Zn-induced Regulation of *E. Coli* Orinithine Transcarbamolyase

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금속 아연 이온에 의한 대장균 오니틴 트랜스카바밀라제의 제어통제

이 선 주

요 약

요소생합성 사이클에 속하는 오니틴 카바밀라제는 원래 allosteric 효소가 아니다. 그러나 금속이온 아연이 오니틴의 효소의 결합에 있어서 반응속도론적인 비선형적 곡선을 유발시킨다는 것이 발견되었다. 이 연구에서는 형광스펙트럼 결과들이 금속이온 아연이 효소와 allosteric하게 결합하여 효소의 구조를 변화시킴과 동시에 느리며 비가역적으로 결합하여 효소활성의 변화를 유발하는 한편, 효소와 인산 카바밀이 결합된 복합체에는 아연이온이 오니틴에 대한 가역적 경쟁관계의 제어제로서 작용한다는 것을 증명하였다.금속과 오니틴의 결합자리는 site-directed mutagenesis를 이용하여 알아 냈는 데 효소의 Cys-237이 아미노산 결합리간드중의 하나로 작용한다는 것을 발견하였다. 이사실은 같은 결합리간드에 대해서 금속과 오니틴이 경쟁적으로 행동한다는 것을 제시하며 그 결과 오니틴의 효소와 의 상호작용에 있어서 비선형적 S 곡선을 나타내게 되는 것이다. 종합적으로 이 연구 결과의 생물학적인 행동을 고찰하여 보면 금속아연 이온은 1) allosteric cofactor 2) slow, tight building inhibitor로 작용하여 오니틴 트랜스카바밀라제를 제어 통제한다.

INTRODUCTION

Ornithine transcarbamoylase (ornithinecarbamoyltransferase, carbamoyl phosphate : L-ornithine carbamoyl phosphate, EC 2.1.3.3) is a trimeric protein consisting of three identical 37,000 Mr subunits. This enzyme catalyzes the synthesis of L-citrulline by the transfer of carbamoyl group from carbamoyl phosphate to L-ornithine and displays Michaelis-Menten kinetics.

Although ornithine transcarbamoylase catalyzes the first step of urea cycle in the mitochondria of eukaryotes, the anabolic enzyme is not known to be allosteric. The aim of this study is to scrutinize the interactions between ornithine transcarbamoylase and zinc. To elucidate the mechanism of regulation of ornithine transcarbamoylase by Zn(II), fluorescence data on the complexation of enzyme and zinc in the absence of substrates are presented to demonstrates that zinc binds ornithine transcarbamoylase co-operatively and that site-site interactions are responsible for the observed sigmoidal saturation curves. Kinetic data are also presented to show that the type of inhibition pattern of the enzyme with its ligands prior to the onset of substrate turnover. To locate and identify the metal sites, site-directed mutagenic methods have been applied to remove plausible protein ligands to the metal.

MATERIALS AND METHODS

The following reagent grade chemicals were purchased from SIGMA Chemical Company and used without further purification: dilithium carbamoyl phosphate, L-ornithine, L-citrulline, 2,3-butadiene monoxime, Tris base, EDTA. Analytical reagent grade zinc acetate and zinc standard for the atomic absorption measurements were purchased from Baker Chemicals. Ornithine transcarbamoylase were purified from *E.coli* K-12, and TB 2 cells containg mutant enzyme as described (Kuo *et al.*, 1985).

Kinetic assays were conducted as described (S. LEE *et al.*, 1990). Fluorescence emission data were collected on a Perkin-Elmer MPF-44A spectrofluorometer in the "ratio" mode using an excitation wavewlwngth of 280nm. The *E.coli* K-12 *argI* gene, which codes for ornithine transcarbamoylase was subcloned to M13mp19 to yield the DNA template for sequencing and site-directed mutagenesis. Site-directed mutag-enesis was conducted as described (Kuo *et al.*, 1988). Details for the chemical modification of the cysteine residue and gel filtration of zinc ion was described previously (S. Lee *et al.*, 1990).

RESULTS AND DISCUSSION

Effects of zinc on the activity of ornithine transcarbamoylase depends on the preincubation pattern. At single point assay, the activity of ornithine transcarbamoylase observed in the absence and presence of zinc is drmatically different. In the absence of metal, the initial velocity of the enzyme is constant over time. When the enzyme is preincubated with zinc prior to the onset of substrate turnover, the reaction velocity decreases slowly as a function of preincubation time. The loss in initial velocity is pseudo-first order. The effect of zinc on the fluorescence of the enzyme is same as that on the enzyme activity. The decrease in fluorescence emission of the enzyme to its final intensity as a result of zinc binding is a slow process in order of minutes. The resultant effects of ornithine transcarbamoylase upon the binding of metal is due to the isomerization of protein. Addition of metal chelators do not recover enzyme activity and the loss in fluorescence intensity. The isomerized enzyme contains 3 molecules of zinc according to the result of gel filtration. Therefore, zinc is called irreversible, slow tight binding inhibitor (Morrison & Walsh, 1988). The initial velocity of the loss of fluorescence intensity at different metal concentration has been measured to determined the mode of metal binding on the enzyme (Figure 1). The data shows that Hill constant is 1.5, which indicates that metal binds enzyme positive cooperatively.

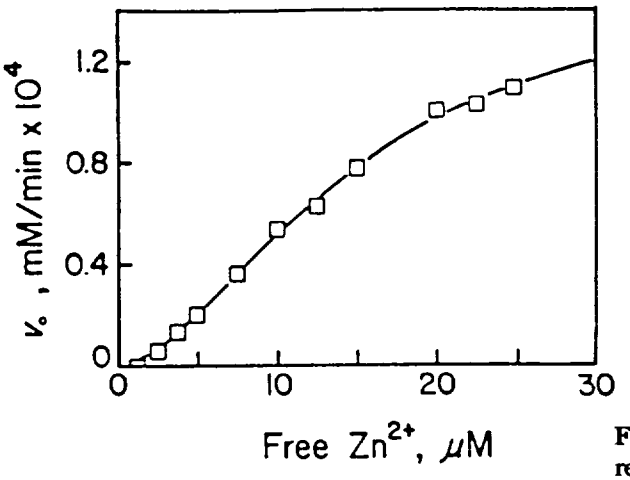

Figure 1. Initial velocity of emission fluorescence decrease of ornithine transcarbamoylase as a function of free zinc ion oncentration. Free zinc ion concentrations were calculated from total added metal concentration based on the composition of the reaction buffer used.

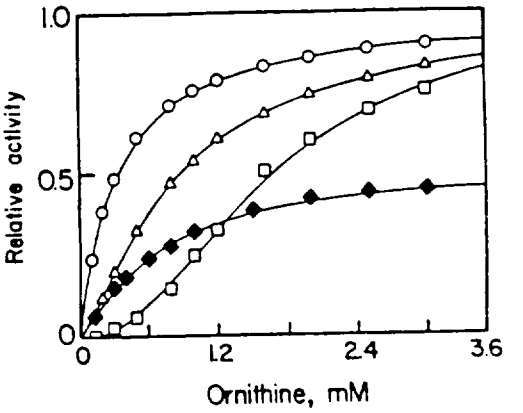

Figure 2. Initial velocity ofthe forward reaction catalyzed by ornithine transcarbamylase as a function of L-ornithine concentration.

However, in the presence of carbamoyl phosphate prior to the addition of metal in the reaction solution, metal does not induce time dependent loss in activity and fluorescence intensity. It behaves as a classical, reversible inhibitor. Ornithine saturation curve has been obtained under the reversible condition. The data shows that the shape of curve is sigmoidal and the Hill constant depends on the concentration of metal ion (Figure 2). The maximum Hill constant reaches 2.5. Metal behaves as a competitive inhibitor of ornithine. The specificity of metal has been determined. The results show that only zinc ion exerts significant effects on the enzyme as mentioned.

To delineate a possible ligand of metal binding, site directed mutagenic methods have been applied. Since sulfur group of cysteine residue in proteins is one of strong ligands of metal, 4 Cysteine groups of ornithine transcarbamoylase have been mutated to alanine, which has no charge but similar size to cysteine. Correct mutation have been confirmed by DNA sequencing, spectroscopic and chemical modification results. Kinetic properties of each mutant enzyme have been obtained and compared to those of the wild type enzyme. Among 4 mutant enzymes, only Cys-273 mutant enzyme has effect on the affinity of L-ornithine and Zn(II). Affinities of L-ornithine, L-norvaline and zinc have decreased about 14-, 10- and 3 folds, respectively (Table 1). The Hill constant of

the Cys-273 mutant enzyme (1.6) is statistically the same as the wild-type enzyme (1.5), but the concentration of zinc needed to achieve a 50% saturation increases from 0.15mM to 0.47mM when the thiol group of Cys-273 is mutated. Thus only the binding affinity of zinc is diminished in the mutant enzyme. Furthermore, Cys-273 mutant enzyme is expected to display similar cooperative behavior at pH 8.5 as the wild-type at a reduced pH. This is indeed the case. At pH7.5, the metal induced cooperativity of the enzyme is similar to that of the Cys-273 mutant enzyme. When fluorescence intensities of 4 mutant enzymes are measured in the presence of zinc, only Cys-273 mutant enzyme shows remarkably slow decrease in the fluorescence intensity. However, final intensities of fluorescence emission of 4 mutant enzymes in the presence of zinc are same over time. The results indicate that Cys-273 residue is a ligand of the L-norvaline, L-ornithine and zinc, and that the residue is not involved in the transmitting process of allosteric conformational change and in the inactivating isomerization upon the binding of zinc to the free enzyme.

The combined results suggest that zinc regulates ornithine transcarbamoylase via two routes: (1) as an allosteric cofactor of the substrate-bound enzyme in mediating site-site interactions, and (2) as a slow, tight binding inhibitor of the free enzyme in inactivation.

Summary

Ornithine transcarbamoylase in the urea cycle is not known to be allosteric. However, it has been shown that Zn(II) can introduce sigmoidal kinetics in L-ornithine binding. In this work, fluorescence spectroscopic results show that

Table 1. Kinetic and binding constants of ornithine transcarbamolyase and its site-directed Cys-Ala point mutants.

Enzyme	Optimal pH	K_{cat} x 10^5	Michaelis constants		Norvaline binding
			Km^{cp} mM	Km^{orn} mM	Ki^{nor} mM
wild-type	6.5-8.5	1.41(.02)	0.05(.001)	0.32(.01)	0.06(.004)
Ala 60	6.5-8.5	1.36(.04)	0.05(.002)	0.32(.03)	0.06(.005)
Ala 191	6.5-8.5	1.33(.02)	0.04(.003)	0.24(.02)	0.05(.003)
Ala 201	8 -10	0.64(.05)	0.04(.003)	0.36(.02)	0.07(.003)
Ala 273	8 -10	0.68(.01)	0.04(.001)	4.2 (.3)	0.56(.07)

Zn(II) adds allosterically to the free enzyme and induces conformational change over time resulting in loss of activity. When the enzyme is first bound with carbamoyl phosphate, steady-state kinetic results show that metal ion is a classical competitive inhibitor against L-ornithine. The binding site of metal and L-ornithine has been delineated by site-directed mutagenic methods. The Cys-273 mutant enzyme is found to be a ligand of L-norvaline, L-ornithine analog, and metal. As a result of competition between Zn(II) and L-ornithine for the same site on the enzyme, the L-ornithine saturation curves sigmoidal. Therefore, zinc regulates ornithine transcarbamoylase: (1) as an allosteric cofactor and (2) as a slow, tight binding inhibitor.

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