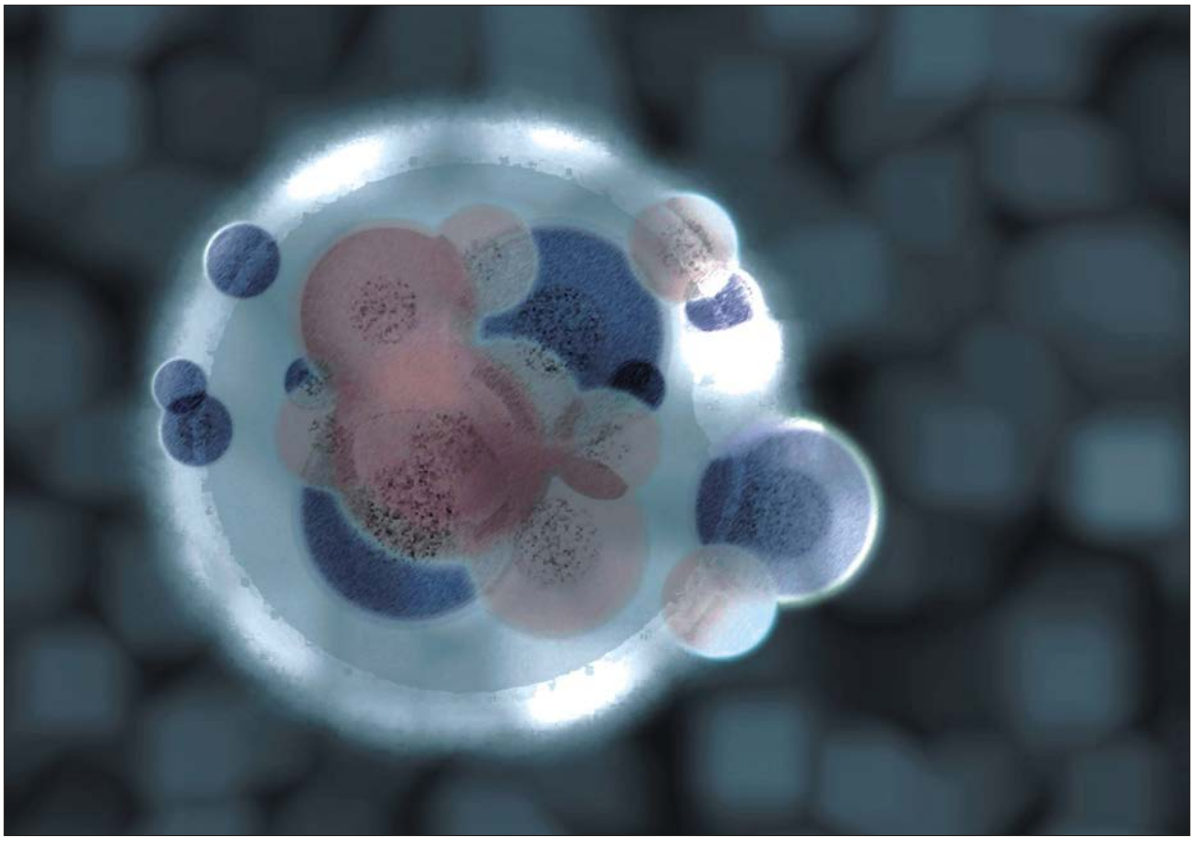

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Research Article

Crystallizability of an Engineered Monomeric Mutant of FK506-binding Protein from *Shewanella* sp. SIB1: Preliminary Diffraction Data Analysis

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Abstract

Background and Objective: A 22 kDa FK506-binding protein from a psychrophilic bacterium *Shewanella* sp. SIB1 (SIB1 FKBP22) is a member of peptidyl prolyl *cis-trans* isomerase (PPIase). This protein is homodimer with a V-shaped form, consisting of N and C-domains, that are connected through a long α-helix, responsible for dimerization and PPIase activity, respectively. Understanding on structural mechanisms behind the function of SIB1 FKBP22 is limited by unsuccessful attempts on crystallization of the full length SIB1 FKBP22 homodimer due to its flexibility and low stability. Despite the isolated N-domain, with the absence of α-helix and C-domain was successfully crystallized and structurally solved, the comprehensive structural arrangement of SIB1 FKBP22 remains missing. The objective of this study is to construct a crystallizable SIB1 FKBP22 derivatives consisting of N and C-domains with its α-helix that reflect full length of SIB1 FKBP22 and a platform for comprehensive structural analysis. **Materials and Methods:** A monomeric mutant of SIB1 FKBP22 was constructed by combining two gene fragments encoding Met 8-Ile 205 and Met 1-Ala 60 of the first and second monomer of SIB1 FKBP22, respectively, with three glycine residues. This design yielded the mutant has tandemly repeated N-domain connected to C-domain through a long α-helix hence designated as NNC-FKBP22. **Results:** The NNC-FKBP22 was monomeric in solution implying that it did not form a V-shaped dimeric structure. The crystallization of NNC-FKBP22 was attempted under sitting-drop vapor diffusion. The crystals were obtained under 1.0 M sodium citrate with 10 mM CHES/sodium hydroxide at pH 9.5. The crystal was in the space group of P212121 with unit cell dimension a = 94.635, b = 92.479 and c = 337.327 Å. A complete native data was collected from a rotating anode source to a resolution of 3.5 Å at 100 K with an R_{merge} value of 25.50. **Conclusion:** The NNC-FKBP22 was successfully crystallized implying that stabilization of SIB1 FKBP22 under protein engineering platform is promising approach to decipher its atomic structure. Protein engineering technique used in this study would also be applicable for other cold-adapted proteins with high structural flexibility and low stability.

Key words: FK506-binding protein, *Shewanella* sp. SIB1, peptidyl prolyl *cis-trans* isomerase, crystallization, protein engineering

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The capacity of organisms to thrive in extreme environment including low temperature has recently led to an aggressive of ecology, biology and biotechnology[1-5]. Yet, molecular mechanism regarding adaptability of the organisms to extreme environments remains enigmatic. In respect of low temperature environment, cold-adapted enzymes are thought to be involved in the adaptation by accelerating chemical rate at low temperature[6], in consequence of exponential decreasing of the reaction rate due to temperature decreasing[5]. The involvement of cold-adapted enzymes in cold adaptation of microorganisms is deciphered based on genomic, transcriptomic and proteomic studies[1,7-9]. The detailed functional mechanism of cold-adapted enzymes and its relationship to the cold adaptation of microorganisms remain to be understood.

A 22 kDa FK506-binding protein (FKBP22) from psychrophilic bacterium *Shewanella* sp. SIB1, designated as SIB1-FKBP22 hereafter, is thought to be involved in cold adaptation of *Shewanella* cells[10,11] based on its high expression level when the cells grow at 4°C[10]. This protein belongs to peptidyl-prolyl *cis/trans* isomerase (PPIase, EC 5.2.1.8) based on its amino acid sequence identity of 56% to *Escherichia coli* FKBP22 (Accession No. AAC77164)[12], 43% to *E. coli* FkpA (Accession No. AAC76372)[13] and 41% to *Legionella pneumophila* MIP (Accession No. S42595)[14]. These enzymes catalyse the isomerization of *cis-trans* prolyl bond[15-24], which is considered as the rate limiting step of protein folding[25]. The presence of these enzymes prevents protein misfolding caused by the incorrect *cis-trans* prolyl bond isomerization and slow folding rate[26,27]. In cold adaptation, SIB1 FKBP22 is thought to accelerate the *cis/trans* isomerization during the folding course of prolyl bond containing proteins in *Shewanella* cells[10,11]. This assumption is supported by the temperature dependence of PPIase activity of SIB1 FKBP22 for both peptide (Ala-Leu-Pro-Phe) and protein (Rnase T_1) substrates, which revealed that the optimum activity was observed at relatively low temperature (10°C)[10]. To our knowledge, SIB1 FKBP22 is the only cold-adapted PPIase family member that has been well studied so far.

The SIB1 FKBP22 forms a homodimer structure based on ultracentrifugation analysis. Each monomer consists of C-catalytic domain connected to N-domain through a long α-helix. While, C-domain is highly similar to a 12 kDa FKBP from human (hFKBP12) and responsible for PPIase activity, the N-domain displays similarity to N-domain of *L. pneumophilia* MIP and it is important for dimerization[10,11,28]. The presence of N-domain promotes SIB1 FKBP22 to exhibit chaperone-like activity as indicated by binding to a folding intermediate protein substrate[29] and prevent insulin aggregation[30]. Both PPIase and chaperone-like activities are structurally and functionally independent[29,30].

Three-dimensional structural model of SIB1 FKBP22, which was built based on *L. pneumophilia* MIP crystal structure[31], showed that this protein forms a V-shaped homodimeric structure[11,29]. In this model, N-domains from two monomers dimerize and provide a platform for the V-shaped form, while C-catalytic domains from two monomers are located at the tips of the V-shaped form with face-to-face orientation. The attempts to clarify the full-length structure of SIB1-FKBP22 have not been successful so far, which might be due to the instability of C-domain and flexibility of the long α-helix. Isolated C-domain with a long α-helix has also been attempted to crystallize, yet remain unsuccessful. However, a 1.9 Å crystal structure of N-domain with half of the long α-helix has successfully been solved confirming the V-shaped structure formed by the dimeric form of N-domain[28]. The crystal structure also revealed that the dimerization is facilitated by a Val-Leu zipper located at the helical structures of both monomers.

The role of V-shaped forms has been firstly proposed by Hu *et al.*[32], in which the author suggested a "Mother's arm" model to explain the chaperone-like activity of *E. coli* FkpA, a homologue of SIB1 FKBP22, based on its structure. Solution structure of *E. coli* FkpA displays similar structure to that of *L. pneumophila* MIP, in which V-shaped dimeric structure is formed through dimerization of the N-domains. Thus, it is speculated that "Mother's arm" model might a general model for FKBPs family proteins with V-shaped dimeric structure. To provide experimental evidence for this model, researchers have engineered a monomeric mutant of SIB1 FKBP22, so-called NNC-FKBP22, consisting of N-domains tandemly repeated through glycine linker and connected to C-domain through α-helix[11,33]. The NNC-FKBP22 is shown to be a monomer in solution according to the size exclusion chromatography and the ultracentrifugation analysis suggested that this monomeric mutant is not capable of forming the V-shaped structure[33]. Further biochemical analysis showed that despite NNC-FKBP22 retains its PPIase activity towards peptide substrate, this monomeric mutant exhibited remarkably lower PPIase activity towards protein substrate and chaperone-like activity. This suggests that the V-shaped form is important for efficient binding to protein substrates, hence generating an optimum PPIase activity for protein substrate and chaperone-like activity[11,33].

The NNC-FKBP22 provides another alternative for in-depth structural study of SIB1 FKBP22 since this monomeric mutant contains fully folded C and N-domains as well as a long α-helix. Structural analysis of NNC-FKBP22 would provide structural features of N and C-domains arrangements through a long α-helix. Furthermore, the structure of C-domain of NNC-FKBP22 would provide the atomic arrangement of catalytic site to support its catalytic mechanism as previously proposed[30]. As the SIB1 FKBP22 is a cold-adapted enzyme, the structural study of NNC-FKBP22 might explain the cold-adapted mechanism of this enzyme at atomic level.

Here researchers have attempted to crystallize NNC-FKBP22. The best crystals are obtained and diffracted to a resolution of 3.5 Å. Further discussion on the crystallization condition and quality as well as the preliminary diffraction data are provided.

MATERIALS AND METHODS

Cloning, over-expression and purification: Plasmid containing fragment encoding His-tagged NNC FKBP22 (pNNC-FKBP22) was prepared as previously described[33]. The pNNC-FKBP22 was further transformed into *E. coli* BL21 (DE3) [F^- ompT hsdSB (rB^-mB^-) gal (kcl857 ind1 Sam7 nin5 lacUV5-T7gene1) dcm (DE3)] (Novagen) and followed by over-expression as described previously[33]. Purification was performed using Ni-NTA affinity chromatography followed by size exclusion chromatography based on previous studies[33]. The purity was confirmed by SDS-Page[34], while protein concentration was determined from the UV absorption on the basis that the absorbance at 280 nm of 0.1% (1 mg mL^{-1}) solution is 0.61. These values were calculated by using ε = 1576 M^{-1} cm^{-1} for Tyr and 5225 M^{-1} cm^{-1} for Trp at 280 nm[35].

Crystallization: Preliminary screening of crystallization was performed using the sitting-drop vapor-diffusion method with Crystal Screen, PEG/Ion and Wizard kits (Hampton Research). The crystallization experiments were consisted of 1.0 µL of 5 protein in 5 mM tris-HCl at pH 8.0 containing 150 mM NaCl mixed with 1.0 µL reservoir solution and were equilibrated against 100 µL reservoir at 277, 283 and 293 K. After about 10 days, small crystals of NNC-FKBP22 were observed using a reservoir condition consisted of 1.0 M sodium citrate with 10 mM CHES/sodium hydroxide at pH 9.5. Optimization was performed using this reservoir with different concentrations of NNC-FKBP22. The best crystal was observed with 6.6 mg mL^{-1} final concentration of NNC-FKBP22 incubated at 277 K for about a week.

Data collection and processing: The x-ray diffraction data were collected from a single crystal of NNC-FKBP22 at BL38B1 beam line in SPring-8, Hyogo, Japan. The crystal was cryo-protected by soaking it in the reservoir solution containing 18% (v/v) ethylene glycol. The mounted crystal was flash-frozen in a nitrogen gas stream at 100 K. A total of 180 images were collected at a wavelength of 1.0 Å with an exposure time of 20 sec per image and an oscillation angle of 1.0°. The images were processed and scaled to 3.5 Å using HKL2000 suite.

RESULTS

The NNC-FKBP22 was designed so that the two N-domains were tandemly repeated and connected through a covalent bond consisting of three glycine residues. The bond connected Met-Ala 60 of the first N-domain of SIB1 FKBP22 to Met 8-Ileu 205 of the full-length SIB1 FKBP22 (Fig. 1). This mutant was successfully over-expressed under *E. coli* BL21 (DE3) system and purified under the same condition as the full length SIB1 FKBP22 homodimer. One liter culture yielded about 18 mg of NNC-FKBP22 which was comparable to that of the full length SIB1 FKBP22 homodimer.

Upon crystallization, clustered cube-shaped crystals appeared within 10 days under high pH (9.5) and low temperature (4°C). Yet, the crystals were too small for diffraction analysis. To confirm whether the crystals are formed by the mutant protein, some crystals (about 10-20 crystals) were collected and loaded into an SDS-PAGE in which yielded a single faint band with the size corresponded to NNC-FKBP22. Further, the optimization was performed through varying the incubation temperature and concentration of proteins and salts. The size of the crystals was improved in which the best crystal (Fig. 2) was observed within a week under 1.0 M sodium citrate with 10 mM CHES/sodium hydroxide at pH 9.5 with 6.6 mg mL^{-1} mutant protein in its final concentration.

The x-ray diffraction data set was collected from a single crystal at 3.5 Å resolution (Fig. 3). The NNC-FKBP22 crystals usually grow up to 0.2 mm in the longest dimension, which is within the acceptable size to provide a sufficient volume of crystal lattice and then can be exposed to the beam[36]. The crystal space group was determined to be an orthorhombic

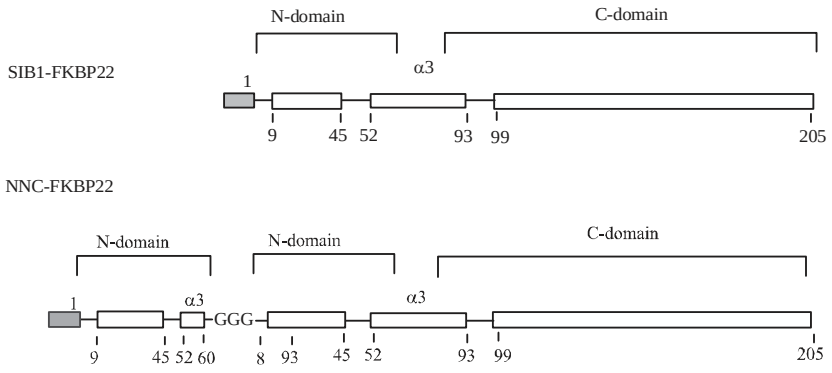

Fig. 1: Schematic representations of the primary structures of SIB1 FKBP22 and NNC-FKBP22. A His-tag attached to the N-termini of the proteins is represented by shaded box. The α-helix connecting N and C-domains is designated as α3 which was predicted based on tertiary model of of SIB1 FKBP22[33]. These secondary structures are arranged based on tertiary models of SIB1 FKBP22 and NNC-FKBP22. Numbers indicate the positions of the residues relative to the initiator methionine residue of the proteins without a His-tag. The ranges of the N and C-domains are also shown. In NNC FKBP22 design, flexible linker of three Gly residues are indicated as GGG connecting Ala 60 and Met 8 from first and second N-domains, respectively

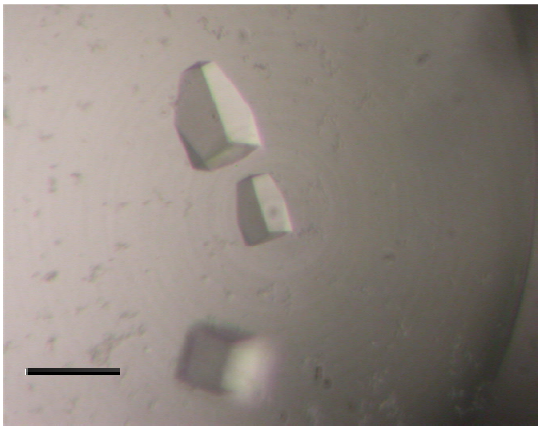

Fig. 2: Crystals of NNC-FKBP22 grown by sitting-drop vapor diffusion method. The bar corresponds to 0.1 mm is displayed for scaling[33]

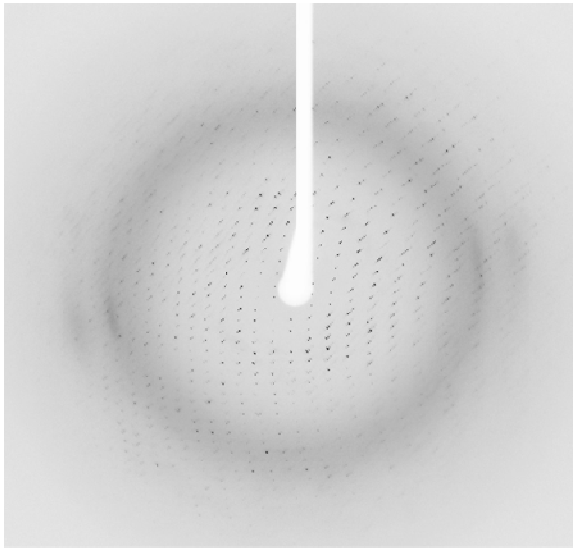

Fig. 3: X-ray diffraction pattern of NNC-FKBP22. The crystal was exposed at 100 K after soaking in artificial mother liquor containing 20% ethylene glycol for cryoprotection. The photograph shows a single frame of 1.0° oscillation with an exposure time of 20 sec per image and a crystal-to-detector distance of 170 mm. The crystal diffracted to 3.5 Å (the edge of the plate)

space group P212121 with the unit-cell parameters a = 94.64, b = 92.48 and c = 337.33 Å. This space group is different to that of N-domain with half of α-helix (SN-FKBP22), which belongs to P3221 space group[28]. The statistics of data collection are shown in Table 1. Given the volume cell calculated based on its space group was 2.95×10^6 Å^3 and molecular weight of NNC-FKBP22, the highest possibilities for Matthews coefficient was 2.28 Å^3/Da with 46% solvent contents and contained 12 identical monomeric NNC-FKBP22. Despite the coefficient was within the acceptable range[37], the number of molecules per cell unit was extremely high. When it was assumed one or two molecule(s) per asymmetric unit, the calculated coefficient was 27.34 or 13.67 Å^3/Da, respectively, which was extremely high and beyond the overall ranges[37]. While, the redundancy and the completeness shown in Table 1 were acceptable for further processing, the

Table 1: Statistic of data collection

Parameters	Values
Space group	P212121
Unit cell (Å)	a = 94.635, b = 92.479 and c = 337.327
(°)	a = 90.00, b = 90.10 and c = 90.00
Wavelength (Å)	1.0
Resolution range (Å)	50.00-3.55 (3.61-3.55)
Observed reflections	12535825
Unique reflections	70869
Redundancy	5.4 (5.7)
Completeness	98.3 (100.0)
I/δ	8.37 (1.91)
R_{merge} (%)	25.50 (77.90)

Values in parentheses are for the highest resolution shells

signal-to-noise ratio in the last resolution shell was slightly low (<2) but remained acceptable since the resolution was higher than 3 Å. Besides, the resolution of diffraction data might be higher than the actual measured. Indeed, the R_{merge} value was considerably high which is probably due to the relatively low multiplicity of our diffraction data.

DISCUSSION

Protein crystallization is an ordered process by which a biomacromolecule changes from a random state in solution to a regular crystal state, rather than to an amorphous state[38]. This has mostly regarded as a stochastic event over which the investigator has little or no control and regarded as the rate-limiting step in x-ray diffraction studies of macromolecules, including proteins[39]. In respect to SIB1 FKBP22, comprehensive understanding on the function of this protein is limited by the lack of structural information of full length SIB1 FKBP22 homodimer. Series of crystallization attempts on the full length were so far unsuccessful yielding an assumption that high flexibility and low stability of this protein account for difficulties of in the crystallization[11]. The success of crystallization on *L. pneumophilia* MIP[31], a homologue of SIB1 FKBP22 does not necessarily imply the crystallizability of SIB1 FKBP22. It is because of SIB1 FKBP22 is a cold-adapted enzyme with lower stability as compared to *L. pneumophilia* MIP as a non cold-adapted enzyme. Li *et al.*[38] indeed proposed that thermodynamic and structural stability seriously affect crystallization process.

Given the afore-mentioned issues, construction of crystallizable SIB1 FKBP22 derivatives are unavoidable to provide a model for the structural studies. The ideal model should contain fully folded of N and C-domains with its α-helix. Given homodimeric form always failed to be expressed in correct folded state, monomeric mutant is considered as an alternative option. The attempts to construct a monomeric mutant consisting of a single N-domain connected to C-domain through α-helix (NC mutant) were unsuccessful due to the insolubility of protein. The presence of two N-domains essential for folding as indicated by the mutation of Val-Leu zipper at this domain[28]. Monomeric mutant NNC-FKBP22 was designed based on the 3D structural model of SIB1 FKBP22. The design used for NNC-FKBP22 construction allows the mutant to fold correctly into a monomeric form[33] revealed. The Differential Scanning Calorimetry (DSC) analysis revalead N and C-domains of NNC-FKBP22 folded as in their full length SIB1 FKBP22 homodimer[33]. This implied that crystallization of this mutant should enable to provide atomic coordinate of N and C-domains that reflect the full length environment.

While full length SIB1 FKBP22 homodimer was unable to be crystallized so far, NNC-FKBP22 was crystallized in this study. Crystallizability of NNC-FKBP22 might be due to stabilization of the mutant that further reduced its flexibility. Thermodynamic parameters calculated from DSC curve revealed that NNC-FKBP22 was slightly stable compared to the dimeric form of SIB1 FKBP22[33]. This might be contributed by the covalent bonds formed by glycine residues, a linker of repeated N-domains. The stabilization of N-domain further reduced the flexibility of α-helix that was connected to C-catalytic domain. It is speculated that low stability of SIB1 FKBP22 and its high flexibility due to α-helix are the main reasons for unsuccessful crystallization attempts of the wild type. Increasing the stability and reducing the flexibility of α-helix in NNC-FKBP22 has led the proteins to be more crystallizable. The flexibility of α-helix connecting N and C-domains was shown in *E. coli* FkPA, a homologous SIB1 FKBP22 protein, under NMR spectroscopy[32]. This flexibility has been placed as the reason of unsuccessful crystallization attempts of full length SIB1 FKBP22 homodimer, beside its low stability[11].

The diffraction data was not-refinable, yet the crystallizability of NNC-FKBP22 provided a new window for more comprehensive structural studies of PPIase family member involving in the cold-adaptation of a psychotropic bacterium. The NNC-FKBP22 crystal implies that NNC-FKBP22 was in correct folding and an appropriate model for deciphering structural and functional relationship of a V-shaped form of SIB1 FKBP22 has been unsuccessful to be crystallized so far. Current study is now dealing with optimizing the condition of NNC-FKBP22 crystallization for obtaining better crystal and refinable diffraction data. Nevertheless, this study also demonstrated application of protein engineering to enhance crystallizability of proteins.

CONCLUSION

A monomeric mutant of SIB1 FKBP22 containing fully folded of N and C-domains with a long α-helix, termed as NNC-FKBP22 was successfully constructed, expressed and purified. This mutant was crystallized under sitting-drop vapor diffusion with 1.0 M sodium citrate with 10 mM CHES/sodium hydroxide at pH 9.5. The crystal was in the space group of P212121 with unit cell dimension a = 94.635, b = 92.479 and c = 337.327 Å. A complete native data was collected from a rotating anode source to a resolution of 3.5 Å at 100 K, with an R_{merge} value of 25.50. Yet, the data is not sufficient to build an atomic structure of NNC-FKBP22. Nevertheless, crystallizability of this mutant provides new hope for a model in structural studies of SIB1 FKBP22. Besides, protein engineering approach used in this study might also promising to be used to address crystallization issue in other cold-adapted enzymes with high flexibility and low stability.

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