行政院國家科學委員會補助專題研究計畫成果報告

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氧化鯊烯環化酵素之導向演化的研究
及其光親和標記的設計與合成

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一、中文摘要

本計畫對於利用導向演化的策略以研究氧化鯊烯環化酵素之環化反應機構已獲得初步結果。經由利用組合基因學針對離氨基酸-448 及白胺酸-443 將其轉變為其他不同胺基酸的結果顯示白胺酸-443 的胺基酸置換對於酵母菌之存活與否具有決定性的影響。經由管柱與薄層色層分析法分離產物的結果發現上述置換無法催化反應的進行而停留在反應物。另一方面，經由分子模擬的結果顯示在三度空間中白胺酸-443 與天門冬酸-456 可能分別位於受質環氧基之兩側而在反應過程中負責穩定碳-2 上所形成的第一個帶正電的高能中間過渡產物以幫助 A 環的合成。由以上的研究結果，預期對增進氧化鯊烯環化酵素之環化反應機構，以及其酵素之特定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，羊毛硬脂醇，定點突變，組合基因學，導向演化，胺基丙酸掃描法，光親和標記

Abstract

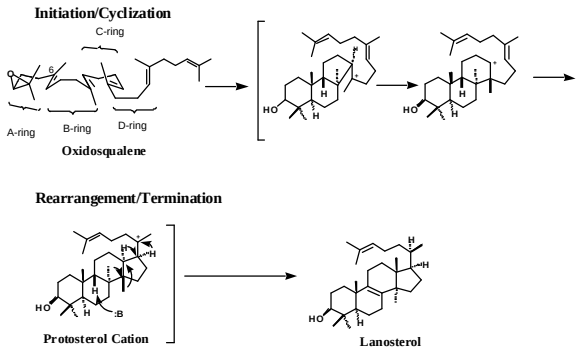
The short-term objectives towards understanding the active site architecture and cyclization mechanism of oxidosqualene cyclase from *Saccharomyces cerevisiae* have been accomplished. Two saturated mutant libraries corresponding to Lys-448 and Trp-443, respectively, were constructed and assayed for their ability to complement the genetic disrupted ERG7 gene of *S. cerevisiae* strain, CBY57, by plasmid shuffle strategy. Among the 9 non-tryptophan mutants, 7 of them failed to complement the ERG7 disruption. Analysis of the cyclase product revealed that the cyclization reaction stopped at oxidosqualene, indicating that Trp-443 replacement might be crucial for cyclase activity. Homology modeling of the cyclase also revealed that the Trp-443, located in opposite position with Asp-456 around the epoxide of the substrate in 3-D structure, might be involved in stabilizing the high-energy C-2 cation intermediate during the A-ring formation. In conclusion, in combination of saturated mutations and analysis of their cyclization products as well as the molecular modeling results, would facilitate better understanding regarding the cyclization/rearrangement mechanism and structure-function relationships of amino acid residues of oxidosqualene cyclases.

Keywords: 2,3-oxidosqualene cyclase, site-directed mutagenesis, directed evolution, photoaffinity label, combinatorial genetics, cassette mutagenesis, lanosterol, cycloartenol

二、緣由與目的

The conversion of (3*S*)-2,3-oxidosqualene (OS) into sterols and tritepenes represents one of the most remarkable and fascinating biotransformations found in nature. The reaction is catalyzed by oxidosqualene cyclases (OSCs) which transform OS to lanosterol in mammals and fungi versus cycloartenol in algae and photosynthetic

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 active site architecture and cyclization mechanism of oxidosqualene cyclase from *Saccharomyces cerevisiae*. We have constructed two saturated mutant libraries of Lys-448 and Trp-443, to study the relationships between active site architecture and cyclization/rearrangement reaction mechanism. The effect of mutations on oxidosqualene-lanosterol cyclase activity was examined by plasmid shuffle technique through assayed for their ability to complement the genetic disrupted ERG7 gene of *S. cerevisiae* strain, CBY57. On the other hand, we tried to design, synthesize several substrate mimics and high-energy intermediates for site-specific labeling.

三、結果與討論

Generation of Saturated Mutants in the Wild Type of *S. cerevisiae* ERG7 Gene.

Two saturated mutant libraries of Lys-448 and Trp-443, respectively, were constructed through replacement of the wild type cyclase gene with the corresponding random primers. 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. Nine non-tryptophan and non-lysine mutants, respectively, were identified through sequencing the 443-448 region of the cyclase gene.

Characterization of Saturated 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 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 saturated mutants, 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 Trp-443-Leu and Trp-443-His 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 Trp-443-Leu and Trp-443-His 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, cyclase mutants bearing Trp-443-Ala, Trp-443-Ser, Trp-443-Pro, Trp-443-Tyr, Trp-443-Asn, Trp-443-Thr, and Trp-443-Arg mutations were unable to complement the ERG7 disruption. The result indicates that above mutations either abolish oxidosqualene-lanosterol cyclase activity completely or reduce it below some threshold level needed to support growth of the ERG7 knockout. In order to identify the possible intermediate generated by Trp-443 mutations, the yeast mutant strains were grown in large quantity and the ergosterol biosynthetic pathway fractions were extracted and assayed by thin-layer chromatography. Analysis of the nonsoponifiable lipid by TLC revealed that the cyclization reaction halted at oxidosqualene, indicating that Trp-443 replacement might be crucial for cyclase activity especially for the closure of the A-ring or oxirane cleavage.

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 Trp-443, in order to gain a more detailed understanding of the structure-function relationships. Homology modeling of the cyclase revealed that the Trp-443, located in opposite position with Asp-456 around the epoxide of the substrate in 3-D structure, might be involved in stabilizing the high-energy C-2 cation intermediate during the A-ring formation.

In summary, we have applied saturated mutagenesis and plasmid shuffle strategies and used them to determine the active site architecture of oxidosqualene-lanosterol cyclase. Among the 9 non-tryptophan mutants constructed, 7 of them failed to complement the ERG7 disruption. Analysis of the cyclase product revealed that the cyclization reaction stopped at oxidosqualene, indicating that Trp-443 replacement might be crucial for cyclase activity.

四、計劃成果自評

We have successfully accomplished the preliminary objective to study the active site architecture and cyclization/rearrangement reaction mechanism of oxidosqualene-lanosterol cyclase. New data and insights have been obtained to facilitate the understanding of the oxidosqualene cyclization reaction, specially in the initiation step. Nine non-tryptophan and non-lysine mutants, respectively, were constructed and tested for their effect on oxidosqualene-lanosterol cyclase activity. The plasmid shuffle and homology modeling results of saturated mutants indicated that Lys-448 residue might be involved in the initiation of oxirane cleavage or A ring cyclization. Furthermore, Trp-443 might be involved in stabilizing the high-energy C-2 cation intermediate during the A-ring formation through spatial interaction with the Asp-456 residue located on the opposite side of the substrate.

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 or A-ring formation. The scientific value of this research result provides important viewpoint relevant to understand the active site architecture and the cyclization/rearrangement mechanism of oxidosqualene cyclase.

五、參考文獻

(1) Abe, I.; Rohmer, M.; Prestwich, G. D. *Chem. Rev.* **1993**, *93*, 2189-2206.

(2) Schroepfer, J. G. J. *Ann. Rev. Biochem.* **1982**, *51*, 555-585.

(3) Mullheirn, L. J.; Ramm, P. J. *J. Chem. Soc. Rev.* **1972**, *259*, 259-291.

(4) Nes, W. R.; McKean, M. L. *University Park Press, Baltimore, MD* **1977**, pp. 229-270.

(5) Ourisson, G.; Rohmer, M.; Poralla, K. *Ann. Rev. Microbiol.* **1987**, *41*, 301-333.

(6) Ourisson, G. *Pure & Appl. Chem.* **1989**, *61*, 345-348.

(7) Corey, E. J.; Virgil, S. C.; Cheng, H.; Baker, C. H.; Matsuda, S. P. T.; Singh, V.; Sarshar, S. *J. Am. Chem. Soc.* **1995**, *117*, 11819-11820.

(8) Corey, E. J.; Cheng, H.; Baker, C. H.; Matsuda, S. P. T.; Li, D.; Song, X. *J. Am. Chem. Soc.* **1997**, *119*, 1289-1296.

(9) Corey, E. J.; Matsuda, S. P. T. *J. Am. Chem. Soc.* **1991**, *113*, 8172-81.

(10) Lesburg, C. A., Caruthers, J. M., Paschall, C. M., Christianson, D. W. *Curr. Opin. in Struc. Biol.* **1998,** *8*, 695-703.

(11) Back, K., Chappell, J. *Proc. Natl. acad. Sci. U.S.A.* **1996**, *93*, 6841-6845.

(12) Griffin, J. H., Buntel, C. J., Siregar, J. J. *Proc. Natl. acad. Sci. U.S.A.* **In Press**

(13) Wendt, K. U., Poralla, K., Schulz, G. E. *Science* **1997**, *277*, 1811-1815.

(14) Sato, T., Hoshino, T. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 2189-2198.

(15) Kusano, M.; Abe, I.; Sankawa, U.; Ebizuka, Y. *Chem. Pharm. Bull.* **1991**, *39*, 239-241.

(16) Abe, I.; Bai, M.; Xiao, X. Y.; Prestwich, G. D. *Biochem. Biophys. Res. Comm.* **1992**, *187*, 32-38.