Mixed Function Oxidases in Sterol Metabolism

SEPARATE ROUTES FOR ELECTRON TRANSFER FROM NADH AND NADPH*

Roy D. Crowder and Donald R. Brady†
From the Department of Chemistry, Memphis State University, Memphis, Tennessee 38102

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Oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholesterol-7-en-3-one and oxidative demethylation of [30,31-14C]4,4-dimethyl-5α-cholesterol-7-en-3β-ol by rat liver microsomes have been shown to be required for oxidative demethylation. Thus, although it is logical to assume that the participation of either NADH or NADPH in oxidative demethylation might be explained in terms of interaction between a terminal oxidase and the known electron transport components mentioned above, there is no evidence to support such an assumption.

We have been interested in the manner in which NADH and NADPH transfer electrons to methyl sterol oxidase and have attempted to answer two questions. First, can NADH- and NADPH-supported oxidative demethylation be separated either physically or functionally? If so, this would imply a system comparable to the known electron transport systems in which separate and distinct components exist each specific for one of the two cofactors. Second, does NADPH-cytochrome b5 reductase or NADPH-cytochrome c reductase participate in oxidative demethylation?

We have also attempted to answer these same two questions with regard to oxidative deformylation of 4-hydroxy[14C]-methylene-5α-cholesterol-7-en-3-one (Fig. 1). Deformylation is catalyzed by an oxidase different from methyl sterol oxidase but with similar properties (7, 8). A comparison of oxidative deformylation and oxidative demethylation in this regard should provide greater insight into the process of mixed function oxidation occurring in the terminal steps of cholesterol biosynthesis.

EXPERIMENTAL PROCEDURES

Isolation of Microsomes—Microsomes were isolated by the procedure described previously and either used directly for further treatment or frozen in liquid nitrogen and stored at −28°C (7). Fresh or frozen microsomes were treated with Triton WR 1339 and sodium deoxycholate (10 mg/ml) as described previously (7). These microsomal preparations will be referred to as deoxycholate-treated microsomes and were used in all experiments in which NADH-supported oxidative demethylation was measured. This allowed measurement of activity at high concentrations of NADH which is inhibitory using other microsomal preparations (7).

For some studies involving the use of mersalyl, glutathione was omitted from the buffer used in the final suspension of the deoxycholate-treated microsomes.

Subtilisin Treatment of Microsomes—Frozen microsomes were treated with subtilisin by the procedure of Comai and Gaylor (10). Treatment was carried out at 4°C for 30 min. Following centrifugation at 105,000 x g for 1 h the pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 10 mM nicotinamide and 2 mM glutathione. Protein concentration was ~20 mg/ml. The second centrifugation carried out by Comai and Gaylor was omitted (10).

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† To whom reprint requests should be sent.
FIG. 1. Reactions of oxidative demethylation and oxidative deformaty-
lation. Reactions A, B, and C are catalyzed by methyl sterol
oxidase and involve conversion of di and monomethyl sterols through
mylation. Reactions A, B, and C are catalyzed by methyl steroi
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mylation.
are treated with subtilisin at 4°C by the procedure of Comai and Gaylor (10) and NADH as well as NADPH-supported oxidase activity is compared, a marked difference in the sensitivity to this treatment is observed for both oxidative deformylation and oxidative demethylation (Table I). NADPH-supported activity is diminished significantly, whereas NADH-supported activity is affected to a much lesser degree. NADPH-supported deformylation activity decreased to about 30% of control and NADPH-supported demethylation to about 13%. NADH-supported oxidative demethylation remained constant while oxidative deformylation supported by NADH decreased slightly to 70% of control activity.

Mersalyl Inhibition—Methyl sterol oxidase has been shown to be inhibited by mercurial reagents (5). Mersalyl served a useful purpose for investigating the role played by NADH-ferricyanide reductase or NADPH-cytochrome c reductase in oxidative deformylation and demethylation (Figs. 3 and 4). When reductase activities were inhibited significantly to 20 to 40% of control, oxidase activity was either stimulated or not affected. For oxidative deformylation, 25 nmol of mersalyl/mg of microsomal protein had little effect on NADH-supported oxidase activity and stimulated NADPH-supported oxidase activity 1.2 to 1.4-fold (Fig. 3). For oxidative demethylation, 25 nmol of mersalyl/mg of microsomal protein had little effect on the activity using either cofactor (Fig. 4). This concentration of mersalyl, however, inhibited NADH-ferricyanide reductase and NADPH-cytochrome c reductase to activities less than 40% of control (Figs. 3 and 4).

While using mersalyl we found that it inhibits the NADH- and NADPH-generating systems. Since saturating amounts of NADH and NADPH were used, this was not a critical problem. ²

**Inhibition by 2'-AMP and Antibody to NADPH-Cytochrome c Reductase**—The role played by NADPH-cytochrome c reductase in oxidative deformylation and demethylation was investigated further by comparing reductase activity and NADPH-supported oxidative activity in the presence of 2'-AMP (Table II) and antibody to NADPH-cytochrome c reductase.
chrome c reductase activity. Aliquots were removed and assayed for NADPH-supported oxidative demethylation and demethylation as well as NADPH-cytochrome c reductase (see "Experimental Procedures"). Experiments were performed using duplicate determinations of oxidase and single determinations of reductase activity.

### Table II

**Inhibition by 2'-AMP**

Washed microsomes (~1.5 mg) were assayed for NADPH-supported deformylation and demethylation as well as NADPH-cytochrome c reductase activity as described under "Experimental Procedures" and Fig. 3. 9 mM 2'-AMP was included in the assay vessel for both oxidase and reductase assays. Values in parentheses are the number of determinations made.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Addition</th>
<th>Oxidase activity</th>
<th>Reductase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmol/min/mg</td>
<td>nmol/min/mg</td>
</tr>
<tr>
<td>Oxidative deformylation</td>
<td>None</td>
<td>1.39 ± 0.07 (4)</td>
<td>198 ± 6 (4)</td>
</tr>
<tr>
<td></td>
<td>2'-AMP</td>
<td>0.99 ± 0.23 (4)</td>
<td>23.6 ± 5.8 (4)</td>
</tr>
<tr>
<td>Oxidative demethylation</td>
<td>None</td>
<td>1.23 ± 0.19 (4)</td>
<td>105 ± 2 (4)</td>
</tr>
<tr>
<td></td>
<td>2'-AMP</td>
<td>1.62 ± 0.18 (4)</td>
<td>20.7 ± 4.9 (4)</td>
</tr>
</tbody>
</table>

### Table III

**Inhibition by antibody to NADPH-cytochrome c reductase**

Washed microsomes were treated with antibody to NADPH-cytochrome c reductase. Aliquots were removed and assayed for NADPH-supported oxidative deformylation and demethylation as well as NADPH-cytochrome c reductase (see "Experimental Procedures"). Experiments were performed using duplicate determinations of oxidase and single determinations of reductase activity.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Experiment no.</th>
<th>Addition</th>
<th>Oxidase activity</th>
<th>Reductase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>nmol 14CO2/mg</td>
<td>nmol/min/mg</td>
</tr>
<tr>
<td>Oxidative deformylation</td>
<td>1</td>
<td>Nonimmune serum</td>
<td>1.74</td>
<td>199</td>
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<tr>
<td></td>
<td>2</td>
<td>Antibody serum</td>
<td>1.67</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>Nonimmune serum</td>
<td>2.44</td>
<td>244</td>
</tr>
<tr>
<td></td>
<td>Antibody serum</td>
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<td>24.4</td>
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</tr>
<tr>
<td>Oxidative demethylation</td>
<td>3</td>
<td>Nonimmune serum</td>
<td>1.04</td>
<td>98.2</td>
</tr>
<tr>
<td></td>
<td>Antibody serum</td>
<td>0.948</td>
<td>32.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Nonimmune serum</td>
<td>1.22</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>Antibody serum</td>
<td>0.879</td>
<td>90.7</td>
<td></td>
</tr>
</tbody>
</table>

* Milligrams of serum protein/mg of microsomal protein was 6.6.

For all other experiments the value was 3.8.

1 Oxidase activity was assayed in one step rather than two steps as described under "Experimental Procedures." Both NADPH (0.1 mM) and NADH (1 mM) were incubated with microsomes and substrate. 14CO2 was collected as usual.

2 D. R. Brady, unpublished observations.

3 Evidence has been presented in this paper which supports two conclusions. First, for both oxidative deformylation and oxidative demethylation, NADPH-supported oxidative activity can be distinguished from NADH-supported activity. In the presence of 10 μM cytochrome c, NADH-supported activity in both cases remained essentially at 100% of control. NADPH-supported activity, on the other hand, decreased significantly (Fig. 2). Subtilisin treatment of microsomes removed the majority of NADPH-supported oxidative deformylation and demethylation while most of the NADH-supported activity survived treatment (Table I).

Differences between NADH- and NADPH-supported oxidative activity have been noted for other systems. Both NADH and NADPH serve as cofactors for fatty acyl desaturation (17). Cytochrome c, however, inhibits NADPH-supported activity to a greater extent than NADH-supported activity. Also, NADP+-supported activity is inhibited by menadione to a greater extent than is NADH-supported activity (17).

The second conclusion arrived at from the evidence presented concerns NADPH-cytochrome c reductase and NADH-ferricyanide reductase and the role each may play in oxidative deformylation and demethylation. Although the evidence is not conclusive, it strongly suggests that neither reductase, particularly NADPH-cytochrome c reductase, participates in oxidative deformylation and demethylation in a manner similar to that in which the reductases participate in drug metabolism, fatty acyl desaturation, and other mesosomal oxidative activities. Numerous studies using the same or a similar approach as presented in this report have implicated participation of the reductases in several oxidase systems by demonstrating inhibition of an oxidative activity under conditions in which the reductase activity would be inhibited (17-24). However, on no occasion in which reductase activity was inhibited did the oxidative activities investigated in this report respond in a similar manner. NADH-supported deformylation was unaffected and NADPH-supported deformylation is stimulated under conditions in which NADH-ferricyanide reductase and NADPH-cytochrome c reductase are inhibited significantly (Fig. 3). NADH- and NADPH-supported oxidative demethylation are unaffected while reductase activities are reduced to low levels (Fig. 4). Furthermore, 2'-AMP, a known inhibitor of NADPH-cytochrome c reductase (25), reduces reductase activity markedly while stimulating oxidative demethylation and reducing oxidative deformylation only slightly (Table II). Finally, antibody to NADPH-cytochrome c reductase diminishes reductase activity considerably while affecting the oxidative activities little (Table III).

This evidence is in agreement with the results of Miyake and Gaylor (26) who demonstrated that oxidative demethylation activity, using α-NADH as a source of electrons, was stimulated almost 2-fold over the activity observed using β-NADH. NADH-cytochrome c reductase activity, using α-NADH, however, dropped to 10% of the activity observed in the presence of β-NADH.

Final proof as to whether or not either or both reductases participate in either or both oxidative activities must await isolation and purification of each oxidase or at least a complete isolation and the assumption that the interaction between cytochrome c
and NADPH-cytochrome c reductase or ferricyanide and NADH-ferricyanide reductase is affected by the inhibitors to the same extent that interaction of a component of either oxidase system with either of the reductases might also be affected. In other words, it may well be that the reductases have more than one site for interacting with oxidizing agents and the lack of parallel effects could be due to different sites which have differential susceptibility to the inhibitors. Different sites for cytochrome P₄₅₀ and artificial electron acceptors which interact with NADPH-cytochrome c reductase have been implied (27). On the other hand, that these data imply that neither reductase is rate limiting for either oxidative activity, i.e. deformylation or demethylation, is strengthened by the fact that mersalyl, a sulfhydryl reagent, inhibited both reductases without inhibiting either oxidative activity (Figs. 3 and 4). Mersalyl was most likely blocking interaction of NADH or NADPH with the reductases since it is known that p-chloromercuribenzoate inhibits NADH-ferricyanide reductase by reacting with a sulfhydryl group essential for NADH binding (28, 29). Furthermore, NADPH-cytochrome c reductase is known to be protected from mersalyl inhibition by the addition of NADPH, implying that a sulfhydryl group is essential for binding of NADPH (18). Also, 2'-AMP is known to be a competitive inhibitor of NADPH-cytochrome c reductase (30). This implies, of course, that it blocks NADPH binding to the reductase. It, too, inhibits NADPH-cytochrome c reductase significantly without appreciably affecting oxidative deformylation or demethylation (Table II). Thus, even if both NADH-ferricyanide reductase and NADPH-cytochrome c reductase were to have sites for binding the artificial electron acceptors different from those for binding possible components involved in the two oxidative activities, the observed lack of inhibition in the presence of mersalyl and 2'-AMP, both of which act presumably by blocking NADH and NADPH binding to the reductases, strongly suggests that neither reductase, if involved, is rate limiting in oxidative deformylation or demethylation.

The work presented in this and earlier reports (7, 8) might be summarized by the scheme in Fig. 5. Thus, two terminal oxidases, each specific for one substrate, bind the substrates while electron carriers shuttle electrons from NADPH and NADII to the terminal oxidases. Cytochrome c would inhibit each oxidative activity by interacting with the electron carriers. The data consistent with this scheme would be, first, that the formyl sterol substrate inhibits, noncompetitively, demethylation of the methyl sterol substrate (7). This would imply the existence of two sites or two terminal oxidases each specific for one substrate. Furthermore, the different responses seen for NADH- and NADPH-supported oxidative activity in the presence of cytochrome c regardless of which substrate is used (Fig. 2) suggest at least two different electron carriers are involved, each specific for one of the cofactors. Finally, cytochrome c inhibits deformylation and demethylation differentially in the presence of NADH (7), but similarly in the presence of NADPH (8), implying that the electron carrier of the NADH-supported route may have two sites, each specific for one of the terminal oxidases. The binding of cytochrome c to the electron carrier may affect the binding of the terminal oxidase at one site more than at the other. For the route involving NADPH, cytochrome c does not distinguish between two sites on the electron carrier if such exist. The modest stimulation by mersalyl of NADPH-supported oxidative deformylation as compared with NADPH-supported demethylation (Fig. 3) may imply the presence of two sites for the electron carrier in the NADPH route. However, since this effect might also result from a reaction between mersalyl and the terminal oxidases, it would be premature presently to propose two sites. It should be borne in mind that such a proposed scheme (Fig. 5) greatly simplifies a very complex system. Other schemes consistent with the data might also be proposed. The scheme should be considered principally as a focal point to provide some perspective in understanding the experiments performed and the direction future research might take.

The metabolic significance of there being two oxidase systems is not currently apparent. The oxidase responsible for demethylation would, of course, participate in cholesterol biosynthesis. The metabolic role played by the oxidase catalyzing deformylation is less clear since no metabolic role has been demonstrated for the formyl sterol. As further studies are conducted in which these two activities can be compared in additional ways, the function of this oxidase should become more clear.

REFERENCES