Structural requirements for substrate recognition of *Mycobacterium tuberculosis* 14α-demethylase: implications for sterol biosynthesis

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Abstract Sterol 14α-demethylase (14DM) is a cytochrome P-450 involved in sterol biosynthesis in eukaryotes. It was reported that *Mycobacterium smegmatis* also makes cholesterol and that cholesterol is essential to *Mycobacterium tuberculosis* (MT) infection, although the origin of the cholesterol is unknown. A protein product from MT having about 30% sequence identity with eukaryotic 14α-demethylases has been found to convert sterols to their 14-demethyl products indicating that a sterol pathway might exist in MT. To determine the optimal sterol structure recognized by MT 14DM, binding of 28 sterol and sterol-like (triterpenoids) molecules to the purified recombinant 14α-demethylase was examined. Like eukaryotic forms, a 3β-hydroxy group and a 14α-methyl group are essential for substrate acceptability by the bacterial 14α-demethylase. The high affinity binding of 31-norcoenzyme A without detectable activity indicates that the Δ8-bond is required for activity but not for binding. As for plant 14α-demethylases, 31-nor-sterols show a binding preference for MT 14DM. Similar to enzymes from mammals and yeast, a C24-alkyl group is not required for MT 14DM binding and activity, whereas it is for plant 14α-demethylases. Thus, substrate binding to MT 14DM seems to share common features with all eukaryotic 14α-demethylases, the MT form seemingly having the broadest substrate recognition of all forms of 14α-demethylase studied so far.—Bellamine, A., A. T. Mangla, A. L. Dennis, W. D. Nes, and M. R. Waterman. Structural requirements for substrate recognition of *Mycobacterium tuberculosis* 14α-demethylase: implications for sterol biosynthesis. *J. Lipid Res.* 2001, 42: 128–136.

Supplementary key words P-450 • type I binding spectrum • sterol analogs • substrate binding

Sterol 14α-demethylases (14DM) are P-450 monoxygenases involved in one of the key steps in sterol biosynthesis, the removal of 14α-methyl group in Δ8-sterols via three successive oxidation steps (1) (Fig. 1A). This function is considered the most conserved amongst the P-450 superfamily (2). In mammals, 14α-demethylase substrates are 24,25-dihydrolanosterol and lanosterol, which is also a yeast substrate, while fungi preferentially use 24-methyl-ene dihydrolanosterol. Plant forms, however, have a more strict substrate specificity and demethylate only obtusifoliol into its 14-demethyl product (3), although plant 14α-demethylases can bind lanosterol and 24-methylene dihydrolanosterol (4).

Sterol substrate requirements are reported to be common to all 14α-demethylases. Studies of maize and *Saccharomyces cerevisiae* show that the 3β-hydroxy group of sterols is critical for the 14α-demethylase activity, presumably by allowing a correct orientation of the 14α-methyl group to the heme center (5, 6). Thr-315 is thought to form a hydrogen bond with the 3β-hydroxy group of 24-methylene dihydrolanosterol, as suggested by a *Candida albicans* 14α-demethylase modeling study (7) and site-directed mutagenesis (8). The Δ8-bond in the lanosterol ring system is also important for substrate metabolism, probably by maintaining a pseudoplanar conformation of the ring system favorable for the enzyme-substrate interaction (5, 9, 10). The importance of the sterol side chain, however, seems to be different from one 14α-demethylase species to another. The structure and the length of the side chain as well as unsaturation at the C-24 alkyl group are important for yeast 14α-demethylase lanosterol metabolism (11, 12). Conversely, plant 14α-demethylase is able to catalyze demethylation of both obtusifoliol and dihydroobtusifoliol with similar efficiency, suggesting that plant forms are less sensitive to unsaturation at the C-24 alkyl group but require the presence of a methyl group at C-24 (5). However, because those studies were performed by measuring catalytic activities, it was not clear if those sterol requirements are also important for the binding.

We have shown that a gene cloned from *Mycobacterium tuberculosis* (MT) encodes a P-450 14α-demethylase (13).
The MT recombinant enzyme expressed in *Escherichia coli* is able to convert sterols to their 14-demethyl product; making 14α-demethylase the only P-450 (CYP) family member found in four phyla (animals, plants, fungi, and now bacteria). The MT 14DM was found to bind and preferentially metabolize obtusifoliol and 24,25-dihydrolanosterol; lanosterol being the poorest substrate despite similar binding as 24,25-dihydrolanosterol (13). Herein, we report a detailed analysis of substrate binding requirements for MT 14DM. Using type I binding spectra (14) (Fig. 1B), $K_v$ values reflecting substrate recognition were determined. Because of the availability of highly purified recombinant MT 14DM, these studies are the first to characterize in detail the $K_v$ values for a sterol biosynthetic enzyme. We have examined the binding of 28 sterols and sterol-like molecules. Results show that the bacterial 14-demethylase requires the presence of a 3β-hydroxy group. Decreased affinity of MT 14DM for $\Delta^2$- and $\Delta^7$- or $\Delta^5$-bond sterol analogs suggests that the particular conformation of the ring system provided by the $\Delta^5$-bond is strongly favored. 31-Nor-sterol derivatives show better binding for the MT 14DM than their 4,4-dimethyl analogs. However, alklylation at C-24 does not affect the binding. Thus, MT 14DM has requirements for substrate binding reported to be important for 14α-demethylase orthologs from all the phyla, suggesting that MT has the same origin as eukaryotic 14α-demethylases. Although no binding was detected for several 9β,19-cyclopropyl sterols such as cycloartenol, dihydrocycloartenol, and 24-methylene cycloartenol, the high affinity observed for the 31-norcycloartenol suggests that specific cyclopropyl analogs might be intermediates in the bacterial sterol biosynthetic pathway. However, 31-norcycloartenol is not metabolized by MT 14DM, indicating that the $\Delta^5$-bond is important not only for substrate recognition but also for the catalytic activity of 14α-demethylase, as previously suggested (9).

**MATERIALS AND METHODS**

**Enzymes and sterol sources**

MT 14DM was expressed in *E. coli* and purified by two passes on an Ni$^{2+}$-nitroloacetic acid affinity column as previously described (13). MT ferredoxin was prepared as previously described (13). Flavodoxin and flavodoxin reductase were kindly provided by C. Jenkins (15, 16). Spinach ferredoxin reductase was a generous gift from D. O’Keefe and tritiated 24,25-dihydrolanosterol (24,25-[2H$_2$]dihydrolanosterol) was a generous gift from J. Trzaskos. The 28 sterols and triterpenoids included in this study (boldface numbers), obtained from the Nes collection (17, 18), are summarized in Table 1. They are shown in Figs. 2–5.

**Type I binding spectra**

P-450 MT 14DM in 0.1 M Tris-HCl (pH 7.5), 0.1 mM EDTA, 20% glycerol, diluted to 10 μM in a 1-ml final volume of 20 mM morpholinopropanesulfonic acid (pH 7.5), 50 mM KCl, 5 mM MgCl$_2$, 10% glycerol was titrated with saturating amounts of sterols (15 to 140 μM depending on the sterol). Spectra were recorded between 350 and 450 nm, revealing changes at 390 and 420 nm characteristic of a P-450 type I binding spectrum (see Fig. 1B). Equal concentrations of P-450 were placed in one sample and one reference chamber of two tandem cuvettes and an equal volume of P-450-containing buffer was placed in the other chambers. The baseline was recorded with a double-beam spectrophotometer (AMINO DW-2 UV/VISIBLE; Spectronic Uni...
TABLE 1. $K_s$ values of sterols and triterpenoids used in this study

<table>
<thead>
<tr>
<th>Sterol</th>
<th>$K_s$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24,25-Dihydrolanosterol (1)</td>
<td>1 ± 0.5e</td>
</tr>
<tr>
<td>3-Keto-24,25-dihydrolanosterol (2)</td>
<td>n.d.</td>
</tr>
<tr>
<td>3-Acetoxy-24,25-dihydrolanosterol (3)</td>
<td>n.d.</td>
</tr>
<tr>
<td>3-Deoxy-24,25-dihydrolanosterol (4)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Obtusifoliol (5)</td>
<td>0.35 ± 0.15</td>
</tr>
<tr>
<td>Dihydroobtusifoliol (6)</td>
<td>1.9 ± 0.65</td>
</tr>
<tr>
<td>31-Norlanosterol (7)</td>
<td>2 and 2.5</td>
</tr>
<tr>
<td>24-Methylenecholphenol (8)</td>
<td>12.5 ± 5.5</td>
</tr>
<tr>
<td>4α-Methyl zymosterol (9)</td>
<td>n.d.</td>
</tr>
<tr>
<td>31-Norcycloartenol (10)</td>
<td>0.88 ± 0.38</td>
</tr>
<tr>
<td>Fusic acid (11)</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>Lanosterol (12)</td>
<td>1.5 ± 0.5e</td>
</tr>
<tr>
<td>Lanosta-7,24-dienol (13)</td>
<td>14 ± 6</td>
</tr>
<tr>
<td>Cycoartenol (14)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Zymosterol (15)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Desmosterol (16)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cholesterol (17)</td>
<td>n.d.</td>
</tr>
<tr>
<td>24,25-Dehydrodolastanol (18)</td>
<td>18.5 ± 14.5</td>
</tr>
<tr>
<td>Agnostosterol (19)</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>Parkel (20)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cucurbita-5,24-dienol (21)</td>
<td>n.d.</td>
</tr>
<tr>
<td>24-Methylene cycloartenol (22)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Dihydrocycloartenol (23)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Euphol (24)</td>
<td>23 ± 6</td>
</tr>
<tr>
<td>Ticralol (25)</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>24-Methylene dihydrolanosterol (26)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Diplopterol (27)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tetrahydromam (28)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$K_s$ values determined by type I binding spectra are the means of three or four experiments except for 3-deoxy-24,25-dihydrolanosterol (4), 31-norlanosterol (7), 4α-zymosterol (13), cholesterol (15), parkel (20), and tetrahydromam (28), for which $K_s$ measurements are duplicated. n.d., No detectable binding or such weak spectral change that $K_s$ cannot be determined. e $K_s$ measurement previously performed (15).

RESULTS

Role of the 3β-hydroxy group in MT 14DM substrate binding

To evaluate the role of the 3β-hydroxy group in substrate recognition, binding of three 24,25-dihydrolanosterol analogs (1, Fig. 2) was examined. Those having a ketone (2, Fig. 2) or acetoxy (3, Fig. 2) group at the 3-position carbon showed no binding. The 3-deoxy-24,25-dihydrolanosterol (4, Fig. 2) also failed to bind to MT 14DM. Yeast and maize 14α-demethylases are also unable to use either 3-methyl or the 3-acetoxy analogs as substrates (5, 6). The yeast form, however, is reported to metabolize 3-keto-24,25-dihydrolanosterol with low efficiency (6). Because those reports were based on the activity, it was not clear if the 3-hydroxy group facilitates substrate recognition or is also involved in the catalytic activity. This group is presumed to form a hydrogen bond with a threonine residue conserved in all forms of 14α-demethylase (8), leading to a correct positioning of the C-14 methyl over the heme center. A methyl or an acetoxy group at this position could result in steric hindrance preventing correct positioning. The low activity reported for the keto substrate with the yeast enzyme (6) might be the result of an impaired hydrogen bond formed between the carbonyl oxygen and the conserved threonine, allowing a low level of 14α-demethylase activity. Our results suggest that the hydroxy group at C-3 is important for MT 14DM substrate binding, which seems to be a common requirement for efficient substrate binding to all 14α-demethylases.

Effect of the 4β-methyl group on MT 14DM substrate binding

In general, the MT 14DM showed better binding with 31-nor-sterols (missing the 4β-methyl group at C-4) (Fig. 3A) such as obtusifoliol (5, Fig. 3A), dihydroobtusifoliol (6, Fig. 3A), and 31-norlanosterol (7, Fig. 3A), than with 4,4-dimethyl sterols (Fig. 3B). The 4β-methyl group seems to have an inhibitory effect on the binding because 31-norcycloartenol (10, Fig. 3A) binds despite the presence of the
cyclopropyl ring system (which seems to inhibit binding as described below). However, the presence of the 4β-methyl group does not preclude binding in the case of lanosterol ([12], Fig. 3B), which does not have the cyclopropyl ring. In the case of cycloartenol ([14], Fig. 3B), two inhibitory effects are summed (presence of 4β-methyl group and cyclopropyl ring system), which led to a loss of binding. 31-Norcycloartenol, however, binds with lower amplitude, indicating that the binding is not optimal although the affinity is the same as that of lanosterol ($K_0 = 0.88 \pm 0.38 \mu M$). However, the inhibitory effect of the 4β-methyl group on the binding is not seen when 31-norlanosterol ([12], Fig. 3B) is compared with lanosterol ([12], Fig. 3B). 24-Methylenelophenol ([8], Fig. 3A), which is the D7-analog of obtusifoliol but missing the C-14 methyl group, showed an altered type I binding spectrum, having a broad maximum and minimum shifted to longer wavelengths compared with a typical substrate binding spectrum. The estimated $K_0$ value is about one order of magnitude higher than that for obtusifoliol (12.5 ± 5.5 vs. 0.35 ± 0.15 µM) and similar to that of lanosta-7,24-dienol ([13], Fig. 3B), which has both the 4β- and the C-14 methyl groups and demonstrates a spectral alteration similar to that of 24-methylenelophenol. Absence of both of the methyl groups at C-4 (Fig. 3C) led to no detectable binding with zymosterol ([15], Fig. 3C), desmosterol ([16], Fig. 3C), and cholesterol ([17], Fig. 3C). 4α-Methyl zymosterol ([19], Fig. 3A), which is the 31-norlanosterol 14α-demethylated product, did not show any binding either. 24,25-Dehydropollinastanol ([18], Fig. 3C), which does not have a C-4 methyl group, showed some binding with a broad maximum (similar to that seen for 24-methylenelophenol and lanosta-7,24-dienol). The estimated $K_0$ is about 18.5 ± 14.5 µM. These results suggest that the 4α- and C-14 methyl groups are required for the spin shift of the heme. The difference in binding observed between 24-methylenelophenol ([8], Fig. 3A) and the other C-14 demethylated sterols (zymosterol, 4α-zymosterol, desmosterol, and cholesterol) can probably be explained by other effects such as the Δ7-bond and C-24 alkylation as discussed below. These results further emphasize the preference of MT 14DM for 31-nor-sterols ([13]), making this bacterial isozyme more plantlike rather than fungal/animal-like ([3], [5]). The inability of maize 14α-demethylase to metabolize 4,4-methyl group sterols such as lanosterol, 24,25-dihydrolanosterol, or 24-methylene dihydrolanosterol has led to

![Fig. 3. C-4 methyl group sterol isomers (highlighted in obtusifoliol (5)). A: C-4 monomethyl sterols. B: C-4 dimethyl sterols. C: C-4 demethyl sterols. $K_0$ = n.d., no detectable binding observed. *$K_0$ measurement previously performed ([13]).](image-url)
the assumption that the plant enzyme active site might contain a cleft accommodating only one C-4 methyl group (3, 5). Lanosterol and 24-methylene dihydrolanosterol have been reported to bind with a 14α-demethylase with apparent $K_s$ values of 2.2 and 2.5 μM, respectively, but with a lower amplitude of spectral shift than that of obtusifoliol. However, no metabolism of either can be detected (4). Although preferentially binding 31-nor-sterols, like the plant forms, MT 14DM showed a high affinity and activity for several 4,4-dimethyl substituents (13) (Fig. 3B), suggesting that this isoform has an active site structure intermediate between that of the plant and fungal/animal isoforms. Fusidic acid (11, Fig. 3A) is a 31-nor-sterol-like molecule having substitutions at C-8, C-11, C-13, C-16, and C-20 and its side chain “left-handed” compared with lanosterol (12, Fig. 3B). This compound showed an altered type I binding spectrum, with its maximum and minimum shifted to longer wavelengths compared with a typical binding spectrum. This spectral change can be titrated with increasing amounts of fusidic acid, demonstrating a binding constant of 28 ± 5 μM, approximately one order of magnitude higher than that of lanosterol (12, Fig. 3B). The alteration of the type I binding spectrum might be the result of the side-chain orientation and/or the absence of the C-13 methyl group in fusidic acid. It might also be the result of the additional carboxy group at C-20 and/or acetate group at C-16.

**Influence of the ring system structure in MT 14DM substrate binding**

To investigate the effect of the ring system conformation and the role of the $\Delta^5$-bond on substrate recognition, $\Delta^6$, $\Delta^7$, and $\Delta^8$-lanosterol analogs, as well as isoforms having the 9β,19-cyclopropane rings, were used (Fig. 4). MT 14DM showed a low affinity for $\Delta^7$-sterols such as lanosta-7,24-dienol (13, Fig. 3B), agnostosterol (19, Fig. 4), and 24-methyleneophenol (8, Fig. 3A) with estimated $K_s$ values of 0.88 ± 0.38 and 18.5 ± 14.5 μM, respectively. Cycloartenol (14, Fig. 3B), 24-methylene-cycloartenol (22, Fig. 4), and dihydrocycloartenol (23, Fig. 4), which are plant sterol intermediates (20, 21), showed slight spectral changes that did not lead to determination of $K_s$ values. The lower affinity observed for the 9β,19-cyclopropyl analogs compared with the $\Delta^5$-sterols (compare lanosterol with cycloartenol, 24,25-dihydrosterol with dihydrocycloartenol, and 24-methylene dihydrosterol with 24-methylene cycloartenol) is probably due to the presence of the 9β,19-cyclopropane ring (22). The high affinity observed for the 31-norcycloartenol can be attributed to the absence of the 4β-methyl group. This compound, however, binds with lower amplitude, as mentioned above, indicating that the binding is probably not optimal although the affinity is the same as for lanosterol. The 9β,19-cyclopropane ring seems to inhibit binding while the absence of the 4β-methyl group seems to favor it. Thus, one possible explanation for the difference seen between cycloartenol and 31-norcycloartenol could be the combination of the two effects. The binding observed with dehydropollinastanol is approximately an order of magnitude weaker than that of lanosterol and 31-norcycloartenol and might be explained by a combination of the 9β,19-cyclopropane ring effect and the absence of the two methyl groups at C-4. These differences might lead to a change in the overall environment of the protein, possibly because of a freer movement of the sterol molecule in the active site but less affinity for the enzyme because of the critical role of the 4α-methyl group in such an interaction (see above). This explanation is supported by the altered type I spectrum observed with dehydropollinastanol, possibly revealing an impaired enzyme-sterol interaction (not shown). Maize 14α-demethylase is unable to metabolize cycloeucalenol, a 9β,19-cyclopropyl analog of obtusifoliol (5).

**Side-chain structure requirement for MT 14DM substrate binding**

Although having similar $K_s$ values, 24,25-dihydrosterol (1, Fig. 2) was found to be a better substrate than...
lanosterol (12, Fig. 3B) (13), as previously shown for human 14DM (3), suggesting that the double bond between C-24 and C-25 might affect MT 14DM activity. Euphol (24, Fig. 5) and tirucallol (25, Fig. 5) are two lanosterol isomers in their side chains. Both have the hydrogen atom on C-20 in the same plane as C-18. The C-22 is cis-oriented (“left-handed”) to C-13 in euphol and trans-oriented to it (“right-handed”) in tirucallol (23). Euphol showed an altered type I spectrum, having a broad maximum and with a smaller amplitude than in the case of a typical substrate (Fig. 5). The $K_s$ value is $23 \pm 6 \mu M$, approximately one order of magnitude higher than for lanosterol and similar to that of fusidic acid (11, Fig. 3A, $28 \pm 5 \mu M$), which also has its side chain in the same orientation as euphol. Tirucallol, however, did not show any binding. These results suggest that the MT 14DM active site can preferentially accommodate side chains having the same orientation as that of lanosterol. The difference observed between euphol and tirucallol can be explained by greater steric hindrance of the tirucallol side chain than that of euphol, which would fit better in the active site. Sterols having a methylene group at C-24, such as 24-methylene dihydrolanosterol (26, Fig. 5), obtusifoliol (5, Fig. 3A), and dihydroobtusifoliol (6, Fig. 3A), bind to the enzyme with $K_s$ values of the same order of magnitude as for lanosterol while 24-methylene dihydrolanosterol has a slightly lower affinity. Obtusifoliol and dihydroobtusifoliol did not show much difference in their binding constants, suggesting that chiral substitution at C-24 is not required for MT 14DM binding. The high $K_s$ value observed for 24-methyleneenolophenol (8, Fig. 3A) might be the result of the presence of a $\Delta^7$-bond and/or absence of a C-14 methyl group. Yeast 14a-demethylase metabolizes sterols with or without an alkyl group at C-24 (24-methylene dihydrolanosterol, lanosterol, 24,25-dihydrolanosterol, and obtusifoliol) but fails to use 24-dihydroobtusifoliol as a substrate (12). Rat 14a-demethylase was reported to metabolize both C-24-alkylated (24-methylene dihydrolanosterol) and nonalkylated (lanosterol and 24,25-dihydrolanosterol) sterols (10). Absence of the C-28 methyl group in 31-norlanosterol, however, precludes plant 14a-demethylase activity, suggesting that the plant form probably has a specific apolar binding site for this group in the side chain (5). In this respect, MT 14DM seems to be more fungal/mammal-like than plantlike. Diplopterol (27, Fig. 5) and tetrahymanol (28, Fig. 5) are a hopanoid and hopanoid isomer, respectively, playing the same role as sterols as membrane inserts in lower eukaryotes and some bacteria (24). These sterol-like molecules have their side chain cycled into a fifth ring and show no binding spectrum (a slight spectral change was observed for tetrahymanol), probably because of the absence of the $\Delta^8$-bond or a smaller space occupied by hopanoids in the active site compared with sterols. Although more representatives of this class of molecules need to be studied in order to clarify the possible role of MT 14DM in hopanoid biosynthesis, our results suggest that MT 14DM is involved in a sterol biosynthetic pathway. Sterol biosynthesis is suggested by the discovery of C$_{28}$–C$_{40}$ steranes in archean fossils 2,700 million years old, believed to be cyanobacterial in type (25). Mycobacterium smegmatis, a mycobacterium closely related to MT, can synthesize cholesterol from radiolabeled mevalonic acid (26), although the deoxyxylulose 5-phosphate pathway, an alternative mevalonate-independent pathway, was found in some bacteria and yeast (27–30). It is possible that in mycobacteria, as is the case in plants and other bacteria (27–29), both pathways coexist.

**Catalytic activity**

From these binding studies, it appears that sterols missing the 4β-methyl group show higher affinity for MT 14DM. To investigate the effect of this group on activity, 24,25-dihydrolanosterol (1, Fig. 2) and obtusifoliol (5, Fig. 3A) were selected for a time course analysis. Both were previously reported to be MT 14DM substrates (13) and are different in their 4β-methyl group in addition to an alkyl group at C-24 in obtusifoliol, which does not seem to affect the binding [compare obtusifoliol with 31-norlanosterol (7, Fig. 3A)]. The 14a-demethylation rate is 0.18 and 0.14 nmol/min/nmol of P-450 for obtusifoliol and 0.13 and 0.074 nmol/min/nmol of P-450 for 24,25-dihydrolanosterol (values from two different experiments). These values are similar, suggesting that the 4β-methyl group does not affect the rate of the 14a-demethylation reaction. The values are close to the $V_{max}$ reported for human and fungal 14a-demethylase and are approximately one order of magnitude lower than those for the plant isoform (3, 5). It is important to remember, however, that the endogenous reductase system is not known for MT 14DM, while P-450 reductase serves this role for all eukaryotic isoforms. Thus, activities of all experiments using MT 14DM and flavodoxin/flavodoxin reductase probably underestimate in vivo activities. Because 31-norcycloartenol showed a high affinity for MT 14DM, we decided to examine whether this 9β,19-cyclopropyl sterol was actually a substrate. After an overnight incubation, no metabolism was seen with 31-norcycloartenol, whereas obtusifoliol was fully metabolized by MT 14DM under the same conditions. The present result demonstrates that the $\Delta^8$-bond is important for catalytic activity in addition to its effect on binding by maintaining a pseudoplanar conformation of
the ring system. Our results, as well as others (9), suggest that the presence of a double bond in the ring system near the C-14 methyl group is a general requirement for sterol 14α-demethylase activity. If we estimate a relative catalytic efficiency as the catalytic rate versus $K_a$ values, this value would be 0.45 for obtusifoliol and 0.068 for 24,25-dihydrolanosterol, indicating that obtusifoliol is the best-known substrate for MT 14DM. Because the reductase for MT 14DM is still unknown and because of the paucity of other sterol analogs, we were unable to determine the relative catalytic efficiency for each of the sterol and sterol-like analogs. Further studies are needed to determine the best substrate for MT 14DM among all the molecules presented in this work.

**DISCUSSION**

On the basis of catalytic activities, it has been suggested that the 14α-demethylase preferentially holds an 8-lanostene conformation of sterols in the active site mainly by hydrophobic contact with the β-surface of the sterol molecule (9) with residues in the protein (31). Thus, the side-chain orientation and its structure may play an important role in this hydrophobic interaction. Such a framework might require participation of C-10 and C-13 sterol methyl groups in hydrophobic interactions. The presumed hydrogen bond between the 3β-hydroxy and the conserved threonine residue in the protein might orient the substrate in the right direction for 14α-demethylation activity (8, 9). Effects of the 4β-methyl group and the side chain seem to be different from one 14α-demethylase species to another. From this study, it appears that the soluble MT 14DM fits in the general hypothetical model of 14α-demethylases where a pseudoplanar conformation of the sterol ring system is required and where the substrate interacts with the enzyme by its 3β-hydroxy group and its side chain facilitating the substrate binding. Although molecules presented in Fig. 2 show clearly that the 3β-hydroxy group is required for binding, other sterols having this group but differing in more than one feature (9, 14, 15, 16, and 17 of Fig. 3; 20, 21, 22, and 23 of Fig. 4; and 25 and 28 of Fig. 5) do not bind. In general and for a given pair of sterols, the binding seems to result from the combination of several effects. It appears from this study that the molecular features of sterols shown to be important for the catalytic activity of eukaryotic 14α-demethylases are rather important for substrate recognition, the first step of catalysis. The 4β-methyl group was found to have, in general, an inhibitory effect on the enzyme-sterol interaction but does not seem to affect the MT 14DM activity, while the 4α-methyl group is required for such interaction as shown for plant 14α-demethylase activity. The presence of the C-24 alkyl group, however, seems to be less critical for MT 14DM binding and activity.

Clearly MT 14DM is a sterol 14α-demethylase having similar substrate structural requirements as eukaryotic 14DM. The function of this enzyme in MT, however, is still an open question. It is possible that this enzyme, which is expressed in MT (13), is involved in a sterol biosynthetic pathway, as is the case for eukaryotic isoforms. Indeed, other enzymes in the *S. cerevisiae* sterol pathway show significant sequence homologies to MT gene products: Rv3823 (50% to HMG-CoA reductase), Rv1745 (46.9% to isopentenyl pyrophosphate isomerase), Rv3383c (39.1% to far-nesyI pyrophosphate synthase), Rv3397 (39.1% to squalene synthase), and Rv1814 (24% to sterol C5-desaturase) (26, 32). The existence of a functional sterol pathway was shown in *M. smegmatis*, a bacterium closely related to MT (26). Why would mycobacteria make sterols, when most bacteria do not? One report shows that cholesterol is essential for the entry of MT into macrophages during phagocytosis, an essential step during the tuberculosis infection (33). Assuming that MT synthesizes sterols, our results can be extrapolated to a more general picture for the MT sterol biosynthetic pathway. It was reported that maize 14α-demethylase is unable to metabolize cycloeucalenol, the 24-methyl isomer of 31-norcycloartenol (5). Cycloartenol, however, is the best substrate for sunflower sterol methyl transferase (SMT), another enzyme involved in sterol biosynthesis in plants and fungi (34). Knowing that the 9β,19-cyclopropyl sterols are obtusifoliol precursors in higher plants (20, 21), the SMT activity should occur before the 14α-demethylation in the plant sterol biosynthesis sequence. The plant 14α-demethylase preference for 31-nor-sterols suggests that the 4β-demethylation might occur before 14α-demethylation and SMT activity (34). Conversely, yeast *S. cerevisiae* SMT shows a preference for zymosterol, suggesting that this activity probably takes place after 14α-demethylation and 4-demethylation in the ergosterol biosynthesis (18). On the basis of our demonstration that MT 14DM prefers 31-nor-sterols, it can be predicted that 4β-demethylation takes place before 14α-demethylation in mycobacteria. The 31-norcycloartenol is not an MT 14DM substrate, but its tight binding would suggest the presence of 9β,19-cyclopropyl sterols as intermediates in the MT sterol biosynthetic pathway, as in higher plants (20, 21). In mammals and fungi, and with the exception of an *S. cerevisiae* mutant strain, that metabolizes radiolabeled cycloartenol with low efficiency (35), no evidence of cyclopropyl sterol intermediates has been reported; lanosterol and 24,25-dihydrolanosterol are the 14α-demethylase substrates and are derived directly from squalene cyclization (36). The cycloartenol pathway occurs only in photosynthetic organisms and can be used as a marker to identify the phyligenic origin of some organisms (37). Taken together, this would suggest that the sterol biosynthetic pathway in MT can use lanosterol as well as cycloartenol as intermediates and leads to the assumption that the MT pathway shares a common ancestor with eukaryotic sterol pathways. MT 14DM, however, is unable to bind or metabolize 9β,19-cyclopropyl isoforms, suggesting that 14α-demethylation might take place before Δ8-cyclopropyl isomerization and after 4β-demethylation.

Fusidic acid initially isolated from the fungus *Fusidium coccineum* is used therapeutically as an antimicrobial agent against gram-positive bacteria (38). This compound was previously reported to be effective against *Mycobacterium*...
leprae infection, but shows only low activity against MT, and therefore is believed to be of no clinical importance in the treatment of tuberculosis (39). A more recent report, however, showed that fusidic acid has rather low MIC values (8 to 32 mg/l) for some M. tuberculosis and Mycobacterium bovis strains (40). It was reported that fusidic acid inhibits protein synthesis (41) by interfering with the “G” elongation factor involved in translocation of rRNAs and mRNA on the ribosome (38). Because this compound binds to MT 14DM, perhaps MT sterol biosynthesis might also be a target for fusidic acid.

In conclusion, this study clearly shows that MT 14DM has a broad sterol binding capacity because triterpenoids such as euphol as well as fusidic acid bind to the enzyme. We also found that the Δ8-bond is not a requirement for sterol binding, but it is for the 14α-demethylation activity. Finally, knowing the structure of sterols required to bind MT 14DM with high affinity, but that are not demethylated, as is the case for 31-norcycloartenol, might be of assistance in drug design for treatment of tuberculosis. Although there is no evidence implicating sterols in the viability of MT, or proof of a functional sterol pathway, it has been shown that cholesterol plays an essential role in MT infection (33), raising the possibility that this P-450 and its MT sterol pathway are associated with tuberculosis infection.

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REFERENCES


