Mechanism of C-5 Double Bond Introduction in the Biosynthesis of Cholesterol by Rat Liver Microsomes

EVIDENCE FOR THE PARTICIPATION OF MICROSOMAL CYTOCHROME \(b_5\)

Vangala V. R. Reddy,*, David Kupfer,‡ and Eliahu Caspi§

From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

(Received for publication, August 12, 1976)

The dehydrogenation reaction of cholest-7-en-3\(\beta\)-ol (I) to cholesta-5,7-dien-3\(\beta\)-ol (II) in the presence of NADH was studied in rat liver microsomes and in microsomal acetone powder preparations, using \([3\alpha\,\text{H}]\)cholest-7-en-3\(\beta\)-ol. It was found that the reaction was inhibited by menadione, adenosine diphosphate, potassium ferriyanide, and cytochrome \(c\) while \(p\)-cresol had no effect. These results indicated the participation of a microsomal electron transport system in the dehydrogenation of cholest-7-en-3\(\beta\)-ol. The conversion of cholest-7-en-3\(\beta\)-ol to cholesta-5,7-dien-3\(\beta\)-ol was also observed in the absence of NADH when ascorbic acid was included in the incubation mixture. However, the ascorbic acid-catalyzed dehydrogenation was not inhibited by potassium ferriyanide.

Immunological evidence that microsomal cytochrome \(b_5\) is involved in the dehydrogenation of (I) to (II) was obtained. Antibodies specific for rat liver microsomal cytochrome \(b_5\) were elicited in rabbits. The anticytochrome \(b_5\) immunoglobulin fraction inhibited rat liver microsomal NADH-cytochrome \(c\) reductase but not NADPH-cytochrome \(c\) reductase. Also, the extent of reduction of cytochrome \(b_5\) was not affected by the antibodies. The conversion of (I) to (II) by rat liver microsomes was inhibited (73%) by anticytochrome \(b_5\) immunoglobulin at a ratio of microsomal protein:immunoglobulin of 1:5.6.

These results are consistent with the participation of microsomal cytochrome \(b_5\) in the introduction of the C-5 double bond in cholesterol biosynthesis. A close analogy of the microsomal dehydrogenation of fatty acids and of cholest-7-en-3\(\beta\)-ol is apparent and this suggests a possible similarity in the mechanisms of the two reactions.

* Present address, Department of Medicine, Division of Endocrinology and Metabolism, Hahnemann Medical College, 230 N. Broad Street, Philadelphia, Pennsylvania 19102.
‡Recipient of Grants GM 22688 and ES 00834 from the National Institutes of Health.
§Recipient of Grant AM 12156 from the National Institutes of Health.
we show that the pathway of the electron flow in the Δ'-sterol dehydrogenation is apparently similar to that in stearyl-CoA desaturase system (18) and in other microsomal dehydrogenations (22, 24).

MATERIALS AND METHODS

NAD, NADH, NADP, NADPH, ADP, menadione, and cytochrome c were purchased from Sigma Chemical Co. The inhibitor of the reduction of C-7 double bond of cholesta-5,7-dien-3β-ol AY9944 (25) was a gift from Dr. I. Dvorvnik (Ayerst Laboratories, Montreal). Thin layer chromatography was carried out on plates coated with silica gel (HF 254-366). Recording spectrophotometer (Cary model 14 equipped with a light scattering accessory and a 0.1 absorbance slide wire) was used for cytochrome b₅ estimations. Female Sprague-Dawley rats (Charles River Breeding Laboratories) and male albino rabbits (Margaret's Home Forme) were used [5α,7(H)-cholest-7-en-3β-ol] was prepared as previously described (5). The radioactivity was determined (5) in a liquid scintillation counter (Mark II, Nuclear Chicago Co.).

Enzyme Preparation and Incubations—Female rat liver microsomal acetone powder was prepared as described earlier (5, 9, 17). Acetone powder (250 mg) was suspended in 2 ml of Tween m, pH 7.4 containing MgCl₂ (4 mM), EDTA (0.1 mM), and AY9944 (0.05 mM) and distributed equally in eight flasks. NADH (3 mg in buffer (0.5 ml)) and [5α,7(H)-cholesterol 7 en-3β ol (2.5 μ Ci, 75 μg) (suspended in buffer (0.5 ml) with Tween 80) were added to each flask as described earlier (5). The indicated amounts of inhibitors were dissolved in 0.5 ml of the above buffer and added to the incubation flasks (Table I). The final volume was adjusted to 5 ml with the same buffer and the flasks were agitated (2 h at 37°) in an atmosphere of air. The obtained [5α,7(H)]cholesta-5,7-dien-3β-ol] was isolated, purified, and its H content determined (5). The dehydrogenation activity was calculated from the amounts of radioactivity found in the recovered [5α-14C]-5,7-diene and that of the incubated [5α-14C]-7-en. The 2.3% conversion of the [5α-14C]-7-en to [5α-14C]-5,7-diene in control experiments was considered to correspond to 100% of the dehydrogenation activity (Table I).

Purification of Cytochrome b₅ from Female Rat Liver Microsomes—The cytochrome b₅ from female rat liver was prepared by the method of Omura and T s u e k a w a (20). From 100 g of liver, 20 mg of cytochrome b₅ with a specific activity of 66 nmol of cytochrome b₅/mg of protein, were obtained. The cytochrome b₅ was subjected to acrylamide gel electrophoresis (27) and showed a single protein band. The spectral properties of the purified cytochrome b₅ were similar to those reported (26). The reduced form showed absorption peaks at 414, 530, and 560 nm. The ratio of the absorption maxima at 414, 530, and 560 nm was similar to that of the oxidized form. The reduced form had an absorption maximum at 280 nm of the oxidized form was 1.25 to 1.30.

Antibodies to Cytochrome b₅—A mixture of the purified rat cytochrome b₅ (15 mg), saline (0.9% NaCl solution) (3 ml), and the complete Freund's adjuvant (Ditco Co.) (3 ml) was thoroughly stirred and injected subcutaneously into four places on the backs of two male albino rabbits, each weighing 5 to 6 kg. Three weeks later, 5 mg of cytochrome b₅ (5 mg) and saline (3 ml) were mixed with incomplete adjuvant (3 ml) and injected into the rabbits near the sites of the earlier injections. After 2 weeks, a third injection of 2 mg of cytochrome b₅ in incomplete adjuvant was similarly injected into each rabbit. A week later, the blood was collected from an ear vein and the presence of antibodies was determined by a precipitin test (28). Ten days after the third injection, blood was collected from the rabbits by heart puncture. The sera were obtained and the immunoglobulin fractions from 10 ml of sera were precipitated with 40% ammonium sulfate. The precipitated globulin fraction (anticytochrome b₅, IgG) was dissolved in 0.3% saline (10 ml) containing phosphate buffer (10 mM, pH 7.4) and dialyzed overnight against the same saline buffer. The dialyzed solution was stored at 4° and used for the precipitation. Control immunoglobulin (Control Ig) was prepared similarly from an untreated rabbit.

Estimation of Reduction of Cytochrome b₅—Rat liver microsomes (29) were suspended in 0.1 M KC1 (8 mg of protein/ml). A mixture of the microsomal suspension (0.25 ml; 2 μg of protein) in sodium phosphate buffer (0.05 M, pH 7.6) containing KCN (1.5 mM), menadione (30 mM), and EDTA (1 mM) was prepared. Aliquots (0.95 ml) of the mixture were placed in two semimicrocuvettes. The reaction was initiated by the addition of a solution of NADH (0.05 μl) in 0.05 ml of buffer to the sample cuvette. Only buffer (0.05 ml) was added to the reference cuvette. The amount of the reduced cytochrome b₅, was calculated from the changes in optical density (50) at 423 nm using ε₅₅₀ = 171.

Determination of NADH-Cytochrome b₅ Reductase (30)—Aliquots (0.7 ml) of a rat liver microsomal suspension (25 μg of protein/0.7 ml of buffer) were placed in two semimicrocuvettes. The cytochrome b₅ (0.17 ml, 0.51 mg) was added to each cuvette and the final volume of the reaction mixtures were adjusted to 1 ml. The reaction was started by the addition of NADH (0.05 ml, 40 μg) to the sample cuvette and the reference cuvette received the same volume of buffer lacking NADH. The reduction of cytochrome b₅ was recorded at 550 nm. Similar incubations were carried out in presence of control Ig (0.5 mg of protein) and anticytochrome b₅, IgG (0.5 mg of protein).

The estimation of NADH-cytochrome b₅ reductase was carried out essentially as described above, except that NADPH was used instead of NADH. Protein was estimated by the method of Lowry et al. (31) using bovine serum albumin as standard.

Inhibition of Dehydrogenation of 5α-[3α-(3H)]cholesta-5,7-dien-3β-ol by Anticytochrome b₅, Immunoglobulin—In Experiment 1, rat liver microsomal acetone powder (200 mg) was suspended in 25 ml of Tris buffer (0.1 M, pH 7.4) containing MgCl₂ (4 mM), EDTA (0.1 mM), and menadione (10 μM) and dialyzed overnight against the phosphate buffer (0.05 M, pH 7.4) containing KCN (1.5 mM) and NADH (0.05 ml) and distributed equally in eight flasks. To each flask was added 3.0 ml of NADH and 5α-[3α-(3H)]cholesta-7-en-3β-ol (2.5 μCi, 75 μg). To evaluate the effect of dilution on the dehydrogenation, 1 ml and 4 ml of phosphate-buffered saline containing AY9944 (0.05 mM) were added to two flasks. The remaining eight flasks were divided into two equal groups. One group of flasks was incubated with increasing amounts of control sera and the other group with increasing amounts of anticytochrome b₅, sera. Different amounts of AY9944 were supplemented to achieve 0.05 mM concentration in all the flasks. The incubation procedure, isolation of sterols, and the calculation of dehydrogenation activity were the same as described above.

In Experiment 2, microsomal pellets (5) (12 mg of protein/pellet) were mixed with increasing amounts of control Ig or anticytochrome b₅, IgG in a phosphate buffer (0.1 M, pH 7.4) containing MgCl₂ (4 mM), EDTA (0.1 mM), and AY9944 (0.05 mM). The mixture was incubated at 25° for 15 min and then centrifuged at 105,000 × g for 1 h. The supernatant fraction which contained unbound globulin was discarded. For the determination of the dehydrogenation activity, the microsomal pellets were resuspended in the above phosphate buffer (0.05 M containing NADH (3 mM) and 5α-[3α-(3H)]cholesta-7-en-3β-ol (2.5 μCi, 75 μg) were added, and the mixture was incubated as described above. The interassay variability was about 5%; in controls Ig product formation varied from 15.1 to 15.8 nmol. Suitable control incubations without added Ig were performed.

RESULTS AND DISCUSSION

Inhibitory Studies on Dehydrogenation of Cholesta-7-en-3β-ol—The following experiments were designed to examine whether cytochrome b₅ is involved in Δ'-sterol dehydrogenation by rat liver microsomes. Menadione and potassium ferricyanide, known to interact with electron transport at the site of flavoproteins e.g. NADH-cytochrome b₅, reductase (32, 33), inhibited the dehydrogenation of cholesta-7-en-3β-ol by the rat liver microsomal acetone powder (Table I). This finding indicated that a flavoprotein, presumably microsomal NADH-cytochrome b₅, reductase, may participate in the dehydrogenation reaction. In addition, adenosine diphosphate at 10 mM concentration was also inhibitory to the reaction. It has been reported that ADP and ADP-ribose inhibit NADH oxidation in the presence of ferricyanide as the electron acceptor (34). The inhibition is presumably due to competition by the ADP derivatives for the NAD sites on the NADH-cytochrome b₅, reductase. It could, therefore, be assumed that in our system, ADP acts similarly. These observations suggested that NADH-cytochrome b₅, reductase is involved in the dehydrogenation of 5α-cholesta-7-en-3β-ol to cholesta-5,7-dien-3β-ol.

The abbreviation used is: Ig, immunoglobulin.
The possibility was considered that a nonspecific substance(s) present in the control serum of the plasmalogen biosynthesis (22) inhibited the reaction. They developed a procedure for the elimination of the nonspecific inhibition of the 5α-cholesterol 7-en-3β-ol dehydrogenation. For this purpose, we used freshly prepared microsomes to which the anticytochrome b1 Ig was added. The microsomes were first preincubated with the Ig fractions (anti-b1 or control) and then recovered by centrifugation. The recovered microsomes, which were pretreated with anticytochrome b1 Ig, showed inhibition of the dehydrogenation reaction, while the control Ig microsomes did not inhibit the process (Fig. 1). The dehydrogenation was inhibited by approximately 73% when 5.6 mg of the anticytochrome b1 Ig protein were used per 1 mg of microsomal proteins. The presented evidence is consistent with the participation of cytochrome b, in the conversion of 5α-cholesterol 7-en-3β-ol to cholest-5,7-dien-3β-ol by rat liver microsomes.

Site of Inhibition of Anticytochrome b1 Ig in Microsomal Electron Transport—We have established that the anti-b1 Ig inhibits the dehydrogenation reaction by approximately 73%.

To further study the role of microsomal cytochrome b1 in the Δ-sterol dehydrogenation, the effect of anticytochrome b1 Ig on the pyridine nucleotide-mediated reduction of cytochrome c by liver microsomes was examined. We observed that anticytochrome b1 Ig inhibited microsomal NADH-cytochrome c reductase (Fig. 2) but not that of NADPH-cytochrome c reductase (Fig. 3). This indicates the specificity of the antibodies and excludes the possibility of a cross-reaction with a flavoprotein not participating in the b1-mediated electron transport.

There was about 70% inhibition of the reduction of cytochrome c by NADH in the presence of anticytochrome b1 Ig. A mixture of microsomal protein and Ig protein at a ratio of 1:20 was used in these incubations (Figs. 2 and 3). The participation of cytochrome b1 in the reduction of added cytochrome c by NADH in microsomal preparations is well established (35). Hence, these observations indicate that the inhibition of NADH-cytochrome c reductase by anticytochrome b1 Ig was at the level of cytochrome b1.

Attempts were then made to explore the site of interaction of anticytochrome b1 Ig with cytochrome b1. The accepted mode of reduction of cytochrome c by NADH in liver microsomes is presented in Scheme 1. We have explored whether the reduction of cytochrome b1 (Site 1) or the reoxidation of reduced cytochrome b1 (Site 2) are affected by anticytochrome b1 Ig.

To test if the reduction of cytochrome b1 is inhibited by anticytochrome b1 Ig, we incubated microsomes (2 mg of protein) with NADH in a buffer solution (pH 7.6) in the presence of control Ig or anticytochrome b1 Ig (20 mg of protein). It was observed that the total amount of cytochrome b1, which was reduced (monitored at 423 nm) was not affected by anticytochrome b1 Ig. As mentioned above, the experiments with cytochrome c clearly show that the anticytochrome b1 Ig inhibits the NADH-cytochrome c reductase (Site 2). These observations suggested that the employed anticytochrome b1 Ig has an affinity for a given binding site and inhibits the transfer of electrons from reduced cytochrome b1 to an electron acceptor, probably cytochrome c. Nevertheless, it is possible that although the overall reduction of cytochrome b1 was not affected by our preparation of anticytochrome b1 Ig, the rate of reduction of cytochrome b1 was. Unfortunately, because of rapidity of the reduction of b1, our methodology could not detect whether the rate of reduction was indeed affected. This aspect will be the subject of a future investigation. In fact, the antibodies to cytochrome b1 prepared by Oshino and Omura (18) were shown to inhibit both the reduction and oxidation of cytochrome b1.

\[ \text{Table I} \]

<table>
<thead>
<tr>
<th>Inhibitor added</th>
<th>Initial activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Menadione (1 mM)</td>
<td>10</td>
</tr>
<tr>
<td>ADP (10 mM)</td>
<td>5</td>
</tr>
<tr>
<td>K_2Fe(CN)_6 (1 mM)</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Cytochrome c (6 mg)</td>
<td>50</td>
</tr>
<tr>
<td>Ascorbate (8 mM)</td>
<td>40</td>
</tr>
<tr>
<td>Ascorbate (8 mM) + K_2Fe(CN)_6 (1 mM)</td>
<td>30</td>
</tr>
<tr>
<td>p-Cresol (6 mM)</td>
<td>120</td>
</tr>
</tbody>
</table>

\[ ^a \text{The conversion of [3α-H]cholest-7-en-3β-ol (2.3 μCi, 75 pg) to [3α-3H]cholesta-5,7-dien-3β-ol in 2.3% yield (4.6 nmol) was taken as 100% of the desaturation activity.} \]

\[ ^b \text{No NADH was added.} \]
Cholesterol Biosynthesis-Cholesta-5,7-dien-3p-ol Formation

The similarity of sterol dehydrogenation and fatty acyl-CoA desaturase system strongly suggests the operation of a similar pathway for electron transfer in both systems. Based on our results, the following scheme for the desaturation of 5α-cholesta-7-en-3p-ol to cholesta-5,7-dien-3p-ol is suggested (CSF, cyanide-sensitive factors):

\[
\text{NADH} \xrightarrow{\text{Ascorbate}} \text{FP} \xrightarrow{\text{cytochrome b}_5} \text{cytochrome c}_5 \xrightarrow{\text{K}_{3}Fe(CN)_{6}} \text{Cytochrome c}
\]

**CONCLUSION**

The similarity of sterol dehydrogenation and fatty acyl-CoA desaturase system strongly suggests the operation of a similar pathway for electron transfer in both systems. Based on our results, the following scheme for the desaturation of 5α-cholesta-7-en-3p-ol to cholesta-5,7-dien-3p-ol is suggested (CSF, cyanide-sensitive factors):

\[
\text{NADH} \xrightarrow{\text{FP}} \text{cytochrome b}_5 \xrightarrow{\text{ox}} \text{cytochrome c}_5 \xrightarrow{\text{red}} \text{CSF} \xrightarrow{\Delta^2\text{-sterol}} \text{K}_{3}Fe(CN)_{6} \xrightarrow{\text{O}_{2}} \text{Cytochrome c}
\]

**REFERENCES**

28. Maurer, P. H. (1971) in Methods in Immunology and Immuno-
Mechanism of C-5 double bond introduction in the biosynthesis of cholesterol by rat liver microsomes.
V V Reddy, D Kupfer and E Caspi


Access the most updated version of this article at http://www.jbc.org/content/252/9/2797

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/9/2797.full.html#ref-list-1