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Validation of protein refolding via 1-dimensional ^{1}H-^{15}N heteronuclear single quantum correlation experiments

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Abstract Many proteins are expressed as an insoluble form during the production using *Escherichia coli* (*E. coli*) system. Although various methods are applied to increase their amounts of soluble expression, refolding is the only feasible way to obtain a target protein in some cases. Moreover, protein NMR experiments require $^{13}C/^{15}N$-labeled proteins that can only be obtained from *E. coli* systems in terms of cost and technical difficulty. The finding of appropriate refolding conditions for a target protein is a time-consuming process. In particular, it is very difficult to determine whether the refolded protein has a native structure, when a target protein has no enzymatic activity and its refolding yield is very low. Here, we showed that 1-dimensional ^{1}H-^{15}N heteronuclear single quantum correlation (1D ^{1}H-^{15}N HSQC) experiment can be efficiently used to screen an optimal condition for the refolding of a target protein by monitoring both the structure and concentration of the refolded protein.

Keywords Blvrb, dialysis using micro-dialyzer, 1-dimensional HSQC experiment, ^{15}N-labeled protein, protein refolding

Introduction

Protein refolding is a well-established method to obtain various target proteins that are expressed as an inclusion body in *E. coli*, and is generally performed by three methods, such as quick dilution, dialysis, and on column refolding.[1-3] Proteins with disulfide bonds including cytokines are generally expressed as an inclusion body in *E. coli* system.[4] During the refolding in solution, denatured proteins face to distinct two competing processes between productive refolding to native structure and fatal aggregation with non-native structure. Detailed refolding process is dependent on specific proteins, and thus it is difficult to rationale an optimal refolding condition. For the refolding of a specific target protein, optimization of additives such as arginine, glycerol, and reduced/oxidized glutathione mixture is required in addition to the determined basic parameters of buffer solutions (pH and ionic strength).[5] The optimization of protein refolding always pursues to increase the productive refolding and to decrease the non-productive aggregation, and the simplest ways are (i) to decrease the intermolecular aggregation by reducing the concentration of the denatured protein and (ii) to decrease the refolding temperature since the hydrophobic interaction is dependent on the entropy of the Gibbs free energy.

The optimization of refolding conditions necessitates the functional validation of the refolded protein. However, the absence of easily accessible enzyme activity in a target protein makes it very difficult to

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optimize the conditions of protein refolding. Since some proteins with non-native conformation do not precipitate and remain as a soluble aggregation,[6] the correct folding of the refolded protein should be confirmed in addition to its amount in the refolding solution. Therefore, the presence of a simple and efficient method to validate the structure of the refolded protein is convenient for the optimization of the refolding conditions. It is well known that the 2D ^{1}H-^{15}N HSQC spectrum of a protein is an excellent reference for protein structures, whether the protein is well structured or not. Amide peaks do not appear well in the ^{1}H-^{15}N HSQC spectrum of the soluble aggregation form. 1D ^{1}H-^{15}N HSQC spectrum, not 2D, is also sufficient indicator for the folding status and concentration of protein and can be recorded for low concentration of ^{15}N-labeled protein (~10 μM) within a practical time limit (10~30 min). Moreover, less background signals of ^{1}H-^{15}N HSQC compared to various huge ^{1}H signals from buffer solution enable us to clearly monitor the structure of the refolded ^{15}N-labeled protein. Most of all, the cost of the production of ^{15}N-labeled protein is not expensive.

Human Blvrb protein is a NADPH-dependent flavin reductase and is a potential drug target to enhancing human platelet counts.[7] Since the some amount of the purified Blvrb protein still contains $NADP^{+}$/NADPH that is already present in *E. coli*, the preparation of the apo-Blvrb protein requires the purification under denatured condition and the refolding process. Here, we developed different purification procedure of the denatured ^{15}N-labeled Blvrb (^{15}N-Blvrb) protein from the previously reported method,[8] and designed the refolding process of ^{15}N-Blvrb that utilized 1D ^{1}H-^{15}N HSQC experiment to validate both structure and concentration of the refolded protein.

Experimental Methods

Preparation of the ^{15}N-Blvrb protein in denatured condition. - Human Blvrb gene was cloned to pET-15b vector by using Nde I and Xho I restriction enzyme sites. The constructed plasmid was transformed into the Rosetta2 (DE3), and then the cells were grown in 1-L M9 minimal media prepared in a 2.8-L baffled flask including 100 μg/ml ampicillin at 37°C. 1 g ammonium chloride (^{15}N, 99%), 5 g D-glucose, and were supplemented as sources of nitrogen and carbon. The trace metals stock solution (0.2 ml per 1-liter) and the MEM vitamin solution (Sigma) were added to the culture in order to increase protein expression.[9] The cells were grown at 37°C until the OD reached an appropriate value (~0.8), and then the expression of the Blvrb protein was initiated by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5-h. The cells were harvested by centrifugation and then were stored at -70°C before the purification.

The cells were re-suspended in buffer (pH 7.5, 50 mM HEPES, 8.0 M urea, 150 mM NaCl, 10 mM 2-mercaptoethanol, 1.0 mM PMSF) and then were disrupted by sonication. The cell debris was removed by centrifugation and then the supernatant was filtrated using 0.45 μm syringe filter. The supernatant was applied to HisTrap HP column (GE healthcare) that was pre-equilibrated with the His-buffer-A (pH 7.5, 20 mM Tris-HCl, 6 M urea, 150 mM NaCl, and 5 mM 2-mercaptoethanol). The Blvrb protein was eluted using the His-buffer-B (pH 4.5, 20 mM Na-acetate, 6 M urea, 150 mM NaCl, and 5 mM 2-mercaptoethanol). For the next ion-exchange column chromatography using Hitrap-SP HP column (GE healthcare), the His-tag elution containing the Blvrb protein was 2~3 fold diluted with the SP-buffer-A (pH 4.5, 10 mM Na-acetate, 6 M urea, and 5 mM 2-mercaptoethanol). The Blvrb protein was eluted by using 100 ml gradient with the SP-buffer-B (pH 4.5, 10 mM Na-acetate, 6 M urea, 1 M NaCl, 5 mM 2-mercaptoethanol). After the determination of the Blvrb concentration using UV absorption at 280 nm, the protein elution was stored at -70°C before the next refolding experiment.

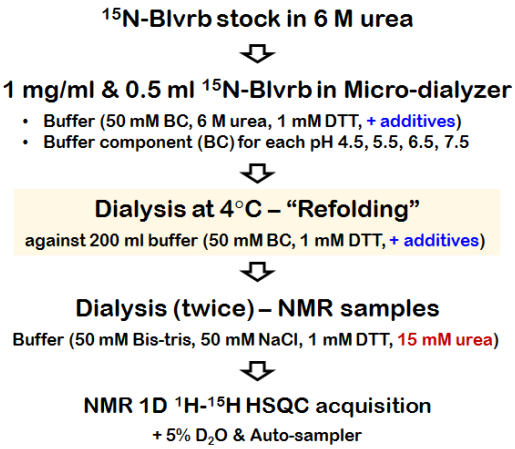
Monitoring the yield of the refolded ^{15}N-Blvrb using 1D HSQC experiment. - The purified ^{15}N-Blvrb (1 mg/ml) was prepared in each denaturation buffer containing 50 mM one of buffer components (pH 4.5, sodium acetate; pH 5.5, sodium citrate; pH 6.5, bis-tris; pH 7.5, HEPES), 6 M urea, 0.4 M arginine, and 1 mM dithiothreitol (DTT). Therefore, small portion of the

buffer and salt components from the ion-exchange column chromatography was remained in the initial denatured ^{15}N-Blvrb protein mixtures. The denatured proteins (500 μl) were placed to the micro-dialyzers (Micro Float-A-Lyzer™, MWCO 3.5 to 5 kD, Spectrum), and then were dialyzed against the same buffer without 6 M urea at 4°C for over-night. After the refolding was finished, the micro-dialyzers containing the protein sample of each different pH value were further dialyzed against the NMR acquisition buffer (pH 6.5, 50 mM bis-tris, 50 mM NaCl, 1 mM DTT, and 15 mM urea). 15 mM urea was used as an internal reference of natural abundant 1D ^{1}H-^{15}N HSQC peak to estimate the concentration of the refolded ^{15}N-Blvrb protein. The samples were transferred to the NMR tubes, and then 5% D_2O was additionally added. The 1D ^{1}H-^{15}N HSQC spectra were recorded using 800 MHz NMR instrument equipped with the TCI cryo-probe and the auto-sampler system.

Results and discussion

The production of ^{15}N-labeled protein as an inclusion body in *E. coli* is not costly process, and the optimization of the refolding condition using 1D ^{1}H-^{15}N HSQC experiments can be practical processes. Although Blvrb has an enzyme activity to reduce various substrates (biliverdin isomers, several flavins including flavin mononucleotide, pyrroloquinoline quinone, and ferric ion) by utilizing the NAD(P)H,[10] we utilized 1D ^{1}H-^{15}N HSQC experiment to monitor the refolding products of the apo-Blvrb protein. First, the Blvrb protein was expressed as the N-terminal His-tagged form, but it was later shown that the native Blvrb protein could also bind His-tag affinity column tightly even after removing the His-tag by thrombin-digestion (data not shown). Therefore, the presence of N-terminal His-tag in Blvrb is not necessary for the His-tag affinity purification.

To completely remove the remaining $NADP^+$/NADPH molecules in the Blvrb protein (K_M, 2.4 μM),[10] we tried to purify the Blvrb protein under denaturation condition (6 M urea) through two different column chromatography methods (His-tag affinity and SP cation-exchange), and then to obtain the clean apo-Blvrb protein through the refolding dialysis. The optimal pH values for the refolding of the ^{15}N-Blvrb protein were first estimated (Scheme 1).



Scheme 1. The refolding optimization of the ^{15}N-Blvrb protein. The ^{15}N-Blvrb proteins prepared in 6 M urea were refolded by dialysis method. The refolded ^{15}N-Blvrb proteins were further dialyzed against the buffer that is suitable for the NMR measurement. 15 mM urea (natural abundance, 0.4%) was added for the internal reference to estimate the concentration of the refolded ^{15}N-Blvrb proteins. Various refolding conditions can be estimated simultaneously simply by increasing the number of the conditions.

After the refolding dialyses of the ^{15}N-Blvrb protein in the different buffers, the yields of the refolding were estimated using 1D ^{1}H-^{15}N HSQC experiment. The presence of 15 mM urea allowed the systematic comparison of the amounts of the refolded ^{15}N-Blvrb protein, since the urea concentration is the same in all different refolded conditions. The 1D ^{1}H-^{15}N HSQC spectra clearly showed that the refolded ^{15}N-Blvrb proteins had a well-folded structure due to less background signals of the ^{1}H-^{15}N HSQC experiment (Fig. 1). It was clear that the optimal pH values for the refolding of the ^{15}N-Blvrb protein were from 5.5 to 6.0. L-arginine amino acid is a well-known chaotropic reagent to suppress aggregation and to enhance protein refolding,[11] and thus we also tested the effect of the presence of 0.4 M arginine during the refolding of the ^{15}N-Blvrb protein. However, no clear improvement of

the refolding yield by the presence of arginine was identified (data not shown).

In conclusion, we were able to monitor the refolding efficiency by utilizing the 1D ^{1}H-^{15}N HSQC spectra of the ^{15}N-Blvrb protein. The application of 1D ^{1}H-^{15}N HSQC experiment using ^{15}N-labeled protein will be practical to monitor the refolding process of a protein at decreased concentration. The systematic evaluation of various refolding conditions is also possible simply by increasing the number of conditions and by automated NMR acquisitions using the auto-sampler equipment.

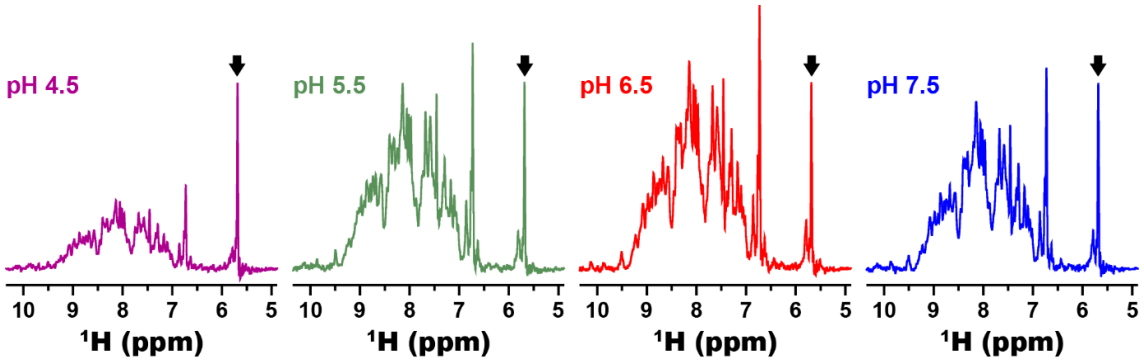


Figure 1. 1D ^{1}H-^{15}N HSQC spectra of the refolded ^{15}N-Blvrb protein at different conditions. The 1D ^{1}H-^{15}N HSQC spectra were recorded with 3017 number of scans. The peak of natural abundant 15 mM urea is indicated with an arrow. The optimal pH values of the buffer seem to be 5.5~6.5 for the optimal refolding of ^{15}N-Blvrb protein. Each refolding buffer contained each 50 mM buffer component, 0.4 M arginine, and 1 mM DTT.

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