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† 八十六年度及以前的一般國科會專題計畫(不含產學合作研究計畫)亦可選擇適用，較特殊的計畫如國科會規劃案等，請先洽得國科會各學術處同意。

氧化鯊烯環化酵素之結構與反應特性之研究及其抑制劑之設計與合成

Structure-Reactivity Studies and Inhibitors Design of Oxidosqualene Cyclases

一、中文摘要

本計畫對於氧化鯊烯環化酵素之環化反應機構及酵素中負責環化之酵素活性區域的氨基酸所扮演角色之確定已獲得初步結果。經由利用氨基丙酸掃描法對天門冬酸-456 上游 7 個柔軟性較高的環狀區域氨基酸中之非氨基丙酸以聚合脢連鎖反應方法進行定點突變，將其轉變成氨基丙酸後轉殖進入染色體上氧化鯊烯環化酵素已缺陷的酵母菌中，以進行互補實驗的研究。實驗結果顯示，離氨基酸-448-氨基丙酸的置換對於酵母菌之存活與否具有決定性的影響。經由萃取酵母菌中麥角脂醇生合成途徑中之成分，進行管柱與薄層色層分析法分離後確認其成份，結果發現上述置換無法催化反應的進行而停留在反應物，此結果顯示離氨基酸-448-氨基丙酸的置換可能部份參與其環化反應的起始作用。經由分子模擬顯示離氨基酸-448 側鏈上之氨基可能與天門冬酸-456 或天門冬酸-454 側鏈上之縮酸基形成鹽橋，進而影響到天門冬酸對氧化鯊烯上之環氧基的作用力。此一結果對增進對氧化鯊烯環化酵素之環化反應機構的了解及所參與之氨基酸所扮演的結構-功能關係的了解將有極大之助益。

關鍵詞：氧化鯊烯環化酵素，氨基丙酸掃描法，聚合脢連鎖反應

Abstract

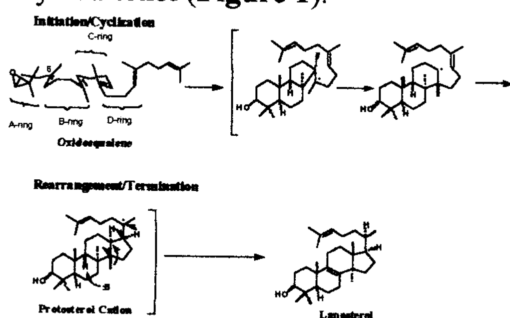
The short-term objectives toward understanding the structure-function relationships of oxidosqualene cyclization reaction has been accomplished. A series of 7 site-directed mutations were made in the ERG7 gene encoding oxiodsqualene-lanosterol cyclase (OSC) from the yeast *Saccharomyces cerevisiae*. Each of the non-alanine residues between amino acid 447 and 453 of OSC was converted to alanine via site-directed mutagenesis of the wild type cyclase gene. The mutants were assayed for their ability to complement the genetic disrupted ERG7 gene of *S. cerevisiae* strain, CBY57, by plasmid shuffle strategy. Among the 7 alanine-scanning mutants, only Lys-448-Ala (K448A) failed to complement the ERG7 disruption. Analysis of the cyclase product revealed that the cyclization reaction stopped at oxidosqualene, indicating that K448A replacement might at least partially involved in the initiation of the cyclization reaction. Homology modeling of the cyclase also revealed that the amino side chain of Lys448 might be involved in the interaction with the carboxylic acid side chain of Asp454 or Asp456 which further affects the interaction between substrate and enzyme. Construction of alanine-scanning mutants and analysis of their cyclization products would give detailed information regarding the cyclization/rearrangement mechanism and structure-function

relationships of amino acid residues of oxidosqualene cyclases.

Keywords: site-directed mutagenesis, oxiodsqualene-lanosterol cyclase, alanine-scanning, plasmid shuffle

二、緣由與目的

2,3-oxidosqualene cyclases (OSCs) catalyze the diverse and complex cyclization/rearrangement of (3*S*)-2,3-oxidosqualene to lanosterol in mammals and fungi versus to cycloartenol in algae and higher plants.[1-4] The formation of lanosterol and cycloartenol is initiated in the pre-chair-boat-chair-boat conformation and starts from an acid-catalyzed oxirane ring opening then followed by a series of discrete conformationally rigid carbocationic transformations until the metastable tricyclic ring formation.[5,6] The five membered cyclopentenyl carbinyl cation then undergoes a ring expansion to a six membered C-ring, leading to the formation of protosterol cation. Additional rearrangements involving transient methyl and hydride migrations, culminated by quenching of the positive charge by deprotonation at C-9 to afford lanosterol versus by ring closure between C-9β and C-19 to form cyclopropyl ring and yield cycloartenol (**Figure 1**).



The objectives of this research program aimed at understanding the structure-function relationships of oxidosqualene cyclization reaction and tried to develop putative inhibitors of oxidosqualene cyclase enzymes as probes of active site structure and as potential antifungal chemotherapeutic agents. We have selected a flexible loop region containing 7 amino acid residues, located upstream of putative active site residue, Asp-456, each of the non-alanine residue was converted to alanine via site-directed mutagenesis, to study their effects on the cyclization/rearrangement reaction. The effect of mutations on oxidosqualene-lanosterol cyclase activity was examined by plasmid shuffle technique. On the other hand, we tried to design, synthesize several electron-poor aromatic compounds as cyclase inhibitors and antifungal agents.

三、結果與討論

Generation of Site-Directed Mutants in the Wild Type of *S. cerevisiae* ERG7 Gene.

Each of the non-alanine residues within the 447-453 sequences of the *S. cerevisiae* oxdiosqualene-lanosterol cyclase was converted to alanine through site-directed mutagenesis of the wild type cyclase gene. Mutants were generated by polymerase chain reaction (PCR), restriction digested with endonuclease restriction enzyme, followed by cloning into a vector to obtain a recombinant plasmid containing a single DNA fragment incorporating the desired mutation sequence and the new restriction site. The presence of the mutations was verified by sequencing of the region of the gene encoding the 447-453 sequence. The plasmids bearing the Thr-453-Ala, Tyr-452-Ala, Gly-451-Ala, Gln-450-Ala, Thr-449-Ala, Lys-448-Ala, and Thr-447-Ala mutations were designated pFHCOSCRS1-7, respectively.

Characterization of Site-Directed Mutagenesis Effects on Oxidosqualene

Cyclase Activity via Plasmid Shuffle

Haploid strain CBY57[pZS11] (ERG7 △::LEU2 ade2-101 his3-△200 leu2-△1 lys2-801 trp1-△63 [pZS11]), a yeast strain bearing both a genomic ERG7-disrupted gene and a URA3 centromeric plasmid with wild type *S. cerevisiae* cyclase gene, allowed the use of a plasmid shuffle to analyze the effects of mutations in the 447-453 region in an ERG7 knockout background. As negative and positive controls for the plasmid shuffle, CBY57[pZS11] was transformed by electroporation with plasmids pRS314 and pTKOSCRS314WT, TRP1 centromeric plasmids bearing no insert and the wild type *S. cerevisiae* oxidosqualene-lanosterol cyclase gene, respectively. Transformants were selected on SD media containing adenine, lysine, histidine and uracil, and pRS314 and pTKOSCRS314WT were recovered from cell-free lysates grown in this media. CBY57[pZS11], CBY57[pZS11][pRS314] and CBY57[pZS11][pTKOSCRS314WT] were then plated on complete media containing 5-fluoroorotic acid (5'-FOA) to counterselect for pZS11. As expected, growth was only observed on plates inoculated with CBY57[pZS11][pTKOSCRS314WT].

CBY57[pZS11] was transformed by electroporation with pFHCOSCRS1-7, selected for growth in the media containing adenine, lysine, histidine and uracil, then re-selected for growth in the presence of 5'-FOA. CBY57[pZS11] transformants carrying pFHCOSCRS1, pFHCOSCRS3, pFHCOSCRS4, pFHCOSCRS5, or pFHCOSCRS7, which bear the Thr-453-Ala, Gly-451-Ala, Gln-450-Ala, Thr-449-Ala, and Thr-447-Ala mutations, respectively, produced colonies upon counterselection for pZS11 with 5'-FOA. These colonies grew at a rate similar to that observed with CBY57[pTKOSCRS314WT]. These results clearly indicate that Thr-453-Ala, Gly-451-Ala, Gln-450-Ala, Thr-449-Ala, and Thr-447-Ala mutants of the *S. cerevisiae* oxidosqualene-lanosterol cyclase are active, and that they have activity qualitatively similar to that of the wild type enzyme. On the other hand, mutant pFHCOSCRS2 bearing Tyr-452-Ala grew slower than that of CBY57[pTKOSCRS314WT]. The result indicates that Tyr-452-Ala mutation may somewhat reduce oxidosqualene-lanosterol cyclase activity but still above the threshold level needed to support growth of the ERG7 knockout.

Cyclase mutant bearing Lys-448-Ala mutation on pFHCOSCRS7 was unable to complement the ERG7 disruption. The result indicates that the Lys-448-Ala mutation either abolish oxidosqualene-lanosterol cyclase activity completely or reduce it below some threshold level needed to support growth of the ERG7 knockout. Based on the assumption that the related sequences between SHC and OSC should possess comparable 3-D structure, a homology modeling approach was applied to determine the possible spatial location of Lys-448, in order to gain a more detailed understanding of the structure-function relationships. Homology modeling of the cyclase revealed that the amino side chain of Lys448 might interact with the carboxylic side chain of Asp454 or Asp456 which

further affects the interaction between substrate and enzyme. The result indicate that Lys-448 (equivalent the residue Asp-368 in SHC) may locate proximal to epoxide or A-ring, possibly involve in the stabilization of the first cation and facilitate the A-ring formation, through the cyclization cascade. In order to identify the possible intermediate generated by Lys-448-Ala mutation, CBY57[pZS11][pFHCOSCRS7] strain was grown in large quantity and the ergosterol biosynthetic pathway fractions were extracted and assayed by thin-layer chromatography. The result indicate that the cyclization cascade can not occur and reaction stopped at oxidosqualene, which is consistent with the proposed model that the closure of the A-ring is concerted with oxirane cleavage.

In summary, we have applied site-directed mutagenesis and plasmid shuffle strategies and used them to determine the structure-function relationships of oxidosqualene-lanosterol cyclase. Among alanine-scanning mutants constructed, the Thr-453-Ala, Gly-451-Ala, Gln-450-Ala, Thr-449-Ala, and Thr-447-Ala mutations complement the disruption and exhibit activities qualitatively similar to that of the wild type enzymes, the mutant of Lys-448-Ala is unable to support the growth of this strain.

計畫成果自評

We have successfully accomplished the preliminary objective to study the cyclization/rearrangement reaction mechanism of oxidosqualene-lanosterol cyclase. New data and insights have been presented on understanding the oxidosqualene cyclization reaction, specially the initiation step. Seven site-directed mutants corresponding to the flexible loop region upstream of putative active site within ERG7 gene of *S. cerevisiae* were constructed and tested for their effect on oxidosqualene-lanosterol cyclase activity. The plasmid shuffle and homology modeling results of site-directed mutants indicated that Lys-448 residue might be involved in the initiation of oxirane cleavage or A ring cyclization. Started from the scratch, we have completed about 80% of the planned objectives within a short 7 months period. The obtained result has been advanced in elucidating the possible role of some amino acid residues present in the enzyme, specially for the residues involved in the initiation of cyclization. The scientific value of this research result provides important viewpoint relevant to understand the mechanism of oxidosqualene cyclization as well as rational designing new drugs for therapeutic purpose.

We are on the way to write a paper and plan to submit it to the international journal in short time.

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