Cloning, Heterologous Expression, and Enzymological Characterization of Human Squalene Monooxygenase

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The cDNA for human squalene monooxygenase, a key enzyme in the committed pathway for cholesterol biosynthesis, was amplified from a human liver cDNA library and cloned, and the protein was expressed in Escherichia coli and purified. Kinetic analysis of the purified enzyme revealed an apparent $K_m$ for squalene of 7.7 $\mu$M and an apparent $k_{cat}$ of 1.1 min$^{-1}$. For FAD the apparent $K_m$ is 0.3 $\mu$M, consistent with a loosely bound flavin. The apparent $K_m$ for NADPH–cytochrome P450 reductase, the requisite electron transfer partner, is 14 nM. The amount of reductase needed for maximal activity is about threefold less than the amount of squalene monooxygenase present in the assay; thus, electron transfer to the monooxygenase is not likely to be rate limiting. Previous reports have implicated inhibition of this enzyme as the cause of a peripheral demyelination seen in weanling rats fed a diet containing tellurium. As no data were available for humans, the ability of a number of tellurium and related elemental compounds to inhibit the recombinant human enzyme was examined. Tellurite, tellurium dioxide, selenite, and selenium dioxide were inhibitory; the tellurium compounds were more potent than the selenium compounds, as indicated by their IC$_{50}$ values (17 and 37 $\mu$M, respectively). Kinetic analysis of the inhibition by tellurite suggests multiple sites of interaction with the enzyme in a noncompetitive manner with respect to squalene.

Elevated serum cholesterol is a major factor in the development of cardiovascular diseases such as coronary heart disease and stroke. An important intervention in the progression of cardiovascular disease is the inhibition of endogenous cholesterol synthesis at HMG-CoA reductase, an early step in the cholesterol biosynthetic pathway. However, inhibition at this early step in the pathway can alter isoprene synthesis, and therefore, isoprene-dependent pathways such as the polyprenylation of cellular proteins involved in development, cell regulation, and mitochondrial function (e.g., Ref. 1). As a result, interest has shifted to the later stages of the pathway, particularly those beyond farnesyl pyrophosphate synthesis, as sites for pharmacotherapeutic intervention. Squalene monooxygenase catalyzes the second step in this “committed pathway” for cholesterol biosynthesis, immediately following squalene synthase and immediately before lanosterol synthase (2,3-oxidosqualene cyclase).

Squalene monooxygenase (EC 1.14.99.7; earlier called squalene epoxidase) is a 64-kDa flavin adenine dinucleotide (FAD)-containing enzyme bound to the endoplasmic reticulum of eukaryotic cells. The enzyme catalyzes the epoxidation of squalene across a C–C double bond to yield 2,3-oxidosqualene in a reaction more typical of cytochrome P450-type oxidations. Squalene monooxygenase, however, does not contain a heme group and is not inhibited by typical inhibitors of the cytochromes P450 such as carbon monoxide and metyrapone (2). However, like the cytochromes P450, this flavoprotein monooxygenase is dependent upon NADPH–cytochrome P450 reductase for reducing equivalents. Although squalene monooxygenase was recognized in the early 1970s as a key enzyme in cholesterol biosynthesis, it has been difficult to purify and characterize, and only recently was cloned from the rat (3). Little is known regarding its enzymological characteristics, and studies on the human enzyme have been limited to those carried out with subcellular prep-
arations from HepG2 cells (4). It is clear that squalene monoxygenase plays an important role in the overall regulation of cholesterol biosynthesis: Addition of cholesterol to cells in culture lowers squalene monoxygenase mRNA levels, suppresses squalene monoxygenase activity, and results in the accumulation of squalene (5). Squalene monoxygenase is directly inhibited by one or more chemical species of tellurium, leading to a transient, peripheral demyelination in weanling rats as a result of inhibition of cholesterol synthesis in Schwann cells (6, 7). The mechanism of inhibition has not been established, and the species of tellurium responsible for the inhibition following the feeding of elemental tellurium is not known.

We used the rat cDNA sequence (3), along with data obtained from The Institute for Genome Research (TIGR) database, to obtain by polymerase chain amplification a cDNA to the human enzyme. Expression of proteins with the IMPACT T7 System (intein–chitin binding domain fusion proteins). Briefly, bacterial cells were lysed by French press in buffer containing 20 mM Tris–HCl (pH 7.4), 500 mM NaCl, 0.1 mM EDTA, and 0.1% Triton X-100. The lysates were then centrifuged at 12,000g. After the supernatant fraction was loaded onto a chitin affinity column, the column was washed with 20 column-volumes of the above buffer. The column was then incubated overnight at 4°C in cleavage buffer consisting of the French press buffer with 30 mM β-mercaptoethanol to promote cleavage. The released squalene monoxygenase protein was eluted and the buffer was changed, by centrifugal dialysis, to a storage buffer containing 20 mM Tris–HCl (pH 7.4) with 0.1% Triton X-100 and then stored at −80°C until use. N-terminal amino acid sequence analysis performed at the Macromolecular Structure Analysis Facility at the University of Kentucky confirmed that the eluted protein was squalene monoxygenase. Enzyme was quantified with the Coomassie Plus Protein Assay Reagent Kit from Pierce (Rockford, IL).

Squalene monoxygenase activity assays. Activity assays were carried out based on the procedure of Bai and Prestwich (2). Standard incubations (200 μL) contained 20 mM Tris–HCl (pH 7.4), 0.1% Triton X-100, 30 μM FAD, 1 mM NADPH, 28 pmol of cytochrome P450 reductase, and 1 μg (19 pmol) of squalene monoxygenase. Reactions were started by the addition of [14C]squalene (20 μM) and carried out at 37°C for 20 min. Reactions were stopped by extraction into methylene chloride and fractionation on silica thin-layer plates with 5% ethyl acetate in hexane. The plates were visualized and quantified by electronic autoradiography (Packard Instant Imager). Cytochrome P450 reductase was purified by affinity chromatography from E. coli cells expressing the cloned rat cDNA (9) and quantified spectrally using an extinction coefficient of 21.4/M at 456 nm. Radiolabeled squalene was synthesized by the Chemical Synthesis Facility, Department of Medicinal Chemistry, University of Utah, at 7 mCi/mmol. All graphs and kinetic constants were generated using Prism 3.0 (GraphPad Software, Inc., San Diego, CA) from measurements made in triplicate or better, with standard deviations of the mean indicated by error bars where appropriate. Enzyme inhibition models were generated with EZ-Fit Enzyme Kinetics (Perrella Scientific, Amherst, NH).

RESULTS

Polymerase chain amplification of a human liver cDNA library with oligonucleotides corresponding to the putative N- and C-termini of human squalene

EXPERIMENTAL PROCEDURES

Cloning and expression of squalene monoxygenase. A human liver cDNA library (Quick-clone) was obtained from Clontech, Inc. (Palo Alto, CA), and amplified with oligonucleotides corresponding to the putative N- and C-termini of human squalene monoxygenase, using Pfu polymerase (Stratagene, La Jolla, CA). The N-terminal primer was obtained from TIGR Human cDNA Database, Level II, with permission, and the C-terminal primer was based on the sequence of the rat enzyme (3). The sequences of these primers are as follows:

**N**, cgaattccatggtggactttttcgggcattgcac

**C**, ccggatcctcagtgaaccagata

Two second, independent amplification reactions were carried out with primers chosen from the putative 5’ and 3’ nontranslated regions of the human squalene monoxygenase cDNA; both primers were obtained from TIGR database:

5’, ctgtctgatccagcaaccttac

3’, gcatgtcccaacaactctac

The products of all three amplification reactions were cloned into the pCRII cloning vector (Invitrogen, San Diego, CA) and a consensus sequence was determined with use of an ABI DNA sequencer at the Molecular Structure Analysis Facility at the University of Kentucky Macromolecular Structure Analysis Facility. A cDNA mapping the consensus sequence was then constructed from the appropriate clones.

The CDNA for squalene monoxygenase was amplified by polymerase chain amplification using primers containing a 5’ Nco restriction site placed immediately before codon 112, incorporating an ATG start codon and replacing Gly111 with Ala, and a 3’ Small restriction site at the C-terminus. These restriction sites were then used to clone the coding sequence into the pTYB4 plasmid (New England Biolabs, Beverly, MA) to create the pTYB4SE110 construct, in which the squalene monoxygenase sequence is in-frame and upstream of the intein sequence and chitin binding domain of the vector (8). This construct was transfected into E. coli strain ER2566, which carries a chromosomal copy of the T7 polymerase gene under control of the lac promoter, and is deficient in the lon and ompT proteases. Twenty-five milliliters of Luria–Bertani broth supplemented with ampicillin (100 μg/ml) was inoculated with a freshly grown colony, grown overnight at 37°C, and then used to inoculate 1 L of terryll of Kentucky. When the cells reached log phase growth, protein production was induced by addition of isopropyl thiogalactoside to a final concentration of 1 mM. Cells were grown overnight at 250 rpm and 30°C and then harvested by low-speed centrifugation.

Purification of squalene monoxygenase. Squalene monoxygenase expressed from the pTYB4 vector was purified according to the protocol provided by New England Biolabs, Inc., for expression of proteins with the IMPACT T7 System (intein–chitin binding domain fusion proteins). Brieﬂy, bacterial cells were lysed by French press in buffer containing 20 mM Tris–HCl (pH 7.4), 500 mM NaCl, 0.1 mM EDTA, and 0.1% Triton X-100. The lysates were then centrifuged at 12,000g. After the supernatant fraction was loaded onto a chitin affinity column, the column was washed with 20 column-volumes of the above buffer. The column was then incubated overnight at 4°C in cleavage buffer consisting of the French press buffer with 30 mM β-mercaptoethanol to promote cleavage. The released squalene monoxygenase protein was eluted and the buffer was changed, by centrifugal dialysis, to a storage buffer containing 20 mM Tris–HCl (pH 7.4) with 0.1% Triton X-100 and then stored at −80°C until use. N-terminal amino acid sequence analysis performed at the Macromolecular Structure Analysis Facility at the University of Kentucky confirmed that the eluted protein was squalene monoxygenase. Enzyme was quantified with the Coomassie Plus Protein Assay Reagent Kit from Pierce (Rockford, IL).

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*Abbreviations used: TIGR, The Institute for Genomic Research; EST, expressed sequence tag.*
monooxygenase yielded a 1600-bp product, which was the expected size for the squalene monooxygenase coding sequence. The encoded amino acid sequence of the 5' and 3' ends closely matched the rat N- and C-terminal sequences, indicating that this was likely to be the human homologue of squalene monooxygenase. To establish the correct sequence of the N- and C-termini of the enzyme, which were encoded by the oligonucleotide primers in the cDNA cloned above, expressed sequence tag data were used to synthesize a second set of primers that corresponded to segments in the 5' and 3' nontranslated regions of the putative human squalene monooxygenase cDNA. Two separate amplification reactions with these primers yielded 2500-bp products. Sequence determination on all three amplification products yielded a consensus sequence of 2199 bp, with a 155-bp 5' nontranslated segment and a 319-bp 3' nontranslated region. This sequence closely matches the available EST database sequences from which the primers were derived.

An open reading frame of 574 amino acids encodes a protein with a molecular mass of 63,927 Da that is 84% identical to the rat (3) and more recently cloned mouse (10) squalene monooxygenase sequences, confirming that a cDNA to human squalene monooxygenase has been isolated (Fig. 1). The mammalian enzymes are only 32% identical with the enzyme from *Candida albicans* (11), and the sequences require the insertion of several gaps for optimal alignment (Fig. 1). Most notably, the yeast sequence lacks the extended N-terminal segment characteristic of the mammalian proteins.

Initial bacterial expression studies with the full-length cDNA did not reveal active enzyme in isolated bacterial membranes, presumably due to very low expression. Similar difficulties with the rat enzyme were overcome by removing the first 99 amino acids (12), and so a similar approach was taken with the human enzyme. The human squalene monooxygenase cDNA

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**FIG. 1.** Amino acid sequence alignment of squalene monooxygenase from human, rat, mouse, and *Candida albicans*. Amino acids differing from the human sequence are indicated by the one-letter amino acid abbreviation. Gaps are indicated by a dash and the lack of an amino acid is indicated by a dot.
lacking the sequence encoding the first 110 amino acids was cloned into the pTYB4 E. coli expression vector and the expressed, truncated enzyme purified by affinity chromatography. This purification system, described in detail under Experimental Procedures, yields a highly homogeneous protein carrying no additional amino acids, such as a hexahistadine tag, nor does it require any postpurification modification, such as enzymatic digestion to remove a fused peptide. As shown in Fig. 2, the eluted protein is the expected molecular mass (51.6 kDa) and is estimated to be at least 95% pure based on SDS–PAGE. The principal contaminant, migrating at approximately 60 kDa, may be the bacterial chaperonin GroEL, which has been shown to be strongly induced in E. coli by squalene monoxygenase expression (data not shown). An additional chromatographic step did not remove this contaminant, suggesting that it is tightly bound to squalene monoxygenase.

The purified enzyme was catalytically active: squalene-2,3-epoxide formation was dependent upon addition of this protein and was linear with protein concentration and time for up to 45 min. Figures 3 and 4 illustrate the enzyme's dependence on Triton X-100 and NADPH–cytochrome P450 reductase, respectively. Enzyme activity was maximal with 0.1% Triton X-100. The amount of P450 reductase required for maximal activity was several-fold less than the amount of squalene monoxygenase in the reaction, such that maximal activity was obtained with a reductase:monooxygenase ratio of about 1:3. Purified cytochrome b5 reductase, a second microsomal electron transport protein, was not able to substitute for cytochrome P450 reductase in these assays.

Experiments were carried out to evaluate some basic kinetic parameters of the enzyme. The substrate-velocity curve for squalene (Fig. 5) shows that the apparent $k_{cat}$ for squalene is 1.1 pmol/min/pmol and that the apparent $K_{m}$ is 7.7 mM. Figure 6 shows the substrate-velocity curve for FAD and indicates an apparent $K_{m}$ of 0.3 μM. The 30 μM data point is included because this is the concentration routinely used in assays, and the inset is included to show the fit of the curve at the lower FAD concentrations. The substrate-velocity curve for P450 reductase is shown in Fig. 7. The apparent $K_{m}$ of the reductase is 14 nM.

Feeding weanling rats a diet containing 1% tellurium produces a transient, peripheral demyelination due to inhibition of squalene monoxygenase in Schwann cells (6, 7). In addition, squalene monoxygenase is inhibited by tellurite in microsomes from...
both rat liver and sciatic nerve (13). As no studies have been reported with the human enzyme, tellurite and a variety of related compounds were tested in our system for their ability to inhibit the purified human enzyme. Tellurite, tellurium dioxide, selenite, and selenium dioxide were all inhibitory (Fig. 8), with the tellurium compounds being more potent than the selenium compounds: The IC50 values for tellurite and selenite are 17 and 37 μM, respectively. Other compounds tested included selenate, telluric acid, arsenite, arsenate, antimonite, sulfite, and sulfate, but none were inhibitory at concentrations up to 1 mM. Tellurite and selenite did not inhibit the reduction of cytochrome c by cytochrome P450 reductase, indicating that the site of action of these chemicals is on squalene monoxygenase.

Further characterization of the interaction of tellurite with squalene monoxygenase suggests a multisite, noncompetitive mode of inhibition. Figure 9 shows the Lineweaver–Burk plot of the inhibition with respect to squalene at 0, 5, 25, and 50 μM tellurite. When the slopes of the lines from the Lineweaver–Burk plot are replotted against tellurite concentration (Fig. 10A), the resulting line is parabolic, rather than linear; the same is true when the Y intercepts are replotted against tellurite concentration (Fig. 10B). These replots suggest both slope-parabolic (s-parabolic) and intercept-parabolic (l-parabolic) inhibition, indicative of two or more binding sites for tellurite on the enzyme.

**DISCUSSION**

Despite its pivotal role in cholesterol biosynthesis, remarkably little is known about squalene mono-ox-
genase. Although first identified in the early 1970s (14, 15), the enzyme is expressed at low levels in most tissues, including the liver, and has been difficult to purify in active form. The first mammalian squalene monoxygenase sequence was reported in 1995 by Teruo Ono’s group (3); they used a novel trans-complementation assay in yeast to select transformants expressing the rat enzyme. The enzyme showed limited sequence similarity to the yeast enzyme (30% identity) and to other flavoproteins. Studies with a truncated form of the enzyme, lacking the first 99 amino acids and containing a hexahistidine tag at the carboxyterminus, permitted expression and purification from bacterial cell lysates by affinity chromatography (12). By using the sequence data of Sakakibara et al. (3) and EST data in a semipublic database, we were able to clone the human enzyme; here we report the sequence, expression, and a detailed kinetic evaluation of human squalene monoxygenase.

Because initial attempts to express the full-length enzyme in E. coli were unsuccessful, we deleted the first 110 amino acids, based on the earlier studies of Nagumo et al. (12) with the rat enzyme. This deletion removes the likely membrane-binding domain (amino acids 28–44) that anchors the enzyme to the endoplasmic reticulum, but does not include the first putative structural feature, the FAD pyrophosphate-binding segment (amino acids 126–154) (3). Notably, the shortened enzyme thus resembles the yeast enzyme that similarly lacks sequence preceding the FAD binding segment (see Fig. 1). Other than anchoring the enzyme to the endoplasmic reticulum, no specific role for this N-terminal segment has been identified, and its deletion is not thought to affect enzyme activity. Indeed, the \( K_m \) of 7.7 \( \mu M \) for squalene reported here with the recombinant human enzyme is very near the \( K_m \) value reported with microsomal preparations from the human hepatoma cell line HepG2 (3 \( \mu M \); Ref. 4), and that reported for purified rat liver squalene monoxygenase (13 \( \mu M \); Ref. 16). The \( K_m \) for squalene with the recombinant, truncated rat enzyme has not been reported.

The turnover number (\( k_{\text{cat}} \)) for our human recombinant enzyme is quite low, at 1.1 pmol/min/pmol. Although this is close to the turnover of 0.33 nmol/min/nmol for the full-length enzyme purified from rat liver (16) and also that of the recombinant rat enzyme (calculated from data in Ref. 12 to be about 6.0 nmol/min/nmol), we cannot exclude the possibility that this low \( k_{\text{cat}} \) reflects the artificial reconstitution conditions of the assay, including the use of Triton X-100 to replace uncharacterized cytosolic factor(s). No catalytic rate studies with squalene monoxygenase from human sources have been reported.

**FIG. 9.** Lineweaver–Burk plot of inhibition of squalene monoxygenase by tellurite. Squalene monoxygenase activity was assayed as described under Experimental Procedures in the presence of various concentrations of tellurite: 0 \( \mu M \), open circles; 5 \( \mu M \), closed boxes; 25 \( \mu M \), open triangles; 50 \( \mu M \), closed triangles. FAD and reductase concentrations were saturating.

**FIG. 10.** Replots of the inhibition data generated by Lineweaver–Burk analysis. (A) The Y axis intercepts from Fig. 9 are plotted against tellurite concentration and fitted to a parabolic curve, \( r^2 = 0.998 \). (B) The slopes of the lines from Fig. 9 are plotted against tellurite inhibitor concentration and fitted to a parabolic curve, \( r^2 = 0.996 \).
As noted in early studies on squalene monooxygenase, FAD is an essential component of the epoxidation reaction (17). This distinguishes squalene monooxygenase from other microsomal monooxygenases, and in particular from the heme-dependent cytochromes P450. The activity of the purified rat enzyme is about 5% in the absence of added FAD (16), indicating that the FAD group in this enzyme is not tightly bound. Similarly, with our purified human enzyme activity in the absence of added FAD is only 4.5% of the maximal rate. This low rate of product formation detected in the absence of added FAD may be attributed to a small amount of enzyme that is purified with bound FAD. The $K_m$ of FAD determined in these studies, 0.3 $\mu$M, is lower than that of the purified rat enzyme (5 $\mu$M) (16).

Initial studies on squalene monooxygenase using rat liver microsomal preparations required the addition of a cytosolic protein fraction (supernatant protein factor) and phospholipids for complete activity (17). It was later observed that Triton X-100 could replace these components in assays containing either microsomes or purified enzyme (15, 16). As shown here, the purified, truncated human enzyme is also dependent on the presence of 0.1% Triton X-100 for maximal activity. The functional role of the supernatant protein factor and of Triton X-100 in the epoxidation of squalene is not known; a role as a substrate carrier protein has been postulated (18), but other effects, such as altering protein conformation to facilitate electron transfer, cannot be ruled out. The present studies indicate that the first 110 amino acids (including the proposed membrane-binding segment) of the enzyme are not involved in this detergent activation.

Cytochrome P450 reductase is also required for activity in the reconstituted system. Maximal activity is attained at a reductase:monooxygenase ratio of less than one, indicating that electron transfer to the FAD group of squalene monooxygenase is not the rate-limiting step in the reaction. In contrast, the cytochromes P450 typically require equimolar or greater ratios of reductase:P450 for maximal activity (19), and electron transfer from the reductase has been implicated as the rate-limiting step with some isoforms (20). Reductase $K_m$ values with P450 enzymes are in the range of 10 to 500 nM, depending on the assay system and the P450 isoform tested. The $K_m$ value of 14 nM determined in these experiments should be interpreted cautiously, as it may be low due to the slow turnover rate of this enzyme. As shown in Fig. 4, only one reductase molecule is needed per 3 monooxygenase molecules to achieve a maximal velocity.

Although, as discussed above, the kinetic constants for this enzyme are consistent with literature values, the possibility that some uncontrollable factors may be affecting activity cannot be ruled out. The deletion of the N-terminal region, for example, may be affecting the observed kinetic values. In addition, as noted earlier, the use of Triton X-100 to activate the enzyme may affect the catalytic activity compared to the in vivo situation, where another protein, “supernatant protein factor” is required for activity.

Feeding rats a diet containing 1% tellurium causes a demyelination of peripheral neurons due to inhibition of squalene monooxygenase in Schwann cells (6, 7). This inhibition blocks cholesterol synthesis, necessary for myelin sheath formation. As no information was available on the ability of tellurium to inhibit human squalene monooxygenase, the ability of tellurium and some related compounds to inhibit the recombinant human enzyme was examined. Two of the tellurium compounds tested, tellurite and tellurium dioxide, are potent inhibitors of the enzyme. The analogous selenium species, selenite and selenium dioxide, were also inhibitory, but sulfite and chemical species in Group VA of the periodic table (arsenicals, antimonite) were without effect. This is the first report that selenium compounds can inhibit this enzyme and is potentially important given selenium’s status as an essential trace element. It was not possible to completely inhibit the enzyme with either tellurite or selenite at concentrations up to 10 mM.

The parabolic nature of the replots from the Lineweaver–Burk graph suggests that tellurite binds to two sites on squalene monooxygenase in a noncompetitive manner with respect to squalene (replots of simple, single-site noncompetitive inhibition yield straight lines). Tellurite is known to interact with sulfhydryls (21), and sulfhydryl reagents such as N-ethylmaleimide also inhibit squalene monooxygenase (13). The presence of seven cysteines in squalene monooxygenase is consistent with a multisite model for tellurite inhibition of this enzyme, and studies aimed at elucidating the specific targets bound by tellurite are currently under way.

It is of interest that garlic is reported to contain relatively high levels of tellurium, as methylated tellurium species, and that garlic is believed to act as a “blood thinner” and lower cholesterol levels. Whether part of the medicinal value of this herb is due to inhibition of squalene monooxygenase and cholesterol biosynthesis in the liver remains to be determined. Recently published data indicate that methylated tellurium species are capable of inhibiting squalene monooxygenase in cultured Schwann cells and in weanling rats (22). We have begun studies to examine the effect of methylated tellurium compounds on the purified human enzyme.

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