ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science **2013** Vol. 23. No. 2. 319~323 DOI : http://dx.doi.org/10.5352/JLS.2013.23.2.319

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Various Aggregate Forms of Tryptophan Synthase α-Subunit

Myung Won Park and Woon Ki Lim*

Department of Molecular Biology, College of Natural Sciences, Pusan National University, Busan 609-735, Korea

Received January 15, 2013 / Revised January 26, 2013 / Accepted January 26, 2013

Protein aggregation can cause diseases and hinder the production of useful recombinant proteins. The present study showed that at least three types of aggregates can be formed from tryptophan synthase α-subunit (αTS) by varying conditions: (1) an opaque white precipitous aggregate, (2) a transparent gel-like precipitous aggregate, and (3) an unprecipitous aggregate. Macroscopically different aggregate types might suggest different mechanisms underlying aggregation processes.

Key words : Opaque white aggregate, protein aggregate, transparent gel-like aggregate, tryptophan synthase α-subunit, unprecipitous aggregate

Introduction

Proteins can fail to form native structures and such failures result in a wide range of symptoms [13]. Pathological states such as Parkinson's diseases and Filaminopathy belong to this category. In addition, proteins are often found in large intracellular aggregates during biotechnical production in bacteria, hindering their utilization. Protein aggregation occurs in competition with the normal folding pathway [2], and it takes place from misfolded and partially unfolded states. Although this area expanded rapidly in recent years, many things, especially when it comes to non-fibrillar protein aggregation, remain largely unknown.

Tryptophan synthase α subunit (αTS) from *E. coli* consists of 268 residues, and has no disulfide bond or prosthetic group. It was shown by X-ray structure to have $(\beta\alpha)_8$ barrel motif, a popular protein fold [5, 10]. The αTS has been extensively studied for its folding property [14], but its aggregation properties were not. The mutational studies on α TS had produced a variety of mutant proteins with different tendency of aggregation [6, 9]. Investigation of aggregation property of these proteins could help to understand underlying mechanisms of protein aggregation. In the course of characterizing such abnormalities, we found different types of aggregates.

***Corresponding author**
Tel : +82-51-510-2289, Fax : +82-51-513-9258.
E-mail : wklim@pusan.ac.kr

Materials and Methods

Chemicals

All chemicals were of reagent grade or ultrapure quality and purchased from Sigma (St. Louis, MO).

Overexpression and purification of wild-type and mutant proteins

Construction of plasmids for wild type, T24A/E49G/F139W and T24A/D60N/F139W αTSs were described [11]. The wild-type and the mutant proteins were overproduced in *E. coli* RB797 and then purified as described elsewhere [6]. Proteins were kept by addition of 85% saturated ammonium sulfate. The pellet was dissolved in 10 mM potassium phosphate (pH 7.8), 0.2 mM EDTA, and 1 mM β-mercaptoethanol and was dialyzed against this buffer. Each purified protein appeared as a major band by SDS-PAGE. The concentration of purified wild-type α-subunit was measured by extinction values using $E^{1\%}_{278nm}$=4.4 [11]. The mutant αTSs were estimated by microbiuret assay [8] using wild-type αTS as a standard.

Nondenaturing- and SDS-PAGE

The purity and homogeneity of the protein were analyzed by nondenaturing and SDS-PAGE [7]. Nondenaturing PAGE was performed using a discontinuous buffer system [3]. Bands were visualized by Coomassie Brilliant Blue R250.

In vitro aggregation reactions

Native forms of wild-type and mutant αTSs in 10 mM potassium phosphate (pH 7.8), 0.2 mM EDTA, and 1 mM β-mercaptoethanol were incubated at varying conditions of

temperature, pH, and trifluoacetic acid (TFA). After incubation, proteins were kept on ice for the analysis.

Results

Previously it was shown that T24A/E49G/F139W and T24A/D60N/F139W mutant αTSs could be induced into various discrete sizes of aggregates in the presence of low concentration of urea [11]. Here we studied further their aggregation properties.

Aggregation of αTSs at 37oC

Given that T24A/ E49G/F139W and T24A/D60N/F139W αTSs could form multimers at mild denaturation conditions [11], it was speculated that they might undergo aggregation even at physiological temperature. The aggregation of wild-type and two triple mutant αTSs (at 10 mg/ml, pH 7.8) were examined at 37°C for lengthy incubation (12, 24, 36, and 48 hr) (Fig. 1). Two mutant αTSs shows quicker transition of a native form into bigger sizes of forms than the wild-type protein. T24A/D60N/F139W shows no native form in 12 hr, but a thick band on top of the gel. T24A/E49G/F139W is less mild, showing complete disappearance of its native form in 48 h. Since then, aggregates seem to continue growing further, because larger sizes of aggregates appearing on the beginning part of the native gels disappear for both mutant proteins in 48 hr. Considering that the total amount of proteins were not altered as shown in SDS-PAGE (Fig. 1, bottom), aggregates may become too big to enter into the native gel.

When the proteins incubated for 48 hr were centrifuged at 10,000× *g* for 30 min, significant amount of pellets were precipitated from two triple mutant αTSs but not from wild-type. Intriguing observation was that these pellets were transparent in contrast to opaque protein pellets in general (see below). The result indicates that different aggregate types are produced in this experiment.

Aggregates at varied pHs

The effects of pH on *in vitro* aggregation of wild-type, T24A/E49G/F139W, and T24A/ D60N/F139W αTSs were investigated. The proteins (at 5 mg/ml) were incubated at 37°C for 48 hr at various pHs of 4.0~9.0 as well as pH 7.8 (Fig. 2). At a first glance they were greatly different in their turbidity to naked eyes. To quantify the pellet amount, the protein solutions were centrifuged. For wild-type αTS, pellets were produced at pH 4.0 and 8.0, but not at other pHs. Their pellets looked white opaque. In contrast, for two mutant αTSs, aggregates were formed in pH range of 4.0~8.0, but not in pH 9.0. Again interesting observation was that the pellets obtained at pH 7.8 was transparent, the pellets obtained from pH 4.0 to 8.0 were white opaque.

Fig. 2 shows aggregate forms produced at different pH by native PAGE. For wild-type and mutant αTSs, large sizes

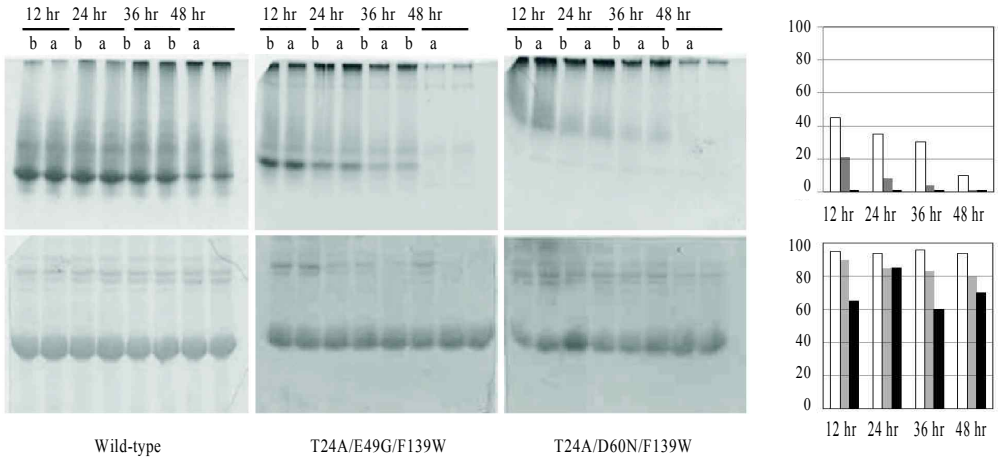

Fig. 1. Aggregation of αTSs at 37°C. Wild-type, T24A/E49G/ F139W, and T24A/D60N/F139W proteins (each 10 mg/ml) were incubated at 37℃ in 10 mM potassium phosphate (pH 7.8), 0.2 mM EDTA, and 1 mM DTT for the indicated time. Their soluble supernatants after centrifugation (a) or their total samples without centrifugation (b) were run on a native PAGE (top panels). To see any possible proteolytic digestion, the amounts of total proteins were monitored on SDS-PAGE (bottom panels). Densitometric amounts of total intact α TS are shown on the right side: open box, wild-type; gray box, T24A/E49G/F139W; black box, T24A/D60N/F139W. For clarity, only (a) data are depicted.

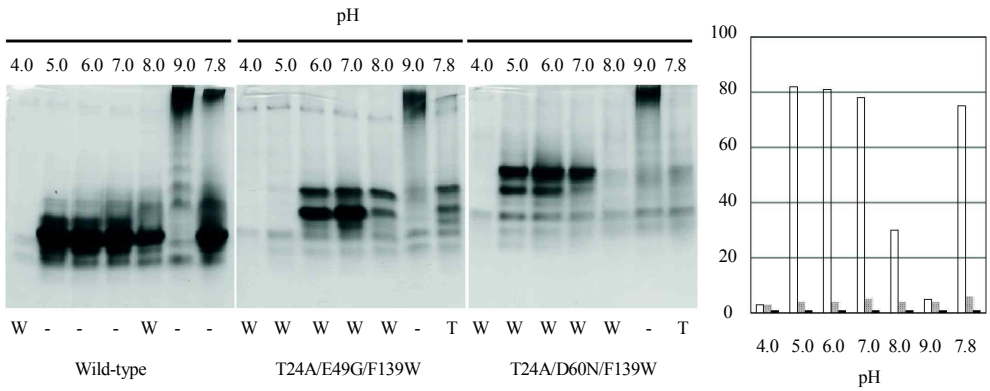

Fig. 2. Effect of pHs on aggregation of α TSs. Wild-type, T24A/ E49G/F139W, and T24A/D60N/F139W proteins (5 mg/ml) were incubated at 37℃ for 48 hr at various pHs (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 7.8). Protein solutions were centrifuged at 10,000× *g* for 30 min, and then their supernatants were run on the native PAGE. The pellets after centrifugation are marked at the bottom: W, white opaque pellet; T, transparent gel-like pellet; -, no pellet. Densitometric amounts of total intact α TS are shown on the right side: open box, wild-type; gray box, T24A/E49G/F139W; black box, T24A/D60N/F139W.

of aggregates were made at pH 9.0, even though the aggregates could be pelleted by centrifugation. No protein bands were shown on the gel for wild-type αTSs at pH 4.0, but large pellets were collected by centrifugation. The same cases were observed for T24A/E49G/F139W at pH 4.0 and 5.0 and for T24A/D60N/F139W at pH 4.0 and 8.0. Aggregates produced at pH 4.0 were too big to even enter into the gel used here. It was observed that a rapid formation of large aggregates at low pH occurred. Acidic pH instantly resulted in turbid suspensions.

Effect of 2,2,2-trifluoroethanol (TFE) on aggregation of mutant αTS

The treatment of TFE causes proteins to become partly folded states [12]. Proteins with the addition of TFE may resemble early intermediates during folding and may be prone to aggregation or misfolding [4]. T24A/E49G/F139W mutant αTS (at 5 mg/ml) were incubated in the presence of TFE at 37°C for 13 hr. Protein aggregates generated at various concentrations of TFE differed greatly in turbidity (Fig. 3). Significant amount of aggregates were produced in 10~30% TFE solutions at both pH 5.0 and 7.8. More amounts of aggregate were produced in the order of 10 >20>30% TFE at pH 5.0, but 20>10 ≈ 30 at pH 7.8. To see the pellets, the protein solutions were centrifuged. The pellets obtained at pH 7.8 with 10 and 30% TFE looked transparent. The pellets formed at pH 7.8 with 20% TFE were a mixture of white and transparent gel-like aggregates. But the treatment of 10, 20, and 30% TFE at pH 5.0 resulted in white pellets. No aggregation was observed

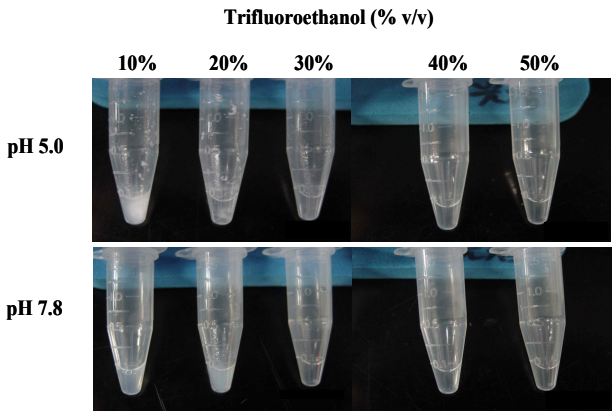

Fig. 3. Effect of 2,2,2-trifluoroethanol (TFE) on aggregation of mutant αTS. T24A/E49G/F139W proteins (at 5 mg/ml) were incubated at 37℃ for 13 hr at various concentration of TEF (10, 20, 30, 40, and 50% v/v) at pH 5.0 or 7.8. See the detailed description in the text.

at higher than 40% TFE. The result suggests that the aggregates were formed from partially denatured structure of protein.

Discussion

It was shown here that at least three types of aggregates could be formed from αTS by varying conditions: (1) opaque white precipitous aggregate, (2) transparent gel-like precipitous aggregate, and (3) unprecipitous aggregate. The opaque white precipitous aggregates were produced from wild-type αTS by incubating at pH 4 and 8, and from tri-

ple mutant proteins by incubating in the range of pH 4 to 8 (Fig. 1) or by adding 10-30% TFE at pH 5 (Fig. 3). The transparent gel-like precipitous aggregates were produced from the wild-type protein by incubating in the presence of 10-30% TFE at 37℃ in pH 7.8 (Fig. 3) or simply incubating at 37℃ for a long period of 7 days (data not shown), and from the triple mutant proteins by incubating at 37℃ in pH 7.8 for 2 days or longer (Fig. 1). The unprecipitous aggregates were made by incubating at pH 9 from both the wild-type and triple mutants (Fig. 2). Previous work reported that the unprecipitous aggregates could be formed from wild-type and mutant proteins by heat or urea treatment [11].

The transparent gel-like precipitous aggregate observed here was rarely reported. Its biological significance is not yet clear. However, this aggregate together with unprecipitous aggregate seem to have discrete structures in contrast to amorphous aggregates observed more often for nonfibril-type protein aggregation. Thus these could enable to further characterize detailed underlying mechanism of protein aggregates. One possible mechanism underlying aggregate formation might be 3D domain swapping [1]. In 3D domain swapping, a structural element of a monomeric protein is replaced by the same element from another protein. This process requires partial unfolding of the closed monomers that is then followed by adhesion and reconstruction of the original fold but from elements contributed by different subunits. This is consistent with the mild conditions here used to create aggregates. Under mild conditions such as 37℃, mid-range of pHs, and TFE addition, intermediate structures required for 3D swapping might be more popular. It has been thought that the aggregates are amorphously clustered nonstructured substances. However, this study suggests that aggregate forms might be more diverse than thought.

Acknowledgment

This work was supported by a 2-Year Research Grant of Pusan National University.

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초록 : 트립토판 합성효소 α 소단위체의 다양한 단백질 덩어리 형성

박명원 · 임운기*
(부산대학교 자연과학대학 분자생물학과)

단백질 덩어리는 질환의 원인이 되기도 하고, 유용한 유전자 재조합 단백질의 생산시 문제를 야기하기도 한다. 본 연구에서는 조건을 달리함으로 트립토판 합성효소 α 소단위체로부터 적어도 3가지 이상 다른 종류의 덩어리가 생길 수 있음을 보여주고 있다; (1) 불투명 흰색 침전 가능한 덩어리 (2) 투명하고 겔 유형의 침전 가능한 덩어리 (3) 불침전 덩어리. 이런 다른 종류의 덩어리 형태는 다른 기작을 통해 일어날 것으로 추정된다.