Active Site Mapping of Affinity-labeled Rat Oxidosqualene Cyclase*

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Rat liver oxidosqualene cyclase (OSC), a 78-kDa membrane-bound enzyme, was purified and labeled with the mechanism-based irreversible inhibitor, \([\text{H}]29\)-methylidene-2,3-oxidosqualene (Abel, I., Bai, M., Xiao, X-Y., and Prestwich, G. D. (1992) Biochem. Biophys. Res. Commun. 187, 32–38). A 6-kDa CNBr peptide was separated by Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted to a polyvinylidene difluoride membrane. The sequence of the first 30 amino acids of this peptide were determined by Edman degradation and showed unexpectedly high similarity to the fungal OSC from Candida albicans (60% identity with Arg(19); Val(44)) and to the bacterial squalene cyclase from Alcyclobacillus (formerly Bacillus) acidocaldarius (37% identity with Lys(96), Leu(96)). Further, radioanalysis clearly established that the two adjacent Asp residues that were labeled is also shared by two other enzyme families important in the terpene biogenesis, the sesquiterpene cyclases and the prenyl transferases.

The remarkable cyclization reaction of oxidosqualene (1α) to lanosterol (3) has intrigued chemists and biologists for more than four decades (1). The cyclization of (3S)-oxidosqualene folded into a pre-chair-boat-chair conformation is initiated by an acid-catalyzed opening of the 2,3-epoxide. Then, sequential ring-forming reactions and backbone rearrangements occur through a progression of rigidly held carbocationic intermediates (2–6). Several oxidosqualene cyclases (OSC)\(^1\) (EC 5.4.99.7) have been purified to homogeneity from vertebrate (7–9), plant (10–13), and yeast (14) sources. Recently, the erg7 gene believed to encode the OSC of Candida albicans was cloned, sequenced (15–17), and expressed in catalytically active form.\(^2\) Comparison of protein sequences deduced from the cDNAs for the C. albicans OSC and two bacterial squalene cyclases (SCs) revealed highly conserved regions with a repeating motif rich in aromatic amino acids,\(^3\) suggesting that vertebrate and fungal OSC evolved from the prokaryotic bacterial SCs.

The first potent mechanism-based irreversible inactivator of OSC, 29-methylidene-2,3-oxidosqualene (29-MOS) (1b, T = 1H) (20), covalently modified enzymatically active OSCs from rat, pig, dog, and human liver, identifying single protein bands in each species with molecular masses ranging from 70 to 80 kDa (8). The mechanism of inhibition required substrate turnover and was postulated to involve the initial cyclization of 29-MOS to the 21-methylidene protosterol cation (2), followed by trapping by an active site nucleophile (Scheme 1).

We describe herein the isolation and sequencing of radiolabeled peptide fragments obtained from purified rat OSC that had been modified by tritium-labeled 29-MOS (1b, T = 3H). The active site of rat liver OSC affinity labeled by this suicide substrate occurred in one of the conserved regions found in the yeast and bacterial cyclases. Moreover, the DDXX(D/E) motif that was labeled is also shared by two other enzyme families important in the terpene biogenesis, the sesquiterpene cyclases and the prenyl transferases.

**EXPERIMENTAL PROCEDURES**

Purified rat liver oxidosqualene cyclase (20 μg of protein in a total volume of 240 μl) was incubated with racemic \([\text{H}]29\)-methylidene-2,3-oxidosqualene (2.3 Ci/mmol, 1.0 μM, 2.6 × 10⁵ dpm) in the presence of 0.1% Triton X-100 for 15 min at 37 °C as previously described (8). The labeled enzyme was chemically digested with 1000 molar excess CNBr (Sigma) in 70% HCO₂H for 24 h at room temperature under nitrogen gas in darkness. After lyophilization and ethanol precipitation (21), the digested peptides were separated using the Tricine SDS-PAGE gel system (16% T, 3% C, 10 mA constant) (22). The radioactivity was monitored on a separate gel by fluorography. Proteins were transferred electrophoretically in 10 mm 3-(cyclohexylamino)-1-propanesulfonic acid, 10 mM MeOH (pH 11.0) to a PVDF membrane (Bio-Rad, TransBlot) in an electrotransfer cell (4 liters) at 50 V, 0.25 A for 1 h (23). The membrane was then stained briefly in 0.1% Coomassie Blue R-250 in 50% MeOH, destained in 50% MeOH, washed with water, and air-dried. The radiolabeled 6-kDa fragment (4.8 × 10⁶ dpm) was excised and sequenced by Edman degradation using a pulsed liquid phase sequenator (Applied Biosystems model 475A). Radiosequencing was carried out separately with an independent preparation of the 6-kDa peptide. In this case, radiolabeled phenylthiohydantoin-amino acids from each sequencing cycle were eluted from the membrane with N-butyl chloride, and the radioactivity was measured using a liquid scintillation counter (Packard, Tri-Carb 1600 TR). Radioactivity was detected in the 18th (1.2 × 10⁶ dpm) and 19th cycle (1.1 × 10⁶ dpm), representing 26 and 24% of the total radioactivity on the PVDF membrane, respectively (Fig. 2). After 25 cycles of Edman degradation, no detectable radioactivity remained on the recovered PVDF membrane.

**RESULTS AND DISCUSSION**

The CNBr digestion of the \([\text{H}]29\)-MOS-labeled rat liver OSC (3) gave a single radioactive fragment (6 kDa), and this peptide could be efficiently resolved by a Tricine SDS-PAGE (22) (Fig. 1). After electrophoretic transfer to a PVDF membrane, the

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\(^1\) The abbreviations used are: OSC, oxidosqualene cyclase; SC, squalene cyclase; 29-MOS, 29-methylidene-2,3-oxidosqualene; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; Tricine, N(2)-hydroxy-L,1,3-bis(hydroxymethyl)glycine.

\(^2\) J. H. Griffin, personal communication.

\(^3\) K. Poralla, A. Hewelt, G. D. Prestwich, I. Abe, I. Reipen, and G. Sprunger, submitted for publication.
Oxidosqualene Cyclase Active Site

**Scheme 1.** Proposed mechanism of irreversible inhibition of OSC by 29-MOS.

![Scheme 1](image)

**Fig. 1.** Chemical affinity labeling of purified rat OSC and separation of the CNBr-digested peptides on Tricine SDS-PAGE. A, purified rat liver OSC was affinity labeled with [3H]29-MOS: lane 1, SDS-PAGE (7.5%) gel stained with Coomassie Blue; lane 2, corresponding fluorogram. B, separation of CNBr-digested peptides: lane 3, Tricine SDS-PAGE (16%) gel stained with Coomassie Blue; lane 4, corresponding fluorogram.

labeled 6-kDa peptide was subjected to sequential Edman degradation to give an unambiguous sequence of 30 amino acids. The sequence of the modified active site showed significant similarity to the previously reported sequence of fungal OSC from *C. albicans* (16, 17) (50% identity with Arg413-Val442) and to bacterial SCs from *Alicyclobacillus* (formerly *Bacillus*) *acidocaldarius* (24) (37% identity with Lys356-Leu385) (Fig. 3). This is consistent with our prediction that the active site of OSC is located in an aromatic-rich region at the conserved C terminus of the protein. The radioactivity associated with the covalently attached irreversible inhibitor was detected in the 18th and 19th cycle (Fig. 2), which clearly established that the two adjacent Asp residues in the most highly conserved region (Asp-Asp-Thr-Ala-Glu-Ala) (Fig. 3) were equally labeled by the suicide substrate.

According to the proposed mechanism of inhibition (8), 29-MOS (1b) would adopt the same conformational folding as (3S)-2,3-oxidosqualene (1a) in the substrate binding site and would be cyclized initially to the 21-methylidene-extended protosterol cation (2). This tertiary allylic cation would then be trapped by nucleophilic attack by the carboxyl group of one of the two adjacent Asp residues, resulting in ester bond formation (3) and concomitant irreversible inactivation of the enzyme (Scheme 1). We suggest that the nucleophilic trapping is most likely to occur at the primary carbon of the allylic cation, since the tertiary C-20 cation is a normal intermediate that does not inactivate OSC. The two Asp residues appear to be equally accessible to the methylidene-extended protosterol cation, based on the observed labeling by [3H]29-MOS. In contrast to vertebrate OSCs, neither yeast nor plant OSCs were labeled with [3H]29-MOS (8), indicating that the appropriate nucleophilic residues may be absent or may not have access to the allylic cation in these cyclases.

The highly conserved binding site sequence, Asp-Asp-Thr-Ala-Glu-Ala, showed significant similarity to the known consensus sequence, Asp-Asp-Xaa-Xaa-Asp ("DDXXD" motif), found in all known sesquiterpene cyclases (25, 27, 28) and prenyl transferases (29–31) (Fig. 3). As each of these enzymes uses an allylic diphosphate as a substrate, the aspartate-rich domain has been proposed to be involved in catalyzing the ionization of the allylic diphosphate to the reactive allylic cation, presumably by coordination via a Mg²⁺-diphosphate bridge. Recently, a site-directed mutagenesis experiment of this domain of farnesyl-diphosphate synthase suggested that the first aspartate residue in the domain is involved in the catalysis (32). The high degree of sequence conservation of the 29-MOS binding site in both eukaryotic and prokaryotic cyclases supports a catalytic or structural function for this domain. Indeed, our result suggests a novel functional role for the DDXXD(E) motif, that is, stabilization of tertiary or allylic carboxylations. Such a "point charge" model has been previously proposed for control of the course of oxidosqualene cyclization by Johnson (18, 19, 33, 34). Finally, it should be noted that the existence of such a consensus sequence in the enzymes involved in terpene and sterol synthesis provides support for Ourisson’s hypothesis for divergent molecular evolution of these enzymes (26).

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4. D. E. Cane, personal communication.
**Fig. 2.** Radiosequencing of the CNBr-digested 6-kDa radiolabeled peptide. The radiolabeled 6-kDa fragment (4.8 x 10^4 dpm) was excised from the PVDF membrane and sequenced by Edman degradation.

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**Fig. 3.** Comparison of the 29-MOS binding site sequence with fungal and bacterial OSC/SCs. Note the alignment of the aspartate-rich consensus sequence ("DDXXD" motif) found in sesquiterpene cyclases and prenyl transferases. Amino acid residues in rat OSC conserved in other enzymes are boxed: no pattern, identical residues; shaded, conserved aromatic residues. Arrows indicate residues modified by [3H]29-MOS.

*Note Added in Proof*—The DDXXD motif is also present in a plant monoterpen cyclase and a diterpene cyclase (Colby, S. M., Alonso, W. R., Katahara, E. J., McGarvey, D. J., and Croteau, R. (1993) J. Biol. Chem. 268, 23016-23024*)