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Review

Sterol 14α-demethylase, an abundant and essential mixed-function oxidase

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Abstract

Sterol 14α-demethylase (CYP51) is the most widely distributed of all members of the cytochrome P450 gene superfamily and the only CYP family found in both prokaryotes and eukaryotes. It is well known as a drug target for microbial pathogenic infections. Studies of CYP51 gene regulation have been carried out primarily in animals because its regulation is similar to those of other genes involved in the cholesterol biosynthetic pathway. The function of CYP51 has been studied widely throughout biology including in animals, plants, yeast/fungi, protozoa, and bacteria. The structure has been determined by X-ray crystallography for the soluble prokaryotic form of CYP51 from *Mycobacterium tuberculosis*. Together these studies provide the most detailed understanding of any single cytochrome P450 and this minireview summarizes this information.

Keywords: CYP51; Sterol 14α-demethylase; Sterol biosynthesis; Gene regulation; Structure–function relationships

For "P450 junkies" like us, the discovery of cytochrome P450 (CYP) genes through recent genomic analysis (more than 5000 today) has been at first glance truly exhilarating. These genes open the door to remarkable and diverse biological functions. Subsequent thinking about this enormous amount of data, however, leads to the daunting challenge of trying to establish the function of each P450. In this effort, a particularly important feature of CYP genes is their sequence identity with other CYP genes which have established function. At the very minimum this relationship can point in an initial direction for studies of substrate specificity and ultimately function. One CYP gene is quite unusual in this respect because its function is easily predicted by sequence in genomes from bacteria, protozoa, yeast/fungi, plants, and animals. This is CYP51, the gene encoding sterol 14α-demethylase, a P450 required for sterol biosynthesis in the above organisms and the most widely distributed cytochrome P450 gene family in biology.

Following cyclization, the sterol biosynthetic pathways involve several steps leading to the essential sterols in different organisms. For example, in animals the 30 carbon lanosterol is converted to the 27 carbon cholesterol via nine steps of this post-squalene portion of the biosynthetic pathway. CYP51 catalyzes a rate-limiting reaction following the cyclization of squalene to form lanosterol or cycloartenol in sterol biosynthesis [1]. This is a three-step reaction whereby the 14α-methyl group of the substrate is converted, first to an alcohol, then to an aldehyde, and finally is removed as formic acid (Fig. 1). Each step requires one molecule of O_2 and one of reduced pyridine nucleotide and this enzymatic activity has been shown to be essential in yeast and fungi, and almost certainly in animals, plants, and protozoa as well [2]. This minireview addresses the general features of three important aspects of CYP51, its gene regulation, activity, and structure.

CYP51 gene regulation

Regulation of CYP51 transcription has been studied extensively in animals. Being cholesterologenic, CYP51 is a housekeeping gene expressed in virtually all animal cells because they all synthesize cholesterol [3]. The human genome contains three CYP51-related genes, one each on

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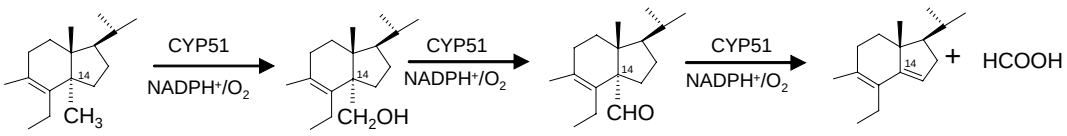

Fig. 1. The three-step 14α-demethylation reaction catalyzed by CYP51. Note that upon removal of the 14α-methyl group a 7/8 trouble bond results. Elucidation of this reaction involved work from several laboratories, a seminal citation being [26].

chromosomes 3, 7, and 13 [4]. The gene on chromosome 7 is the functional one while those on chromosomes 3 and 13 are processed pseudogenes which arise from reverse transcription of mRNA in germ cells and illegitimate recombination into the genome. While this gene is expressed in all cells, it is most highly expressed in the liver [3] which is the major site of cholesterol production. Another site of readily detectable CYP51 expression is male germ cells.

Like other cholesterologenic genes CYP51 responds to cholesterol feedback regulation, being upregulated in sterol limiting conditions and downregulated in cholesterol rich conditions [3,5]. This regulation involves the sterol regulatory elements (SRE) in the promoter of the gene which bind sterol regulatory element binding protein (SREBP) [6]. SREBP and its regulatory proteins are found in the endoplasmic reticulum and require proteolysis to be released from the membrane to find its way into the nucleus where it can upregulate transcription of cholesterologenic genes [7]. The CYP51 promoter also contains a cAMP regulatory element (CRE) known to bind cAMP regulatory proteins CREB/CREM [8]. The CYP51 response to cAMP has been observed to be independent of lipid composition and to be an immediate early gene response [D. Rozman, personal communication]. Studies from the Rozman laboratory have proposed that the CYP51 CRE plays a role in cross-talk of cholesterol biosynthesis with other important biological processes including inflammation and circadian responses. The details of transcriptional regulation of CP51 are being revealed in animal cells, but little is known about this process in other organisms such as plants, yeast/fungi, protozoa, and bacteria. While CYP51 enzymatic activity is shared by all these organisms, it is not at all clear what similarities exist in transcriptional regulation of this gene among different phyla.

The expression of CYP51 in male germ cells has proven to be quite interesting. Northern analysis of testis RNA clearly shows a strong pattern of CYP51 expression [9]. However, when examined more carefully, it is found that elevated expression is dependent on the developmental stage of the germ cells, being most abundant in round and elongated spermatids [10]. This emphasizes that while CYP51 is modestly expressed in Leydig cells in the testis, it is most abundant in this organ in sperm. CYP51 protein is present in the acrosomal membrane of elongated spermatids and mature sperm [11] raising the interesting question of whether CYP51 has an important role in fertilization. This is particularly intriguing because two intermediates in the cholesterol biosynthetic pathway have been reported to accumulate in gonads, 4,4-dimethylcholesta-8(9),14,24-dien-3β-ol (also known as FF-MAS) and 4,4-dimethylcholesta-8/9)24-dien-3β-ol (also known as T-MAS) [12]. FF-MAS was identified in the follicular fluid (FF) of the ovary and is also found in testis. T-MAS is found to accumulate in the testis. They are known to activate meiosis of oocytes (MAS-meiosis activating substance). The accumulation of these meiosis activating sterols in the gonads suggests that they have a function beyond being intermediates in sterol biosynthesis. Therefore, it will not be surprising if CYP51 is found to play a key role in fertilization by producing intermediates that could serve as ligands for nuclear receptors.

CYP51 activity

Fig. 1 illustrates the general CYP51 activity and Fig. 2 shows the four endogenous substrates known for this enzyme. It is seen that these molecules are very closely related with subtle differences in the sterol side chain and the presence of one or two methyl groups at C4. Currently, there are 64 complete CYP51 sequences known in biology and a majority of these will demethylate all 4 substrates although different CYP51s demonstrate different patterns of substrate preference among these four. Plant CYP51s appear to exclusively demethylate obtusifoliol (a single methyl at C4) [13]. Recently, it has been found that CYP51 from the protozoan *Trypanosoma brucei* also exclusively demethylates obtusifoliol but this is not a general pattern in protozoa [14]. In animals the sterol pathway leads to cholesterol, and 24,25-dihydrolanosterol and lanosterol are the favored substrates, in yeast/fungi ergosterol is the product of this pathway, and 24-methylenedihydrolanosterol and lanosterol are the substrates. Obtusifoliol in plants leads to the biosynthesis of phytosterols. In most cases in bacteria, the function of CYP51 is unknown. For example, the first bacterial CYP51 was reported in 1998 when the genome of *Mycobacterium tuberculosis* was solved [15,16]. However, *M. tuberculosis* does not contain a complete sterol biosynthetic pathway and consequently the function of this CYP51 remains unknown. The one case where bacterial CYP51 function is clear is in *Methylococcus capsulatis* where multiple intracellular membranes are present and CYP51 participates in biosynthesis of cholesterol, a component of these membranes [17].

Certainly a major function of sterol biosynthesis in microbes is to produce membrane lipids. In more complex organisms like plants and animals, CYP51 participates not only in sterol biosynthesis but also in the synthesis of

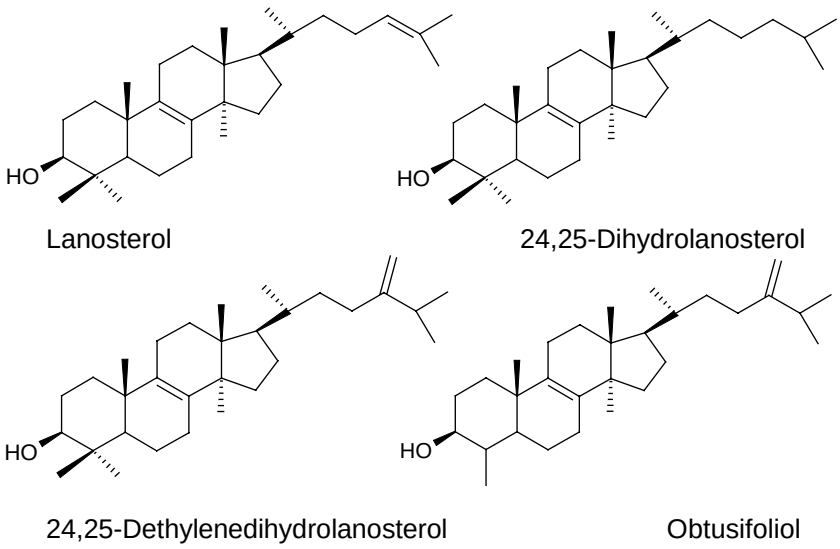

Fig. 2. The four known endogenous substrates for CYP51. As noted in the text, different forms of CYP51 have different substrate specificities.

intermediates like FF-MAS which serve as ligands for nuclear receptors. Also, in these organisms the sterols serve as precursors of important biological compounds like steroid hormones, vitamin D, and regulators of growth and development. As with other members of the P450 superfamily, eukaryotic forms of CYP51 are membrane bound and prokaryotic forms are soluble. Virtually all eukaryotic forms are functional in the endoplasmic reticulum, their activity being supported by NADPH cytochrome P450 reductase (CPR). It may well be that CYP51 activity can be supported by another reductase as well, perhaps involving cytochrome b_5. This idea arises from study of the CPR knockout in mice [18], where it is lethal, but not until about ten days of gestation. For a fertilized egg to develop into a blastocyst of approximately 100 cells, ready for implantation, it seems obvious that cholesterol biosynthesis must occur. Therefore, some reductase system other than CPR must exist, which can support CYP51 activity in the CPR knockout embryo.

Lanosterol is a very nonpolar molecule, even more so than cholesterol. Consequently, it is expected that the substrate for eukaryotic CYP51 enters the active site through the membrane. Details of CYP51 structure will be described in the next section. However, it is reasonable to imagine that CYP51s from protozoa to human function in a very conserved manner. Substrate enters the active site through the ER membrane and demethylation activity is supported by membrane bound CPR. The reductase for most prokaryotic forms of CYP51 is not known. In some cases,it is anticipated to involve a ferredoxin/ferredoxin reductase system because no FMN-binding protein is evident in many bacterial genomes. In *M. capsulatis* CYP51 the reductase is clearly a ferredoxin because this P450 is a fusion protein with a 3Fe–4S ferredoxin [17].

In the future, additional CYP51 genes will be identified through genomic studies, both in prokaryotes and eukaryotes. Two extensively studied phyla, insects and nematodes, are not found to contain a CYP51 gene. Cholesterol in these organisms comes solely from the diet. The most intriguing CYP51 to be discovered through genomic analysis is that in Mimivirus, the largest known virus, which is found in *Acanthamoeba polphaga* growing in the water of a cooling tower of a hospital in England [19]. The function of this unusual example of CYP51 (based only on sequence and not activity) is completely unknown.

CYP51 structure

After Tom Poulos reported the high resolution X-ray structure of $P450_{cam}$ [20] many investigators in the P450 field began to think about structural analysis of their favorite P450. The initial P450 structures were of soluble enzymes from bacteria and the first structure of a membrane P450 did not appear until 2003 [21]. Therefore for many years,structural information on CYP51 was not available. It was not known that there were soluble forms of CYP51 until the genomic structure of *M. tuberculosis* (MT) appeared in 1998. This genome was a revelation to the P450 field because it contained 20 CYP genes [15], 19 of these being members of novel gene families at that time. The 20th resembled sequences of eukaryotic forms of CYP51,except that it did not contain a signal anchor sequence showing that it was a soluble protein. Our laboratory was carrying out studies on mammalian forms of CYP51 and we cloned the MT form and after expression in *Escherichia coli* demonstrated that it catalyzed CYP51 activity [16]. We were subsequently able to solve the high resolution X-ray structure of MT CYP51 [22]. The structure shows the traditional P450-fold but contains two very unique features compared to those of other P450 structures (Fig. 3). First, the I-helix which is the longest helix in P450 molecules is disrupted into two pieces, the N-terminal piece pulling away from the center of the molecule. The second novel feature is the position of the BC loop generating an

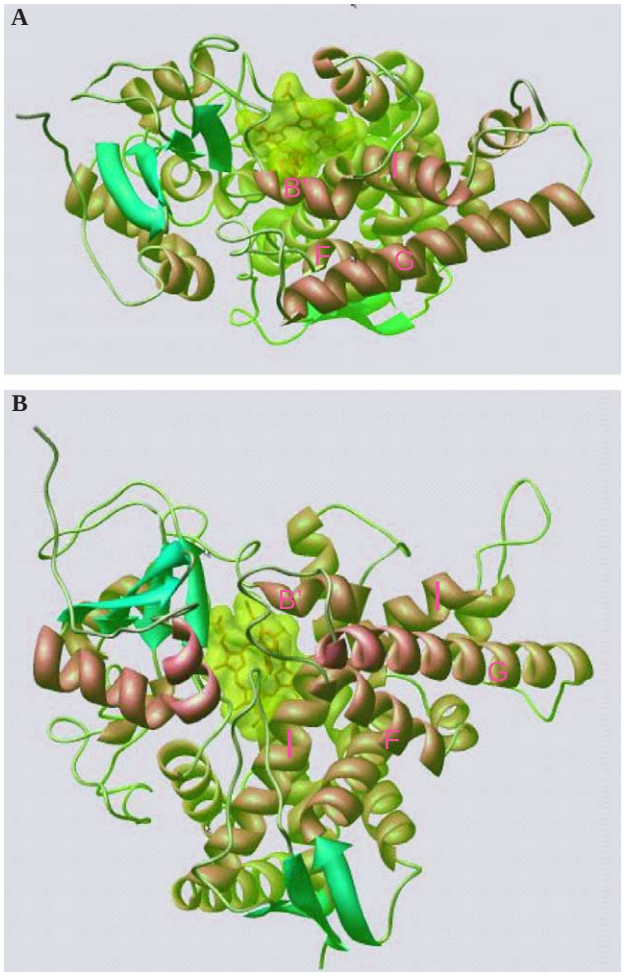

Fig. 3. Schematic representation of the MTCYP51 [1e9x] structure. (A) Upper view. (B) Distal view. The color of the helices changes from yellow-green to purple to represent regions distant and closer to the viewer, respectively. β-sheets are colored light-green. The heme is shown as a backbone surrounded by half-transparent surface. Helices B′, F, G, and I are marked. The figure was prepared in Chimera (http://www.cgl.ucsf.edu/chimera).

open substrate access channel at the top of the molecule. This access channel is the most open of any observed in P450s to date.

The question arises as to whether the structure of MT CYP51 is a good model for eukaryotic forms of this mixed function oxidase. Primary sequence alignment of a large number of CYP51s shows that only approximately 35 amino acids are conserved from the human enzyme to bacterial forms. These amino acids are predicted to represent the minimal requirement for a sterol 14α-demethylase and the conservation of these residues in bacterial and human CYP51s would suggest that the MT CYP51 structure is a good model for eukaryotic forms. However, because eukaryotic forms of CYP51 are membrane bound, structural differences related to cellular location exist between them and the soluble forms. Comparison of conserved residues between MT and human forms of CYP51 by site-directed mutagenesis suggests that in many cases the model is a good one but not in all cases [23]. Further mutagenesis studies will be necessary to better understand the structural relationships between CYP51 in different phyla.

Efforts to crystallize MT CYP51 as a complex with lanosterol have failed, presumably because of the very non-polar nature of this substrate. However, the much more polar sterol, estriol, binds to CYP51 and it has been possible to crystallize this complex although the entry to the heme is less well defined in this structure [24]. Our experimental results from MT CYP51 mutagenesis have shown that the conserved residues in the BC loop and helices F and G (Fig. 3), which are exposed into the substrate binding cavity of MT CYP51 in the structure but located at a distance from the heme, are essential for substrate binding. Substitution of each of them inactivates the enzyme [23]. Therefore, we believe that CYP51 undergoes the conformational change that several P450s do, leading to closure of the entry to the active site upon substrate binding.

It can be expected that several additional CYP51 genes will be discovered in the future by genomic analysis which will lead to further information on the minimal amino acid requirements for a sterol 14α-demethylase. Also, the structure of one or more of the eukaryotic forms of CYP51 will be elucidated and through such studies the conserved structural details of soluble and membrane bound forms of CYP51 will become clear. Combination of this information with studies using site-directed mutagenesis will permit detailed understanding of the structural requirements for sterol 14α-demethylation.

Conclusion

Sterol 14α-demethylase is not the only P450-dependent activity to be present in all biological kingdoms. For example, P450-dependent fatty acid hydroxylation is also found in all phyla. However, the difference between sterol 14α-demethylation and other P450-dependent reactions across phyla is that CYP51 genes are recognizable according to their primary amino acid sequence. Generally speaking, P450s showing about 40% amino acid sequence identity are classified as members of the same gene family [25]. However, while CYP51is highly conserved within the same phylum, the conservation between phyla can be as low as 22%. Nevertheless because of the conserved group of about 35 amino acids [23], genes containing them are readily classified into the CYP51 family. Fatty acid hydroxylases, even looking just at ω-hydroxylases, are grouped into different gene families in different phyla. To date, no amino acids are known to be conserved among all the fatty acid hydroxylases, except, of course, for those conserved in all P450s. So it is an interesting question why amino acid conservation has been maintained in CYP51 over hundreds of millions of years and not in fatty acid hydroxylases.

One possible explanation is that sterol 14α-demethylation is a three-step regio-and stereospecific reaction as seen in Fig. 1. Perhaps, this reaction requires conservation of

amino acids where fatty acid hydroxylation which involves only a single step rather than three does not. Certainly there is much more to be learned about the structure/function relationship in CYP51.

In addition to structure/function, intriguing questions on CYP51 physiological function remain to be answered. What exactly is the role of this enzyme in postmeiotic male germ cells? Why is this gene found in *M. tuberculosis* which does not have a sterol biosynthetic pathway?

Clearly CYP51 is a target for biochemical, biophysical, and physiological studies for years to come. But perhaps most importantly, it is a drug target for treatment of infection by many pathogenic microbes and the detailed studies of this enzyme will lead to design of new drugs which will prove more efficient in killing the pathogens while at the same time being less effective or ideally, ineffective, in initiating drug resistance. The general area of CYP51 research continues to expand and to bring new insights into research on both P450 and sterol biosynthesis.

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