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Role of Ser-33 and Asp-112 Residues in *In vivo* Folding of *E. coli* Tryptophan Synthase α Subunit

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Abstract

In the present report, a possibility of the interaction of Ser-33 and Asp-112 residues in folding of tryptophan synthase α subunit was explored by examining the effect of single or double substitution of these residues on folding of α subunit in *E. coli.* α subunit of which Ser-33 was substituted with Leu (SL33) was accumulated as insoluble aggregate form, when overproduced in *E. coli*, whereas α subunit of which Asp-112 was replaced by Asn (DN112) or Gly (DG112) was accumulated as soluble form to the similar extent as wild type α subunit was. When these alterations were combined into one protein, the synergistic effect of residues 33 and 112 on the amount of aggregate form was shown. The amount of doubly altered SL33/DG112 α subunit as aggregate form was increased 5-13 fold that of SL33 α subunit, and the amount of SL33/DN112 α subunit as aggregate form was decreased 3-4 fold that of SL33 α subunit. Aggregates are derived from the specific association of partially folded or unassembled subunits in the folding process. Therefore, this result suggests that residues 33 and 112 of α subunit may interact during the folding of this enzyme in *E. coli.*

Key words : in vivo folding, tryptophan synthase α-subunit, mutant

Introduction

It is generally accepted that the amino acid sequence of a protein determines its three-demensional structure[1]. However, the mechanism by which the amino acid sequence of a protein directs the folding to the native functional conformation is not known in detail for any protein. This lack of understanding is due in part to the high cooperativity of the folding transition and the concomitant absence of stable intermediates and in part to the rapid rate of folding which precludes the use of high-resolution techniques such as X-ray diffraction or nuclear magnetic resonance (NMR) spectroscopy[2].

The native conformation of proteins is maintained by numerous noncovalent and covalent interactions resulting from hydrogen bonds, salt bridges, van der Waals interactions, hydrophobic interactions and disulfide bonds. Although the stabilization energy contributed by any one of these interactions is small, it has long been known that they act in a cooperative fashion to stabilize a single three-dimensional structure for a given amino acid sequence.

Recent studies showed additional factors involved in in vivo folding of protein : (i) Peptidyl prolyl cis-trans

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isomerase (PPI), which catalyzes the slow isomeration of X-P peptide bonds (where X is any amino acid and P is proline in single-letter amino acid code), can accelerate the refolding rate of proline containing polypeptides *in vitro* and *in vivo*[3]. (ii) The rearrangement of disulfide bond can be promoted by protein disulfide isomerase (PDI) which catalyzes thiol/disulfide interchange reactions[4]. (iii) Chaperones function *in vivo* not as catalysts of secondary structure formation, but rather to recognize and stabilize partially folded intermediates during polypeptide folding, assembly and disassembly[5]. (iv) The ligands increase the stability and affect folding process of protein[6]. For example, the renaturation of dimeric horse liver alcohol dehydrogenase was accelerated by Zn^{++}. (v) The cofactors affect not to functions but to folding and assembly. For instance, the pantothetenate is required for the assembly of cytochrome c oxidase and ATPase/ATP synthase in *Neurospora crassa*[7].

Roles of particular amino acids in protein folding can now be elucidated by using single amino acid replacement[8]. An excellent candidate for this approach is the tryptophan synthase (TSase) α subunit of *Escherichia coli*, since it is a monomer in solution and contains no prosthetic group or disulfide bond[9].

TSase is composed of $\alpha_2\beta_2$ multienzyme complex, catalyzes final two reactions in the biosynthesis of L-tryptophan[9,10]. The α subunit (M_r=28,600) alone catalyzes the cleavage of indole 3-glycerol phosphate (IGP), termed the α reaction.

Indole 3-glycerol phosphate ⇄
indole+D-glyceraldehyde 3-phosphate

The β subunit (M_r=43,500) usually exists as a dimer, contains one molecule of a cofactor pyridoxal phosphate (PLP) per each subunit, and catalyzes the synthesis of L-tryptophan (L-Trp) from indole and L-serine (L-Ser) termed the β reaction.

Indole+L-serine ⇄ L-tryptophan+H_2O

When the α and β subunits combine to form the $\alpha_2\beta_2$ complex, the rates of the α and β reactions and the affinities for substrates in these reactions are increased[11]. This activation is contingent on conformational changes that occur during the assembly of the nature $\alpha_2\beta_2$ complex. The physiologically important reaction catalyzed by the $\alpha_2\beta_2$ complex, termed the αβ reaction, is the sum of the α and β reactions.

Indole 3-glycerol phosphate+L-serine ⇄
L-tryptophan+D-glyceraldehyde 3-phosphate+H_2O

TSase $\alpha_2\beta_2$ complex from *E. coli* has been crystallized, but crystals suitable for x-ray crystallography have not been obtained. In contrast, crystals of the enzyme from *Salmonella typhimurium* yielded good x-ray data[12]. The high homology with the α and the β subunits from *E. coli* and from *S. typhimurium* suggests that the conformations of these two complexes will be quite similar.

Previous studies on the folding of the α subunit have proposed that one or more stable intermediates appear during folding[13]. The structural interpretation of the principal intermediate is based upon the observation that the protein can be converted to two fragments by limited tryptic digestion[13]. The amino region consists of residues 1−188 and carboxyl region of residues 189−268. The isolated fragments can be combined to form active enzyme in solution. The X-ray structure shows that the amino region corresponds to the first six strands and five helices of this α/β barrel proteins, while the carboxyl region corresponds to the last two strands and three helices. The principal intermediate has been proposed to consist of a folded amino domain and an unfolded carboxyl domain on the basis of both difference UV and circular dichroism spectroscopies[14]. The amino domain was shown to be substantially more stable than the carboxyl domain. Also, the unfolding transition of the isolated amino domain is coincident with the second of the two transitions that are observed in the intact protein[15]. Thus, there appears to be a good correlation between the structure of the principal stable intermediate and the state of folding of the structural domains in the native conformation.

Certain mutant TSase subunits containing single amino acid difference from the wild type protein were expressed *in vivo* as large, insoluble aggregates[16]. Aggregates are derived from the specific association of partially folded or unassembled subunits in the folding process, and the end products of aggregation *in vivo* are dense, insoluble protein particles (inclusion body). Such mutant α subunits are of particular interest for structure-function studies because they may represent polypeptides with altered folded properties[14].

When α subunits encoded in plasmids containing *trpA* plus *tac* promoter were overexpressed by lactose, SL33 single mutant enzymes (XY refers to the replacement of X by Y at position 33 in one letter abbreviation) were mainly expressed as insoluble aggregates, whereas wild type, DG112 and DN112 single mutant enzymes were expressed as soluble forms[17].

Differential scanning calorimetry (DSC) was used to compare stability of each mutant protein. The SL33 replacement was shown to decrease the Tm by 7.53℃ ; the DN 112 or DG alteration decreased the Tm's by 3. 08 and 5.08℃, respectively[17]. Nevertheless, these single amino acid replacements don't affect to global structural alterations, because these mutant proteins showed enzymatic activities similar to those of wild type α subunit in α, β, and αβ reactions. Therefore, Ser 33 and Asp 112 residues of *E. coli* TSase α subunit appear to be involved in folding and/or stability of this enzyme.

In order to examine a possible interaction of residue 33 and residue 112 during folding, these two substitutions were combined into one protein, and *in vivo* folding properties of doubly substituted mutant subunits were examined.

Materials and Methods

Chemicals

Ampicillin, chloramphenicol, indole, L-serine, dithiothreitol (DTT), phenylmethyl sulfonylfluoride (PMSF), acrylamide, N,N,-methylene-bis-acrylamide, and D-lactose were purchased from Sigma (USA). Agarose and phenol were purchased from BRL (USA). Restriction endonucleases, Sephaglas™ BandPrep Kit for DNA ligation were purchased from Phamarcia (USA).

Bacterial strain and plasmids

E. coli RB797 (F', *lacI*^{q}, *proL8/arg, nalr, rif, recA, sup, lac, pro*) was served as the host strain for plasmids containing the wild type or mutant *trpA* genes or *trpB* gene. Construction of plasmids and preparation of mutant subunits are described by Milton *et al.*[9].

Wild type and SL33 TSase α subunit genes are in plasmid containing the ampicillin resistant β-lactamase gene. DG112, and DN112 α subunit genes and wild type β subunit gene are present on a chloramphenicol acetyltransferase (*CAT*) gene encoding plasmid. The β-lactamase and *CAT* genes are widely used as selection marker genes.

DNA manipulation

The plasmid DNA was routinely prepared by the alkaline lysis method[18]. Each isolated DNA was digested using Cla I and Sal I restriction endonucleases. After restriction endonuclease digestion of the DNA, the fragments were separated by 1% agarose gel electrophoresis in L-buffer (36mM Tris base, 30mM NaH_2PO_4, 1mM Na_2EDTA (pH 8.0)). Extraction of DNA from agarose gel was carried out by Sephaglas™ BandPrep Kit from Pharmacia. The ligation reaction was done by ligation reaction mixture containing 10x ligation buffer (100 mM Tris-HCl (pH 7.4), 50mM KCl, 1mM DTT, 0.1mM EDTA, and 50% glycerol) 10ng/ml BSA, 1mM ATP, T 4 DNA ligase. and then the mixture was incubated overnight at 12℃.

Transformation of *E. coli* strain RB797 with mutant plasmid was done by the $CaCl_2$ method[18]. Since the double mutant plasmid for SL33 and DG112/DN112 mutant TSase α subunit conferred ampicillin resistance to their host cells, the transformed bacteria were spreaded on TYS plates containing ampicillin (30μg/ml) and

incubated at 37℃ overnight.

Growth of cells and preparation of cell-free extracts

E. coli strains containing plasmid were grown at 37℃ with shaking in TYS broth containing ampicillin (30μg /ml) or chloramphenicol (10μg/ml). 20ml of overnight-grown cells were subcultured into 1ℓ of a modified TYS broth containing appropriate antibiotic. Lactose (1%, final concentration) was added to cells at an OD_{600} of 0.4 to 0.5. Following growth for 20 to 22 hrs for α subunit or for 4 to 5 hrs for β_2 subunit, the cells were harvested by centrifugation at 28,000×g for 20 minutes, and washed twice with 20mM potassium phosphate buffer (pH 7.8) containing 5mM DTT, 5mM EDTA, and 0.2mM PMSF.

The cells were resuspended in 20mM potassium phosphate buffer (pH 7.8) containing 5mM DTT, 5mM EDTA, 0.2mM PMSF and sonicated. The sonicated cells were centrifuged at 28,000×g for 15 minutes. The preparation of crude extracts containing β_2 subunit was similar to those described above except that the buffer was 0.2M potassium phosphate buffer (pH 7.8) containing 10mM β-mercaptoethanol, 5mM EDTA, 0.5mM PMSF, and 0.02mM PLP. Induction of α and β_2 subunit by lactose was ascertained on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[19]. The supernatant was stored at −70℃ until used.

Protein assays

The concentrations of crude extracts were measured by microbiuret method with bovine serum albumin as a standard[22].

Results

Construction of plasmids for double mutant SL33/DG112 and SL33/DN112 of the TSase α subunit

The double mutant vectors were obtained by combining different fragment of expression vectors which have single mutants (Fig.1). Plasmid DNA's containing single mutant *trpA's* were digested by Cla I and Sal I.

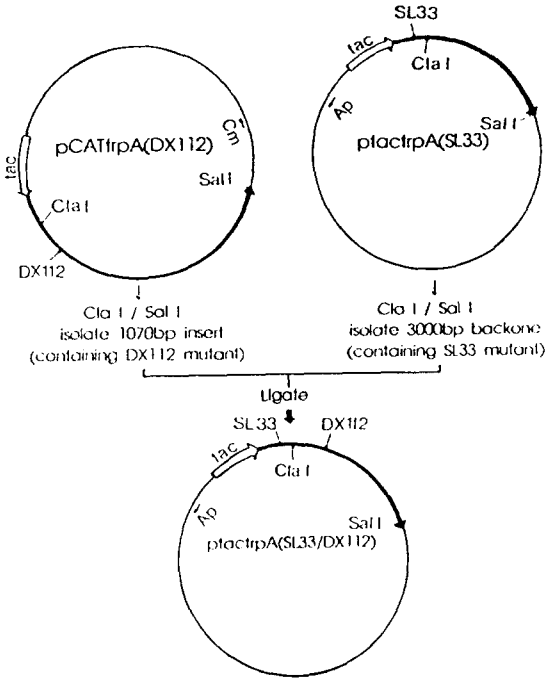

Fig. 1. Construction of vectors for double mutant subunits. The open bar arrow represents the *tac* promoter and indicates the direction of transcription. The solid bar represents the *trpA* gene encoding TSase α subunit. The detailed explanation for the construction of vectors are described in Materials and Methods. Single mutant vectors were prepared previously by Lim *et al.*[16].

1,070bp fragment of the *pCATtrpA* gene containing DX 112 was ligated to *ptactrpA* backbone containing SL33 mutant by T4 DNA ligase. Since the double mutant plasmid for SL33/DX112 conferred ampicillin resistance to their host cells, the transformed bacteria could be selected on TYS plates containing ampicillin. Transformation with backbone alone, which was processed exactly by the same way except omitting insert, resulted in no transformant. Right size of insert was verified on agarose gel after treatment of restriction endonuclease Cla I and Sal I.

In vivo overexpression of wild type, singly or doubly substituted TSase α subunits

The *trpA* genes are under the control of the *tac* promoter and are inducible by lactose[9]. The wild type α subunit constituted more than 30% of total soluble protein and a little amount of aggregated pellets inside cells after 22 hr induction.

Because the altered amount of aggregated pellets may be due to the altered kinetics and (or) pathway of protein folding, the amounts of soluble and aggregated forms of doubly substituted TSase subunits together with wild type and singly altered ones were examined.

Overnight-grown RB797 celles carrying plasmids with various trpA genes were subcultured. When OD at 600 nm reached to approximately 0.4, TSase α subunits were induced with lactose. Cells were harvested after 6 hr and 22 hr, and lysed by sonication. Soluble and pellet fractions were run on SDS-PAGE (Fig. 2). Majority of wild type subunits were overexpressed as soluble form to the extent similiar to that reported previously[9]. In case of singly altered mutants, larger amount of SL33 α subunit was accumulated as insoluble aggregates compared to wild type α subunit, which was prominent after 22 hrs induction, whereas DG112 and DN112 α

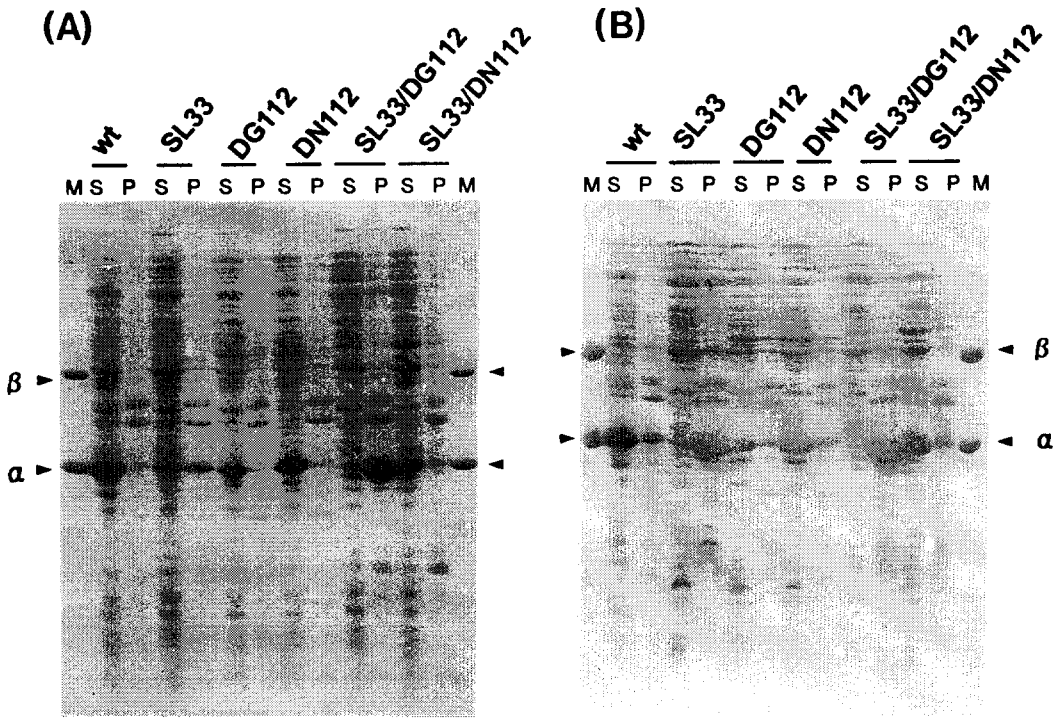

Fig. 2. Amounts of wild type and various mutant α subunits in the soluble or aggregated forms after lactose induction. *E. coli* cells carrying various *trpA* genes were havested after 6hrs (A) or 22hrs induction with lactose (B). The cells were resuspended in 20mM potassium phosphate buffer (pH 7.8) containing 5mM DTT, 5mM EDTA, 0.2mM PMSF, and sonicated, and centrifuged at 28,000×g for 15 minutes. The pellet (P) was resuspended in the same buffer. 100μg of each supernatant (S) was loaded, and the amount of loaded pellets are equivalent to that of supernatant in terms of volumn. α and β (lane M) indicates the mobilities of TSase α and β subunit, respectively.

Table 1. Summary of densitometric scans of gels

TSase	Fraction of total α subunit (%)[a]		Ratio of aggregated α subunit to soluble α subunit[b]	
	6 hr induction	22 hr induction	6 hr induction	22 hr induction
Wild type	56	58	0.04	0.24
SL33	20	18	0.3	1.9
DG112	28	18	0.08	0.59
DN112	39	24	0.07	0.49
SL33/DG112	29	18	3.8	10.2
SL33/DN112	32	41	0.11	0.49

The amounts of protein bands on gels were presumed to correspond to their peak areas of gel scans.

[a] means percentile of total subunits (both soluble and pellet forms) to total cell proteins : this was calculated by following equation $(A+(B\times D)\div 100)/(100+D)$, where A is ratio of soluble α subunit to total soluble protein, B is ratio of aggregated α subunit to total pellet protein, and D is ratio of aggregated α subunit to soluble α subunit.

[b] obtained by following equation $((A\times 100)/B)\times C$, where C is ratio of net amount of α subunit in supernatant and pellet, and A and B are defined as above.

subunit showed wild type like patterns. These are also consistent with the previous report[26]. When SL33 alteration and DG112 or DN112 was combined into one subunit, SL33/DG112 α subunits were more severely converted into aggregate pellets than either SL33 or DG 112 α subunits did, whereas the distribution of SL33/DN112 α subunit was not significantly different from that of SL33 or DN112 α subunit. The amount of subunits were quantitatively estimated by densitometric scanning and summarized in Table 1.

Any mutant α subunit didn't exceed the level of total wild type proteins, even though all mutant α subunits were accumulated to the significantly high levels.

Distribution of α subunits in soluble and pellet forms was varied depending on the kinds of alteration. In case of wild type α subunit, the ratio of aggregated form to pellet one was 0.04 and 0.24 after 6 hr and 22 hr, respectively.

Importantly, it is clear here that SL33/DG112 double substitutions increased the formation of insoluble aggregates of α subunits compared to either SL33 or DG112 singly substituted α subunit. The ratio of aggregated SL 33 or DG112 α subunit to its corresponding soluble protein was 0.3 or 0.08 after 6 hr induction, respectively, and 1.9 or 0.59 after 22 hr induction, respectively. But, the ratios of SL33/DG112 mutant α subunit were 3.8 and 10.2 after 6 hr and 22 hr induction, respectively. This indicates that additional DG112 replacement of SL33 mutant α subunit increased the ratios of aggregated SL33 α subunit to it soluble form, respectively, 12.7 and 5.4 fold after 6 hr and 22 hr induction.

Contrary to this, the additional DN112 substitution of SL33 mutant subunit increased the formation of soluble α subunit. The ratios of aggregated SL33/DN112 subunits to its soluble forms were reduced about 3 fold (0.3→0.11), and about 4 fold (1.9→0.49) after 6 hr and 22 hr induction, respectively, compared to that of SL33.

Discussion

Generally, an amino acid either plays a role in itself or cooperates with other amino acids in the function of

protein. Example of such interactions can be found in classical genetic studies of second-site revertants, e.g., the α subunit of tryptophan synthase[23] and, more recently, the λ repressor/operator system, where the loss of binding due to substitution of one amino acid can be recovered by substitution at a second site[24]. The use of mutagenic method is suitable for this kind of study. Previously it was reported that the functional interaction between two residues can be measured by quantitatively comparing the properties of the two singly substituted proteins and the protein containing both mutations[25].

Similar strategy could be appiled to dissect protein folding process. Cooperativity of two sites in the folding, which are separated in three dimentional structure of a native protein, can be examined by mutagenic approach.

Both residues 33 and 112 of *E. coli* TSase α subunit are located in N-terminal folding domain, but separated distantly enough not to have any direct interaction between two sites composed of residues directly interacting with these two residues. The altered ratio of aggregated α subunit to soluble α subunit may be indicative of changed folding kinetics/pathway. Aggregates is reported to be formed by intrachain interaction of partially misfolded protein in the folding process of protein tertiary structure.

SL33/DG112 double substitutions increased the formation of insoluble aggregates of α subunits compared to either SL33 or DG112 singly substituted α subunit (Table 1 and Fig. 2). Contrary to this, the additional DN112 substitution of SL33 mutant α subunit increased the formation of soluble subunit.

If the sum of the effects of two single substitutions on the folding pattern of the wild type protein equals the change seen in the double mutant, two sites sorrounding these residues may not interact in the folding process. Because SL33/DG112 double mutant α subunit more easily formed insoluble aggregates than SL33 or DG112 single mutant subunit did, Leu-33 and Gly-112 have negative synergistic effects in folding, compared to wild type. Since SL33/DN112 mutant α subunit reduced the formation of insoluble aggregates and increased the amount of soluble form compared with SL33 α subunit, positive synergistic effects are shown.

Therefore, this result suggests that residues 33 and 112 located in N-terminal domain may synergistically play important roles in the folding of N-terminal domain of this protein.

Detailed properties of cooperativity of these two sites remain to be examined further by other methods, for example, urea denaturation folding/unfolding.

In addition, it could be suggested that it may be possible to suppress the aggregation of proteins frequently encountered during *in vivo* overexpression of recombinant proteins by specifically changing residue(s) that plays a critical role in the folding process.

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초록 : 트립토판 중합효소 α 소단위체의 대장균내 구조형성과정에서의 Ser-33과 Asp-112 잔기의 역할

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최근에 발표된 연구 결과에 의하면 대장균 트립토판 중합효소 (tryptophan synthase) α 소단위체에서 33번 잔기의 세린과 112번 잔기의 아스팔산은 이 효소의 구조 형성 과정(folding)에 관여하는 것으로 보여진다. 이들 효소를 대장균내에서 과발현시켰을 때, SL33(잔기 33번의 세린이 류신으로 치환) 효소는 대부분 응집된 덩어리형태의 효소로 생성되고, DG112(잔기 112번의 아스팔산이 글리신으로 치환) 효소와 DN112(아스팔산이 아스파라진으로 치환) 효소는 야생형 효소와 비슷한 양의 수용성형태로 생성된다고 보고된 바 있다. 본 연구에서는 이 효소의 구조 형성 과정동안 33번 잔기와 112번 잔기가 상호 작용하는가를 조사하기 위하여 두 자리 잔기 치환 단백질을 만들어 이들의 성질을 단일 잔기 치환 단백질들과 비교하였다. 이들을 대장균내에서 젖당으로 과생산하여 전기영동으로 조사하여 본 결과 SL33 단일 잔기 치환 효소에 비해 SL33/DG112 이중 치환 효소는 현격히 보다 많은 양이 응집된 형태의 불용성 효소로 생성되며, SL33/DN112효소는 많은 양의 효소들이 가용성 효소로 생성되었다.

Densitometer로 정량한 결과 SL33/DG112 치환 소단위체는 SL33 치환 소단위체에 비해 가용성 형태의 양에 대한 응집된 형태의 양의 비가 6시간 젖당처리시 약 5배, 22시간 젖당처리시 약 13배 증가하였으며, SL33/DN112 치환 α 소단위체는 그 비가 1/4−1/3로 감소했다. 이러한 결과는 33번 위치에서 세린이 류신으로 치환되었을 때 나타나는 구조 형성 과정의 변화를 112번 위치에 새로 치환된 글리신 또는 아스파라진이 영향을 미쳐, 구조 형성 과정 변화가 더욱 더 변화했거나 원상태로 어느 정도 회복되었음을 의미한다.

이 결과는 대장균 트립토판 중합효소 α 소단위체의 N 말단 도메인(N-terminal domain)의 구조 형성 과정시 33번 잔기와 112번 잔기가 상호작용을 하고 있음을 시사해 주고 있다.