Study of Microsomal Mixed Function Oxidative Demethylation and Deformylation of 4-Methyl and 4-Hydroxymethylene Sterols*

(Received for publication, August 29, 1977)

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Oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one catalyzed by rat liver microsomes has been studied using both NADH and NADPH as a source of reducing equivalents. Microsomes treated with a nonionic detergent, Triton WR-1339, will catalyze oxidative deformylation in the presence of NADPH. Both NADH and NADPH will serve as a source of reducing equivalents, however, if the microsomes are treated further with deoxycholate at a concentration of 10 mg/ml. Using deoxycholate-treated microsomes, the oxidase-catalyzing oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one has been compared with methyl sterol oxidase which participates in oxidative demethylation of 4,4-dimethyl-5α-[30,31-14C]cholest-7-en-3β-ol. Oxidative metabolism of both substrates was inhibited in a similar manner upon heating the microsomes at 47°C or including dithiothreitol or CN– in the reaction vessel. Cytochrome c, however, caused marked inhibition (40%) of methyl sterol oxidase when NADH was used. Deformylation was virtually unaffected at concentrations of cytochrome c as high as 10 μM. Furthermore, methyl sterol oxidase was inhibited noncompetitively by 4-hydroxymethylene-5α-cholest-7-en-3-one. Finally, if Triton-treated microsomes are treated with deoxycholate at a higher concentration (20 mg/ml) both oxidative deformylation activity and methyl sterol oxidase activity appear in the 105,000 × g supernatant fraction. However, specific activity of the oxidase catalyzing oxidative deformylation decreases significantly, whereas the specific activity of methyl sterol oxidase increases. The ratio between the two activities changes by as much as 5-fold.

During oxidative demethylation of 4-methyl sterols by rat liver to form cholesteral a microsomal, mixed function oxidase, methyl sterol oxidase, is functional and requires either NADH or NADPH and uses molecular oxygen as the oxidizing agent (1-7). The oxidase system is proposed to catalyze three sequential reactions in which a 4-methyl group is oxidized first to an alcohol, next to an aldehyde, and finally to a carboxylic acid (see Fig. 1). In all, during the conversion of 4,4-dimethyl-5α-cholest-7-en-3β-ol to cholesterol, six substrates could be oxidatively metabolized by the oxidase system. We have been interested in the manner in which substrates interact with the oxidase and have studied oxidative demethylation of 4,4-dimethyl-5α-[30,31-14C]cholest-7-en-3β-ol as well as oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one, a model substrate for the oxidase (see Fig. 1).

Previously, it has been reported that 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one is deformed to form 14CO2 by an oxidase system with properties similar to methyl sterol oxidase, the oxidase participating in demethylation of 4,4-dimethyl-5α-cholest-7-en-3β-ol (8). These previous studies were conducted using NADPH as the source of reducing equivalents. Since NADH also can serve as a source of reducing equivalents for methyl sterol oxidase, we have compared oxidative demethylation of 4,4-dimethyl-5α-[30,31-14C]cholest-7-en-3β-ol and oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one in the presence of NADH. In attempting to establish a system that would allow this, we have observed evidence that oxidative demethylation is different from oxidative deformylation.

EXPERIMENTAL PROCEDURES

Isolation of Microsomes – The isolation procedures reported here are modifications of procedures reported elsewhere (1, 9). Rat livers were placed in 2 volumes of 0.1 m potassium phosphate buffer, pH 7.4, containing 2 mM glutathione and 10 mM nicotinamide. Tissue homogenate was prepared by blending for 3 to 10 s at low speed on a Waring Blender using a cooled Eberbach stainless steel container followed by homogenization in a ground glass Teflon® homogenizer. Mitochondria, nuclei, and cellular debris were removed by centrifugation for 20 min at 10,000 × g. The supernatant fraction was centrifuged for 1 h at 105,000 × g and the microsomal pellet frozen in liquid nitrogen and stored at −28°C.

Frozen microsomes were thawed and treated with Triton WR-1339 as described previously (1). Triton-treated microsomes were isolated by centrifugation at 105,000 × g for 1 h. Triton-treated microsomes were suspended in 0.1 M Tris buffer, pH 7.1, at 25°C, containing 10% glycerol (w/v), and treated with deoxycholate dissolved in 1 ml of water. The amount of deoxycholate added, based on the volume of microsomal suspension, was either 10 mg/ml or 20 mg/ml depending upon the experiment. The volume of Tris buffer used was equal to 1/4 the volume of the supernatant resulting from the initial centrifugation at 10,000 × g. This volume ratio was used for all microsomal suspensions. The deoxycholate-treated microsomes were left at room temperature for 20 min and passed through a Sephadex G-25 column (3 × 13 cm) of medium pore size equilibrated with water. The eluted colored band of protein was collected at 6°. From 8 ml of microsomes, 30 to 35 ml of eluant were collected. The eluant was adjusted to 0.05
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**Table 1**

| Cofactor requirement for oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one |
| Microsomes were incubated aerobically for 10 min with 50 nmol of substrate and either NAD+ (1 mM), a NADH generator, or a NADPH generator (see “Experimental Procedures”). Values in parentheses are the number of determinations made. |
| Microsomal preparation | Cofactor addition | nmol [14CO2]/mg protein |
|------------------------|-------------------|------------------------|
| Triton treateda | NADH (5) | 0.387 ± 0.089 |
| | NADPH (2) | 2.56 ± 0.28 |
| | NAD+ (3) | 1.29 ± 0.25 |
| Deoxycholate-treatedb | NADH (7) | 3.94 ± 0.99 |
| | NADPH (5) | 3.80 ± 0.88 |

*a 2 to 3 mg of protein.

*b 1 to 2 mg of protein.

RESULTS

Cofactor Requirement—4-hydroxy[14C]methylene-5α-cholest-7-en-3-one was incubated with Triton-treated microsomes in the presence of both reduced and oxidized pyridine nucleotides (Table I). The rate of oxidative deformylation in the presence of NADPH was almost 7-fold greater than the rate in the presence of NADH. NAD+—dependent deformylation was also observed. The activity requiring NADPH has been shown to be similar if not identical with methyl sterol oxidase activity (8). The activity in the presence of NAD+ remained to be characterized. A previous report suggested a dehydrogenase was not responsible for the activity, and removal of oxygen has been shown to result in complete loss of activity, suggesting the presence of an oxidase responsible for deformylation (8). Since Bechtold et al. (11) have shown that reducing equivalents needed for methyl sterol oxidase can be generated from added NAD+, we felt it logical to assume that both NADH and NAD+ were present in our incubation system and one or both could be responsible for the activity observed for NAD+ (Table I). Since this activity was present even when a NAD-generating system was used only low levels of NADH appeared to be required. Thus, the effect of varying NADH concentrations upon deformylation was determined.

Removal of Inhibition of Deformylation at High NADH Concentrations—NADH was varied from 5 μM to 80 μM and release of [14CO2] from 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one measured (Fig. 2). The amount of [14CO2] released reached a maximum at approximately 10 μM NADH and then decreased. The inhibited release of [14CO2] at high NADH concentrations was investigated further by attempting to wash out the competing or inhibitory reactions that might be preventing [14CO2] release. Triton-treated microsomes were treated with deoxycholate (10 mg/ml) as described earlier. Deoxycholate-treated microsomes were found to catalyze dehydrogenase reaction of 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one, yielding [14CO2] even at 1 mM NADH (Table I). Using deoxycholate-treated microsomes, we next attempted to characterize oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one in the presence of NADH.

Characterization of Deoxycholate-treated Microsomes—Deformylation of 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one in the presence of NADH obeyed linear kinetics when both microsomal concentration and time were varied. Linearity was observed for up to 10 min. The rate of deformylation was proportional to protein concentration up to 2.5 mg/ml. Fur...
thermore, both NADH and NADPH served as cofactors for this enzymatic process (Table D). Hence, deformylation of 4-hydroxy[14C]methylene-5α-cholesterol-7-en-3-one was similar to demethylation of 4-methyl sterols in that both NADH and NADPH could contribute reducing equivalents to the oxidative process (1). Since 4-hydroxy[14C]methylene-5α-cholesterol-7-en-3-one serves as a model substrate for the methyl sterol oxidase when NADPH is used (8), we investigated the comparable role using NADH.

Deformylation of 4-Hydroxy[14C]methylene-5α-cholesterol-7-en-3-one Compared to Demethylation of 4,4-Dimethyl-5α-[30,31-14C]cholesterol-7β-ol — 4-Hydroxy[14C]methylene-5α-cholesterol-7-en-3-one was incubated aerobically with deoxycholate-treated microsomes and NADH. 14CO2 release was measured. 4,4-Dimethyl-5α-[30,31-14C]cholesterol-7β-ol was incubated aerobically using the two-step assay to measure 14CO2 release. Dithiothreitol, cyanide, heat, and cytochrome c were tested for inhibitory effects upon the oxidative metabolism of these two substrates (Fig. 3). Parallel results were obtained using dithiothreitol, heat, and cyanide. However, when cytochrome c was used, oxidative deformylation was considerably more sensitive to inhibition than it was oxidative demethylation. Demethylation was inhibited to 60% of control by 10 μM cytochrome c whereas deformylation was unaffected. The inhibition due to cytochrome c was investigated using normal microsomes and Triton-treated microsomes as well to assure that deoxycholate treatment had not produced an artificial system not indicative of the true microsomal oxidase activity. Using low levels of NADH (10 μM) so as to observe 14CO2 release from 4-hydroxy[14C]methylene-5α-cholesterol-7-en-3-one, a similar response was observed with microsomes and Triton-treated microsomes.1 Oxidative demethylation of 4,4-dimethyl-5α-[30,31-14C]cholesterol-7-en-3β-ol was incubated much more markedly than oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholesterol-7-en-3-one when NADH was used as a cofactor regardless of the condition of the microsomes. Also, under conditions in which oxidative demethylation was inhibited 80%, the second step of the two-step assay was unaffected.1 Thus, the reactions in the first step catalyzed by methyl sterol oxidase are the ones affected by cytochrome c.

It has been previously shown that deformylation and demethylation are inhibited to the same extent by cytochrome c when NADPH serves as the source of reducing equivalents (8). These studies were done using Triton-treated microsomes and have been verified in our laboratory as well. A similar response was also observed using NADPH and deoxycholate-treated microsomes.1 Thus, deformylation and demethylation can be distinguished as two dissimilar processes regarding inhibition by cytochrome c only when NADH is used regardless of the state of the microsomes.

Inhibitory Effects of 4-Hydroxymethylene-5α-cholesterol-7-en-3-one upon Methyl Sterol Oxidase — Since the inhibitory effect of cytochrome c upon oxidative deformylation is clearly different from that upon oxidative demethylation, the possibility presented itself that two different oxidases might be involved. If so, the two substrates might not be binding to the same oxidase. The hydroxymethylene substrate, however, has been reported to be a competitive inhibitor of oxidative demethylation (8). Accordingly, 4,4-dimethyl-5α-[30,31-14C]cholesterol-7-en-3β-ol was incubated aerobically with deoxycholate-treated microsomes and either NADPH or NADH in the presence of 4-hydroxymethylene-5α-cholesterol-7-en-3-one which was not radioactively labeled. Formation of 14CO2 was measured by the two-step assay as described previously. As seen in Fig. 4, oxidative demethylation was inhibited in a noncompetitive manner.

Selective Extraction — To this point we have been able to demonstrate that oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholesterol-7-en-3-one is functionally different from oxidative demethylation. If two oxidases are present, extraction procedures might affect the two oxidases differentially. This was investigated by extracting Triton-treated microsomes with a higher concentration of deoxycholate (20 mg/ml of microsomes) as described earlier.

Both oxidase activities were found in the supernatant fraction from centrifugation at 105,000 × g; however, oxidative

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**Fig. 2.** Effect of NADH concentration on deformylation of 4-hydroxy[14C]methylene-5α-cholesterol-7-en-3-one. Triton-treated microsomes (2 to 3 mg of protein) were assayed for deformylation of 50 μM 4-hydroxy[14C]methylene-5α-cholesterol-7-en-3-one as described under "Experimental Procedures" using a NADH generator at the concentrations of NADH shown. Results are the average of two experiments.

**Fig. 3.** Effect of inhibitors on methyl sterol oxidase and deformylation. Triton-treated microsomes were treated with deoxycholate (10 mg/ml). The deoxycholate-treated microsomes (1 to 2 mg of protein) were assayed for methyl sterol oxidase (▪) and deformylation activity (□-□) in the presence of the inhibitors shown. 50 nmol of substrate were used in each case. The concentration range for each inhibitor used was as follows: cyanide, 0 to 100 μM; dithiothreitol, 0 to 2 mM; and cytochrome c, 0 to 40 μM. The heating experiment was carried out by heating the microsomes at 47°C for the times indicated. Samples were removed, placed on ice, and assayed for activity. Assay and isolation of microsomes are described under "Experimental Procedures." The number of experiments performed were: cyanide (4), dithiothreitol (1), heat (2), and cytochrome c (4).
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Fig. 4. Inhibition of methyl sterol oxidase by 4-hydroxymethylene-5α-cholest-7-en-3-one. Triton-treated microsomes were treated with deoxycholate (10 mg/ml). Deoxycholate-treated microsomes (1 to 2 mg of protein) were assayed for methyl sterol oxidase activity (O-O) using either a NADH- (A) or NADPH- (B) generating system in the presence of 50 μM 4-hydroxymethylene-5α-cholest-7-en-3-one (●—●). For methyl sterol oxidase assays, 4,4-dimethyl-5α-[30,31-14C]cholest-7-en-3β-ol was varied from 10 to 80 μM. Isolation of microsomes and assay procedures are described under "Experimental Procedures." Results are the average of three experiments.

Table II
Selective extraction of methyl sterol oxidase activity

Triton-treated microsomes were extracted with deoxycholate (20 mg/ml) as described under "Experimental Procedures." Microsomes and supernatant fraction were assayed for 10 min to measure oxidative deformylation and methyl sterol oxidase activity using either a NADH- or a NADPH-generating system and 50 nmol of substrates (see "Experimental Procedures"). Values in parentheses are the number of determinations made.

| Preparation                | Cofactor | Oxidative demethylation | Oxidative deformylation | Deformylation/ demethylation |
|----------------------------|----------|-------------------------|-------------------------|------------------------------|
| Triton-treated* microsomes | NADH     | 1.10 (3)                | 3.12 (2)                | 2.84                         |
|                            | NADPH    | 1.08 (3)                | 2.56 (2)                | 2.37                         |
| Deoxycholate-treated* microsomes | NADH     | 0.20 (2)                | 0.25 (2)                | 1.08                         |
|                            | NADPH    | 0.17 (2)                | 0.23 (2)                | 2.56                         |
| 105,000 × g supernatant* fraction | NADH     | 1.36 (5)                | 0.74 (5)                | 0.54                         |
|                            | NADPH    | 1.50 (2)                | 1.40 (2)                | 0.93                         |

* 2 to 3 mg of protein.
† Value is maximal value of curve in Fig. 2. [NADH] is 10 μM.
‡ 1 to 2 mg of protein.
§ 1 to 2 mg of protein.

...deformylation activity had diminished significantly (Table II). The ratio of oxidative deformylation activity to methyl sterol oxidase activity changes approximately 5-fold when NADH is used and approximately 2.5-fold when NADPH is used. Clearly, the two activities do not change in a similar manner when extracted from the microsomes. Calculations of total activity showed above 50 to 60% recovery of oxidative demethylation activity, whereas only 20% of the oxidative deformylation activity was recovered.

DISCUSSION

Although methyl sterol oxidase appears to function at several steps during demethylation of methyl sterols, little is known about the mechanism by which reactants interact with the oxidase (1-7). Considering the diverse structural nature of the sterol substrates, it would not be surprising for the oxidase to be composed of several distinct components each responsible for interacting with the given substrate during the oxidative process. If such were the case, it would be expected that the oxidative reactions for each substrate might be experimentally separated one from the other, either functionally or by actual separation of physical components. In this report we have demonstrated that oxidative demethylation of 4,4-dimethyl-5α-[30,31-14C]cholest-7-en-3β-ol and oxidative deformylation of 4-hydroxy[14C]methylene-5α-cholest-7-en-3-one can be separated functionally through the use of inhibitors and extraction with deoxycholate. Thus, although deformylation requires either NADH or NADPH (Table I), only low levels of NADPH (10 μM) support oxidative deformylation (Fig. 2 and Table I). Unlike oxidative demethylation, oxidative deformylation is diminished at higher concentrations of NADH. This may, however, not be a property of the oxidase itself, since treatment of the microsomes with deoxycholate (10 mg/ml) yields microsomes capable of catalyzing NADH-dependent oxidative deformylation at rates comparable to oxidative deformylation in the presence of NADPH (Table I). The oxidase catalyzing oxidative deformylation using NADPH has already been shown to be comparable to oxidative demethylation under both inhibitory and stimulatory conditions (8). A similar comparison, in this report, using NADH has yielded different results. Although cyanide, dithiothreitol, and heat all affected oxidative deformylation in a manner which paralleled effects on oxidative demethylation, cytochrome c had a different effect (Fig. 3). At 10 μM cytochrome c, deformylation was unaffected; oxidative demethylation was inhibited to 80% of control. Furthermore, at 20 μM cytochrome c, oxidative de-
methylation was inhibited to 20% of control and deformylation had diminished to only 75% of control. The differences between the two oxidative systems were also seen when oxidative demethylation was assayed in the presence of 4-hydroxymethyl-ene-5α-cholest-7-en-3-one (Fig. 4). The noncompetitive inhibition implies interaction at a separate site for each substrate. Further confirmatory evidence for two oxidative systems was obtained when Triton-treated microsomes were extracted with deoxycholate at a concentration of 20 mg/ml. Upon analysis of the deoxycholate-treated microsomes and the supernatant resulting from isolation of the microsomes, oxidative demethylation occurred predominately in the supernatant and oxidative deformation had diminished to low levels (Table II). The dissimilar nature of the two oxidative systems is seen when the ratio of the two activities is compared before and after deoxycholate extraction. The marked change seen (Table II) indicates that the two oxidases do not extract in a similar manner from the microsomes.

Although oxidative deformylation and oxidative demethylation are separable, they do share some common properties and, therefore, perhaps some common physical components. Both respond in a parallel manner to some inhibitors and stimulators (Ref. 8 and Fig. 3). The noncompetitive inhibition we have observed suggests that different binding sites may be present for each substrate. Herein may lie one principal difference between the two oxidative systems. This interpretation must be considered tentative, however, since an earlier report presented competitive inhibition for these two substrates (8). Triton-treated microsomes were used, however, whereas use of deoxycholate-treated microsomes is reported here. Treatment of the microsomes with different detergents may result in different spatial relationships between membrane-bound components of the oxidase system that could change the observed kinetic properties of the oxidase. A comparison of $K_{m}$ values indicates that the kinetic properties of the oxidase-catalyzing oxidative demethylation do change (Ref. 8 and Fig. 4). Thus, when Triton treated microsomes are used, $K_{m}$ is approximately 115 µM (Ref. 8). $K_{m}$ decreases to 33 µM, however, when deoxycholate-treated microsomes are used (Fig. 4).

Another difference between the two oxidative systems apparently occurs at the level where reducing equivalents are introduced since cytochrome c, known to inhibit oxidase systems by competing for reducing equivalents, inhibits each oxidase differently (Fig. 3). This difference is evident only when NADH is used since in the presence of NADPH, cytochrome c inhibits both oxidases similarly (Ref. 8 and Footnote 1). Reducing equivalents from NADPH are introduced to the oxidase of demethylation by a system which does not require cytochrome b (7). Participation of cytochrome b, in oxidative deformylation has not yet been investigated so our data cannot be interpreted in terms of known electron transport components. The data, however, for cytochrome c inhibition (Fig. 3) imply that in the presence of cytochrome c a rate-limiting step for introduction of reducing equivalents from NADH must be different for demethylation and deformylation. If it is assumed that the rate of demethylation and deformylation is dependent upon the rate of introduction of reducing equivalents, then the rate of demethylation as compared with the rate of deformylation is consistent with the possibility that there are two different rate-limiting components for introducing reducing equivalents. $V_{max}$ for demethylation is 2.22 nmol/mg/10 min (Fig. 4A). This is approximately 2-fold slower than the rate of deformylation in the presence of NADH (Table I). The deformylation rate is not a $V_{max}$ value and the difference between the two rates would presumably be much greater if $V_{max}$ values were compared. A comparison of demethylation and deformylation rates at equimolar substrate concentration (Fig. 4A and Table I) shows deformylation to be approximately 3-fold faster than demethylation. Since a greater rate of deformylation would mean a greater rate of utilization of reducing equivalents, the rate-limiting component for introducing reducing equivalents could be different for the two reactions. This conclusion, of course, is based on the assumption that other factors such as substrate availability are not causing the rate differences. This assumption should be considered with caution, however, since nothing is known about the rate of transfer of the substrate from the detergent suspension to the membrane-bound enzyme.

The observations we present here of two different oxidase systems, one for deformylation, the other for demethylation, are consistent with the diverse nature of oxidases which participate catalytically in the conversion of squalene to cholesterol. Thus, squalene epoxidase and methyl sterol oxidase do not require cytochrome P₄₅₀ for activity, whereas oxidative removal of the 14α-methyl group of lanosterol is believed to require cytochrome P₄₅₀ (12-14). Also, squalene epoxidase utilizes NADPH preferentially as a source of reducing equivalents; methyl sterol oxidase is proposed to use NADH preferentially (12, 14). Methyl sterol oxidase is inhibited by cyanide (1 mM); squalene epoxidase is not (12, 14).

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J. Biol. Chem. 1978, 253:3101-3105.