行政院國家科學委員會專題研究計畫　期中進度報告

烏氨酸異位胺構造及反應機制的研究(1/3)

計畫類別：個別型計畫
計畫編號：NSC91-2320-B-032-001-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：淡江大學化學系

計畫主持人：陳灝平

計畫參與人員：林錦芬、林麗瑩、陳志明、王良哲、陳柏男

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 5 月 23 日

行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※※※

※　鳥氨酸異位脢構造及反應機制的研究**(1/3)** ※

※※※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：☑個別型計畫　□整合型計畫

計畫編號：NSC 91－2320－B－032－001－

執行期間：91 年 8 月 1 日至 92 年 7 月 31 日

計畫主持人：陳灝平 副教授

共同主持人：

計畫參與人員：林錦芬、林麗瑩、陳志明、王良哲、陳柏男

成果報告類型：☑精簡報告

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：淡江大學化學系

中 華 民 國 92 年 5 月 23 日

鳥氨酸異位胺構造及反應機制的研究(1/3)

期中進度報告

計劃編號：91-2320-B-032-001-
執行期限：自民國九十一年八月至民國九十二年七月
主持人：陳灝平 副教授 淡江大學化學系
計劃參與人員：林錦芬、林麗瑩、陳志明 中國醫藥學院生化學科
王良哲、陳柏男 淡江大學化學系

一、中文摘要：

輔胺 B_{12} 需求型的 D-ornithine aminomutase 能夠可逆地將 D-ornithine 轉換成 2,4-diaminopetanoic acid。此酵素是由 S component (13 *Kd*)及 E component (90 *Kd*) 兩個蛋白次單元所構成。由於未來的 EPR, isotope-labeled, kinetic studies…等實驗，需要使用到大量的酵素，在此第一年計劃中，我們首先改進了蛋白質的表現系統，使表現的重組酵素不再需要經過重新摺疊的步驟，使得蛋白質的產率大幅提高。此酵素催化的機制非常複雜，同時有兩種不同的輔胺參與，由於 B12 的碳鈷鍵結，在沒有 B6 存在的情況下不會斷裂，表示酵素的反應受質需先和 B6 形成 Schiff base，B12 才再參與反應。平衡常數經測定為 0.85，反應平衡偏向 D-ornithine 這一方向。我們也針對酵素反應受質的類似物做了探討，只有 2,4-diaminobutyric acid 為一競爭型抑制劑，這些類似物誘發碳鈷鍵結的斷裂，亦尚待進一步的研究。輔胺 B6 是藉由 lysine 629 殘基與酵素結合，由蛋白質一級結構分析，B6-binding motif 緊臨著 B12-binding motif，但是在空間上他們相隔著 B12 的 corrin ring，為了能更明瞭酵素如何同時操縱兩種不同的輔胺，我們構築、表現、純化了突變酵素 OraEX-K629M，不意外的是其並無活性，對此突變酵素 OraEX-K629M 的定性分析，亦正在進行中。

二、前言

There are an increasing number of enzymes known that use organic-based free radicals to catalyze a variety of unusual and important reactions. Many of these reactions have no counterpart in conventional organic chemistry. In comparison with enzymes that catalyze ionic reactions, our knowledge of radical-mediated enzymatic catalysis is still limited. My goal is to understand how enzymes generate free radicals in a controlled manner at the active site, and how these reactive species are used to catalyze novel reactions. I am interested in studying the reversible isomerization of D-ornithine to (2R, 4S)-diaminopetanoic acid, catalyzed by adenosylcobalamin (coenzyme B12) dependent D-ornithine aminomutase, as a model system with which to explore the fundamental problem of how these enzymes generate and exploit free

radicals to catalyze novel chemical reactions. (Fig.1)

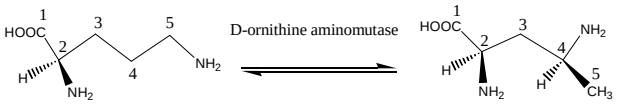


Fig.1 The reaction catalyzed by D-ornithine aminomutase

三、結果與討論：

Protein expression system improvement

We have previously reported that the genes encoding D-ornithine aminomutase, *oraE* and *oraS*, have been cloned, sequenced, and expressed in *E. coli*. (1) However, the majority of OraE protein was expressed in the form of inclusion bodies. Although the enzymatic activity could be restored after refolding, it is still not efficient enough to obtain large amount of proteins in short time for X-ray crystallography, EPR, and isotope-labeled studies. We therefore decided to improve the expression system for OraE and OraS.

Instead to express OraE and OraS in *E. coli* separately, the DNA fragment containing both *oraE* and *oraS* genes was subcloned into pET-28a vector under the control of T7 promoter. The desired plasmid was designated *p*oraESX and used to transform *E. coli* BL21 codon plus. Cultures were grown at 25 ˚C by inoculating 1-ml overnight culture into 1 liter LB medium containing 30 mg/liter kanamycin with a shaking speed of 150 rpm. Incubation was continued until the culture reached an OD_{600} of between 0.8 and 1, at which point the incubation temperature was switched to 20˚C and expression was induced by the addition of 200 mg/liter IPTG.

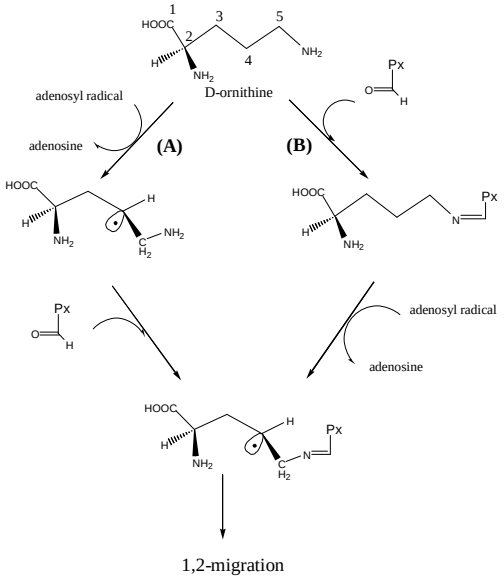
Under this mild condition, a significant portion of expressed proteins is present as soluble, active enzyme in a cell-free crude extract. The recombinant apoenzyme is first purified by a PhenylSepharose High Performance hydrophobic interaction column (26X400 mm). The minor contaminating proteins are further removed by a Q-Sepharose High Performance ion-exchange column (26x200 mm). The pure proteins are stored in –20˚C in the presence of 50% glycerol.

Catalytic steps of the reaction

The equilibrium constant, K_{eq}, of this reaction is measured by NMR analysis. The ratio of D-ornithine to 2,4-diaminopentanoic acid is calculated on the basis of the proton NMR peak area of the corresponding reactant. The equilibrium constant, K_{eq}, is 0.85. The equilibrium direction is in favor of D-ornithine, the less branched one between them.

Fig. 2 Possible chemical routes of D-ornithine aminomutase-catalyzed reaction. (A) The formation of substrate radical is before the formation of Schiff base. (B) The formation of a Schiff base is before the formation of substrate radical.

Whether the formation of the Schiff base between coenzyme B6 and



D-ornithine is essential for the cleavage of the cobalt-carbon bond has not been investigated. The cob(II)alamin molecule, the product of the cleavage of the cobalt-carbon bond of AdoCbl, can be detected by the change of the UV-Visible spectrum. To ensure that most AdoCbl is bound by protein, overwhelming amount of enzyme is used in the measurement. No alteration on the spectrum can be observed when D-ornithine was added into the solution in the absence of coenzyme B6. Therefore, as shown in Fig.2, route (B) is the only pathway occurred in this enzymatic reaction.

Kinetic studies of substrate analogs

Substrate analogs, including D-ornithinol, 2,4-diaminobutyric acid, D-leucine, 5-aminopentanoic acid, 1,3-diaminopropane, and 1,4-diamino- butane, were either purchased from Sigma or synthesized by our group. Only 2,4-diaminobutyric acid is capable of acting as a competitive inhibitor with a K_i of 96.4$\pm$14.1 uM. This result indicates that, within the active site, each function group on D-ornithine plays an important role in the specific binding of the substrate. However, whether these

substrate analogs are able to induce the cleavage of the cobalt-carbon bond of coenzyme B12 is still unknown. To answer this question, several different approaches by employing UV-Vis spectroscopy and tritium-labeled coenzyme B12 is currently conducted in our lab.

Construction, expression, and characterization of mutant enzyme OraEX-K629M

The binding of coenzyme B6 to protein is achieved through the Schiff base formation between a lysine residue of the enzyme and the aldehyde group of the cofactor. By comparison among several nucleotide sequences of PLP-dependent enzyme recently published, the residue lysine 629 appears to be responsible for the binding of PLP (2). Surprisingly, this PLP-binding motif is just situated on the downstream of the B12-binding motif. Because the His-Asp on the B12-binding motif is *under* the corrin ring and the PLP is supposed to locate above the corrin ring, this result strongly implies that the enzyme fine-tunes its activity across the corrin ring through B12- and PLP-binding motif. To further characterize the catalytic mechanism, we therefore decided to construct and express mutant enzyme, OraEX-K629M.

The construction of mutant, *poraEX-K629M*, was carried out using recombinant PCR. Two overlapping, complementary oligonucleotides, gtaatagatattatacatggcggtattgaa and aataccgccatgtataatatctattacttc, were designed to introduce the mutagenic sequence. A 1.9 Kb and 300 base pair region of the *oraE* gene was PCR amplified using pmutEX as template. Both PCR products were gel-purified and assembled in a second round PCR reaction. The PCR product was purified, restricted with *NcoI* and *BamHI*, and ligated with *NcoI/BamHI*-restricted pET-28a vector. The resulting plasmid was designated *poraEX-K629M*. The procedure for expression, and purification of the mutant protein were the same as that of wild type. The mutant protein was refolded in the presence of OraS and AdoCbl. Not surprisingly, the mutant is catalytically inactive.

四、計劃成果自評：

Because I have moved from Department of Biochemistry, China Medical College to Department of Chemistry, Tamkang University on Aug, 2002, it took about one month to set up new lab and lead to a little bit delay in progress. Even so, most experiments have been done as we expected. Now, we are in a good position to continue to investigate the catalytic mechanism of D-ornithine aminomutase. In the next coming year, we will focus on the step of the cobalt-carbon bond cleavage and the role of coenzyme B6. The chemical synthesis of another substrate and reaction

intermediate analogs will be finished before the end of this year. This will also shed some light in the chemical course of this reaction.

伍、參考文獻：

1. HP Chen*, SH Wu, YL Lin, CM Chen and SS Tsay, “Cloning, sequencing, heterologous expression, purification and characterization of adenosylcobalamin-dependent D-ornithine aminomutase from *Clostridium sticklandii”*. (2001) *Journal of Biological Chemistry*, **276**, 44744-44750.
2. KH Tang, A Harms, and PA Frey, “Identification of a Novel Pyridoxal 5’-Phosphate Biding Site in Adenosylcobalamin-Dependent Lysine 5,6-Aminomutase from *Porphyromonas gingivalis*” (2002) *Biochemistry*, **41**, 8767-8776.