± 2.5</td>
</tr>
<tr>
<td>1.5</td>
<td>94.4 ± 1.9</td>
</tr>
<tr>
<td>0.5</td>
<td>86.0 ± 2.4</td>
</tr>
<tr>
<td>1.5</td>
<td>92.0 ± 4.1</td>
</tr>
</tbody>
</table>

*% recovery of protein and enzymic activity in S-1 fraction are values compared to the appropriate microsomal control incubated at the same time.
Sterol 8-Isomerase

Enrichment of 8-Isomerase—The solubilized S-1 was first fractionated by PEG precipitation. 8-Isomerase activity was readily precipitated by treatment of S-1 with PEG 3000 (Table II). Considerably better yields and enrichment were obtained when a single 12–23% PEG precipitation step than when multiple fractionations were performed (see "Experimental Procedures" and Ref. 15). Indeed, minimization of the duration of exposure to PEG generally afforded considerably higher recovery of active enzyme. Precipitation with PEG at 12–23% also afforded resolution of 8-isomerase from 4-methyl sterol oxidase, 24-reductase, and 3-ketosteroid reductase that were removed in the 0–12% PEG fraction. The 12–23% fraction, however, contained about 5% of the original activity of 14-reductase (<0.06 units/mg protein) that is precipitated between 8–16% PEG (15). With the exception of 14-reductase, all enzymes of cholesterol synthesis that were routinely assayed. The 12–23% PEG fractionation resulted in approximately 65% recovery of 8-isomerase activity and a consistent 2-fold enrichment (Table II). Although when in suspension with PEG the 8-isomerase was unstable, the pellet that was collected by centrifugation of the resulting precipitate between 12–23% PEG was found to be quite stable either upon storage at −80 °C for several weeks or at 4 °C for a week as observed previously for the 14-reductase (15).

Further enrichment of the 8-isomerase by chromatography on DEAE-Sepharose is illustrated in Fig. 3. Combined fractions containing 8-isomerase exhibited an average of 14-fold enrichment with approximately 30% overall yield (Table II). At this point in the purification, more than 40 h of exposure to detergents resulted in considerable loss of activity. Correction for loss of enzymic activity upon storage at 4 °C (see Fig. 2) suggests that greater than 40-fold enrichment is theoretically possible.

Attempts to prevent the loss of activity ascribed to detergent and PEG exposure have been only partially successful. EDTA, glycerol (or ethylene glycol), and glutathione (or dithiothreitol) were necessary to minimize the rate of loss of activity, but high concentrations of these agents did not improve the yield. We assume that in the presence of 0.2% OG (w/v) and 0.05% TDCA (w/v), at which optimal chromatographic resolution was observed, the 8-isomerase protein is too labile to slow the rate of loss of activity. At this point in the enrichment, other microsomal enzymes and electron carriers were assayed. NADPH-dependent cytochrome P-450 reductase and cytochrome P-450 were removed chromatographically after PEG precipitation; however, a minor contamination by cytochrome b₅ was still observed spectrophotometrically along with cytochrome b₅ reductase.

The resulting DEAE-Sepharose fractions (15 mg of protein) were concentrated on a Diaflo YM-10 membrane and filtered through a Sephadex G-25 column as described under "Experimental Procedures." The concentrated protein sample obtained from this procedure was then applied to a nucleotide affinity gel (agarose-hexane-ADP column that had been equilibrated with Buffer G) to remove pyridine nucleotide-dependent enzymes and electron carriers. The unbound protein (chromatographic data not shown) fraction contained active 8-isomerase while yellow-colored material remained bound to the gel. Bound proteins were eluted with addition of 0.3 M STDC. The protein was still observed spectrophotometrically as indicated.

### Table II

**Purification of the 8-isomerase from rat liver microsomes**

<table>
<thead>
<tr>
<th>Purification procedure</th>
<th>Total units</th>
<th>Protein content</th>
<th>Specific activity</th>
<th>Purification Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Microsomes</td>
<td>1496</td>
<td>755</td>
<td>1.98</td>
<td>1.00</td>
</tr>
<tr>
<td>II. Solubilized</td>
<td>1300</td>
<td>604</td>
<td>2.15</td>
<td>1.87</td>
</tr>
<tr>
<td>III. PEG (12–23%)</td>
<td>984</td>
<td>246</td>
<td>4.00</td>
<td>2.66</td>
</tr>
<tr>
<td>IV. DEAE-Sepharose</td>
<td>Average</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>442</td>
<td>16</td>
<td>27.6</td>
<td>14.29</td>
</tr>
<tr>
<td>V. Peak</td>
<td>91</td>
<td>2.5</td>
<td>36.4</td>
<td>18.18</td>
</tr>
<tr>
<td>VI. V plus phospholipid</td>
<td>321</td>
<td>7.7</td>
<td>41.4</td>
<td>21.22</td>
</tr>
</tbody>
</table>

*Combined fractions obtained from Step V were incubated with liposomal phospholipid in a ratio of 2:1, (w/w) as described under "Experimental Procedures" and in Ref. 15.

![Fig. 2. Elution profile of PEG precipitates chromatographed on DEAE-Sepharose.](image-url)
Sterol 8-Isomerase

The 8-isomerase resolved on the affinity column with liposomal phosphatidylcholine in a 2:1 ratio of phospholipidprotein (found to be optimal in preliminary tests) resulted in a specific activity of 8-isomerase that was 1.8 times the activity observed in the absence of exogenous phospholipid (Table II). This result also was consistent with similarly enhanced activities of 14-reductase (15) and 5-desaturase (1).

Many alternative protocols for the enrichment of 8-isomerase have been attempted without improvement of either yield or specific activity. HPLC size-fractionation of 8-isomerase did not further enrich activity. Similarly, 8-isomerase was not enriched by chromatography on either Sephadex G-200 or Sepharose CL-4B. As shown in Fig. 3, HPLC-gel filtration, did allow an estimation of the approximate molecular weight of 80,000 for the active 8-isomerase. Activity was only sufficiently stable when phospholipid was present in the elution buffer. However, inclusion of phospholipid in the elution buffer did not alter the chromatographic pattern of standard proteins.

Comparison of Properties of Enriched and Microsomal-bound Enzyme—Enzyme that had been solubilized and enriched approximately 20-fold through the DEAE-Sephacel chromatographic stage was assayed under various conditions that were also used for simultaneous assays of membrane-bound 8-isomerase. Effects of potential inhibitors, changes in pH, and substrate specificity of solubilized and the membrane-bound 8-isomerase were compared to demonstrate that the purified enzyme is responsible for the same activity that has been observed in intact microsomes.

The soluble and membrane-bound forms of 8-isomerase were sensitive to inhibition by HgCl2 with 75 to 80% of activity being destroyed at a 3 mM concentration (Table III). The inhibitory effect of HgCl2 was fully reversed for both enriched and bound enzyme when 10 mM glutathione was added.

As described previously, the microsomal-bound 8-isomerase is particularly sensitive to inhibition by the drug AY-9944 (1). Incubation of either 0.2 mg of enriched enzyme or 1.0 mg of membrane-bound protein with 0.6 μM AY-9944 produced 22 and 29% inhibition of bound and soluble enzyme, respectively (Table III). At 1.2 μM AY-9944, inhibition was approximately 40% for each form of the enzyme. Recent experiments suggest that 8-isomerase from yeast is also sensitive to AY-9944 (26). Triparanol, a hydrophobic substance, was significantly more inhibitory toward membrane-bound 8-isomerase (Table III). The effective concentration of drug in the membrane may have been substantially greater by partitioning into the lipids of microsomes. A similar concentrating effect upon microsomal enzymes of cholesterol biosynthesis was observed with either free fatty acids or acyl coenzyme A thiosters (27). Cyanide ion was not inhibitory (data not shown). Also, addition of either NADPH or NADH did not produce an effect with either soluble or bound 8-isomerase. (This is the only enzyme of the 18-step microsomal synthesis of cholesterol from lanosterol for which there is no requirement for a pyridine nucleotide; see Ref. 28.)

Investigation of substrate specificity of microsomal-bound 8-isomerase has been hitherto limited by GLC assays in which relatively small amounts of sterol substrates and products of 8-isomerase had to be analyzed in the presence of very large amounts of endogenous cholesterol of rat liver microsomes. Throughout these studies (2, 12) zymosterol substrate has been used because both zymosterol and the 8-isomerase product, 5α-cholesta-7,24-dien-3β-ol, are readily resolved from even large amounts of cholesterol. 5α-Cholest-8-en-3β-ol (zymosterol) is not easily separated from cholesterol chromatographically. But, soluble 8-isomerase that is essentially free of cholesterol and the high resolving capability of capillary GC allowed study of a variety of putative cholesterol precursors, steroidal inhibitors, and other similar compounds

![Fig. 3. Estimation of molecular weight of 8-isomerase. 28 mg of protein from DEAE-Sephacel column fractionation (approximately 30 units/mg) were applied to the HPLC column (TSK 3000 SWG, 2.25 × 60 cm) as described under "Experimental Procedures." Values represent means of duplicate samples.](https://example.com/fig3.png)

**TABLE III**

Properties of the enriched and membrane-bound 8-isomerase

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Enriched protein</th>
<th>Membrane-bound protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>Relative rate</td>
<td>Specific activity</td>
</tr>
<tr>
<td>units/mg</td>
<td>%</td>
<td>units/mg</td>
</tr>
<tr>
<td>Control</td>
<td>41.4</td>
<td>100</td>
</tr>
<tr>
<td>+HgCl2 (3 mM)</td>
<td>10.4</td>
<td>25</td>
</tr>
<tr>
<td>+HgCl2 (3 mM) + GSH (10 mM)</td>
<td>39.2</td>
<td>95</td>
</tr>
<tr>
<td>+AY-9944 (0.6 μM)</td>
<td>29.3</td>
<td>71</td>
</tr>
<tr>
<td>+AY-9944 (1.2 μM)</td>
<td>24.4</td>
<td>59</td>
</tr>
<tr>
<td>+Triparanol (1 μM)</td>
<td>27.6</td>
<td>67</td>
</tr>
<tr>
<td>+NADPH (2 mM)</td>
<td>39.5</td>
<td>95</td>
</tr>
<tr>
<td>+NADPH (2 mM)</td>
<td>40.1</td>
<td>97</td>
</tr>
</tbody>
</table>

* EDTA was also omitted from the assay medium.
* Stock solutions of AY-9944 and Triparanol were prepared as described (15). Inhibitors were added into the incubation mixtures followed by a 5-min preincubation prior to substrate addition.
* Because zymosterol is also a substrate of 24-reductase that is active when NADPH is added, the observed specific activity was corrected about 4% to account for loss of isomeric substrate. No correction was needed when NADH was added because the 24-reductase is specific for NADPH.

The samples from HPLC were dialyzed for 3 h against 1000 ml of Buffer A. The 8-isomerase was estimated to be approximately 80,000. The samples from HPLC were dialyzed for 3 h against 1000 ml of Buffer A. The 8-isomerase was estimated to be approximately 80,000.
Sterol 8-Isomerase

(11). Furthermore, steroid isomeric equilibrium studies with microsomes were complicated by the presence of the endogenous isomerization product, 5α-cholesta-7-en-3β-ol (7).

With the 16-23% polyethylene glycol precipitate as a source of enzyme the initial rate of isomerization of zymosterol (0.81 nmol/min/mg) was approximately 17% of the rate of zymosterol isomerization (4.9 nmol/min/mg). Although the initial rate was considerably slower, at equilibrium >90% of both Δ⁴-sterols were converted to the corresponding Δ⁵-sterol products (Fig. 4).

Even with this very sensitive assay and lengthy incubation periods, no isomerization could be observed when lanosterol, 4,4-dimethyl-Δ⁶-cholestenol, 4,4-dimethyl-Δ⁶,14-cholestenol, Δ⁸,14-cholestenol, or Δ⁶,14-cholestadienol were assayed using identical conditions as those used for zymosterol. The assay is capable of detecting as little as 2% conversion (20 pmol/min/mg). These data are in general agreement with that obtained by previous investigations using the microsomal-bound enzyme, and these observations with enriched enzyme strongly suggest that as earlier predicted, with membrane-bound isomerase, isomerization occurs primarily after complete nuclear demethylation in the biosynthetic formation of cholesterol from lanosterol (2). Since Δ⁸,14-cholestenol is not a substrate, the data suggest that this sterol may not be an obligatory precursor of cholesterol. Yabuskii et al. (29) have shown that fecosterol (24-methylene-Δ⁴-cholestenol) can act as an isomerization substrate using yeast extracts. In addition, Pascal and Schroefer (30) have described the isomerization of 24-methyl and 14-hydroxymethyl sterols in preparations of rat liver microsomes. However, because large quantities of microsomes (100 mg) were used in their incubation to overcome the slow rate of conversion (about 0.1 pmol/min/mg; i.e. four orders of magnitude less rapid than zymosterol), it is doubtful if isomerization of 14α-hydroxymethyl sterol could be of consequence in cellular cholesterol synthesis.

The Δ⁴-isomerase is thought to catalyze an equilibrium reaction based on the isotopic equilibration of Δ⁴-cholestenol with tritiated water employing microsomes from rat liver and also from the appearance of radiolabeled 8-cholestenol in similar experiments using yeast extracts (29-33). However, either direct conversion of cholesta-7,24-dien-3β-ol to zymosterol or lahydrosterol into Δ⁴-cholesterol has not been demonstrated, due to presence of endogenous sterols in microsomes. With partially purified 8-isomerase in which cholesterol is at a low concentration, the time-dependent formation of Δ⁴-cholesterol from lahydrosterol was measured directly (Fig. 4). Maximum conversion was only about 2.5 nmol/i.e. 5% of the substrate converted after a 2-h incubation in which only the first 20 min were at a constant velocity. This, along with >90% formation of lahydrosterol from Δ⁴-cholesterol, suggests that the reaction equilibrium is about 19:1 in favor of the Δ⁴-sterol. 5α-Cholesta-7,24-dien-3β-ol was also converted to the corresponding Δ⁴ isomer (1.8 nmol/30 min/mg).

The pH dependence of partially purified and microsomal 8-isomerase was examined in parallel (Fig. 5). Both enzymes showed a sharp peak of maximal activity at pH 7.4.

After establishing optimal conditions for measurement of initial kinetics (Figs. 5 and 6), the observed $K_m$ value for the enriched enzyme was measured with zymosterol 55.5 μM (Fig. 7) which is in excellent agreement with earlier determinations for the microsomal-bound enzyme (52 μM) (12). All of these results are consistent with the conclusion that the solubilized and enriched 8-isomerase is the same enzyme that catalyzes the isomerization of sterol biosynthetic intermediates in microsomes.

Strong inhibition of microsomal 8-isomerase by AY-9944 with concomitant interruption of cholesterol synthesis in liver and adrenal gland has been demonstrated (34-36). Previously, the type of inhibition produced with membrane-bound enzyme could not be easily interpreted due to nonuniform distributions of inhibitor and substrate within microsomal particles that were incubated with variable amounts of substrate and inhibitor; e.g. see “partitioning effect” mentioned above. When partially purified enzyme was studied, the inhibition pattern was found to be of a mixed-type with strong competition between substrate and inhibitor as had been observed for the effect of AY-9944 on solubilized and enriched steroid 14-reductase (15). The mechanistic course of catalysis must
be understood first to interpret such observations more fully. However, based on structural analogy, competitive inhibition certainly was not expected for either 8-isomerase or 14-reductase.

This laboratory has previously reported that microsomal 8-isomerase requires neither cofactors nor metal ions for catalytic activity (2, 12). With the enriched 8-isomerase presumably free of endogenous cofactors, the enzyme assay was carried out in the presence of various potential cofactors: hemin, TPP, ATP, ADP, FMN, FAD, and coenzyme A at various concentrations up to 2 mM. A second set of experiments was performed in Tris-HCl buffer (pH 7.4) in the presence of various metal ions: Ca$^{2+}$, Mg$^{2+}$, Fe$^{2+}$, Cu$^{2+}$, and Mn$^{2+}$ at 0.2 to 4 mM. Neither the cofactors nor metal ions affected enzymic activity. Finally, spectral evidence does not suggest the presence of a prosthetic group. Thus, the isomerase appears to be unique among the microsomal enzymes that catalyze the 18-step conversion of lanosterol into cholesterol in that all of the other enzymes catalyze redox reactions that are dependent upon pyridine nucleotides, and in some steps flavoproteins and hemoproteins are also required.

The present preparation of 8-isomerase is 20- to 40-fold enriched, resolved from other microsomal enzymes of cholesterol biosynthesis, and stimulated by phospholipids. This preparation of enzyme will be used for reconstitution studies in combination with other similarly resolved enzymes and phospholipids.

REFERENCES
Sterol 8-Isomerase

Supplementary Material
Micromolecular Properties of 8-Isomerase from Lanosterol: Solubilization and Purification of Sterol 8-Isomerase

Young-Eui Paik, Jeffrey T. Billheimer, Ronald L. Magdol, and James L. Gaylor

Experimental Procedures

Enzyme Assays. Assay of the 8-isomerase was carried out nonradioactively at 37°C for 5 min with a slightly modified procedure of Yamaguchi and Gaylor (13). The incubation mixture contained 100 mM Tris-HCl (pH 7.4), 100 mM KOAc, 0.1% 3-mercaptoethanol, 0.05% I2, 3% acetonitrile:isopropanol (v:v), 180 mM potassium phosphate buffer (pH 7.0), and 150 mCi of radiolabeled substrate. The reaction mixture was incubated at 37°C for 5 min under nitrogen for 10 min. The reaction was terminated by addition of 20 mM potassium phosphate buffer (pH 7.4). The mixture was centrifuged for 5 min at 15,000 g, the supernatant was transferred to a scintillation vial, and 4 mL of toluene:scintillation fluid (2:1, v:v) was added. The scintillation fluid was added to the vials and counted in a Beckman scintillation counter. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]

The specific activity of the 8-isomerase was determined using the same assay procedure as described above. The specific activity of the 8-isomerase was calculated according to the following equation:

\[ \text{Specific activity} = \frac{\text{Counts per minute}}{\text{Units of enzyme activity}} \]
Gel Filtration of 8-Isomerase on HPLC. In an attempt to estimate the molecular weight of native 8-isomerase, HPLC size-exclusion column separation was performed in the presence of phospholipid and detergent. The combined active fractions obtained from DEAE chromatography were concentrated by YM-10 Diaflo membrane filtration. The concentrated protein solutions were resuspended in elution buffer, which was filtered through a Millipore (0.5 μm) filter system, in 10-20 ml of HPLC elution buffer (protein concentration: 5.0 mg/ml). The elution buffer, which was filtered through a Millipore (0.5 μm) filter system, included 0.4% OG, 0.2% STDC (w/v), and 50 mM potassium phosphate buffer (pH 7.0) and containing 0.4 mg/ml sonicated egg lecithin, 2 mM glutathione, 0.5 mM EDTA, and 10% glycerol (v/v), Buffer B. The protein suspension (10 ml) was directly injected onto a Tosoh TSK gel column (H-D000, SWG, 2.25 x 60 cm) that had been equilibrated with Buffer B. The column was run at 4°C with a flow rate of 1.15 ml/min and a pressure of 200 psi. Column eluent was monitored at 280 nm. A set of standard molecular weight proteins prepared in the same buffer was injected onto the same column.

Sources of Reagents. The following chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO): NADPH (tetrasodium salt), HgCl₂, glucose oxidase (type V, from Aspergillus Niger), glutathione (reduced form), dithiothreitol, phospholipase A₂ (from porcine pancreas). Triparanol was obtained from Merrell Richardson. Polyethylene glycol and Silicagel G 60 were purchased from EM Science (Gibbstown, NJ). The chromatographic gels, Sephadex G-25, DEAE-Sephacel, Hydroxylapatite, and Agarose-hexane HP254 were purchased from Pharmacia Fine Chemical Co. (Milwaukee, WI). N-Octylglucoside and taurodeoxycholic acid were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Egg lecithin and other phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). (All other chemicals were of the best grade.) All protease inhibitors, benzamidine, pepstatin, Leupeptin, and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). Triparanol is 4-chloro-β-[4-(3-diethylaminooethoxy)phenyl]-α-(4-methylphenyl)benzemethanol. AY-9944 (obtained from Dr. D. Dvor- nik, Ayerst Labs, Montreal) is trans-1,4-bis(2-chlorobenzylamino-methyl)cyclohexane dihydrochloride.