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Detection of Structural Changes in a Cofactor Binding Protein by Using a Wheat Germ Cell-Free Protein Synthesis System Coupled With Unnatural Amino Acid Probing

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ABSTRACT **A cell-free protein synthesis system is a powerful tool with which unnatural amino acids can be introduced into polypeptide chains. Here, the authors describe unnatural amino acid probing in a wheat germ cell-free translation system as a method for detecting the structural changes that occur in a cofactor binding protein on a conversion of the protein from an apo-form to a holo-form. The authors selected the FMN-binding protein from *Desulfovibrio vulgaris* as a model protein. The apo-form of the protein was synthesized efficiently in the absence of FMN. The purified apo-form could be correctly converted to the holo-form. Thus, the system could synthesize the active apo-form. Gel filtration chromatography, analytical ultracentrifugation, and circular dichroism-spectra studies suggested that the FMN-binding site of the apo-form is open as compared with the holo-form. To confirm this idea, the unnatural amino acid probing was performed by incorporating 3-azido-L-tyrosine at the Tyr35 residue in the FMN-binding site. The authors optimized three steps in their system. The introduced 3-azido-L-tyrosine residue was subjected to specific chemical modification by a fluorescein-triarylphosphine derivative. The initial velocity of the apo-form reaction was 20 fold faster than that of the holo-form, demonstrating that the Tyr35 residue in the apo-form is open to solvent. Proteins 2007;67:643–652.** © 2007 Wiley-Liss, Inc.

Key words: cell-free translation; unnatural amino acid; chemical modification; flavin; flavoprotein; FMN-binding protein

INTRODUCTION

A cell-free protein synthesis system[1–7] is a powerful tool with which unnatural amino acids can be introduced into polypeptide chains.[8–11] Indeed, several cell-free systems have been utilized to incorporate selenomethionine for X-ray crystal structure analyses[6,12,13] and stable isotope labeling for NMR studies.[6,14,15] However, such structural studies generally require professional techniques and expensive instruments, are considerably time-consuming, and incur high costs. Nowadays, numerous proteins have been structurally studied and their coordinates are available from the Protein Data Bank.[16] In a general laboratory, quick, convenient, and inexpensive methods based on these structural data are desirable to detect structural change(s), protein–protein interactions, and/or protein–small molecule(s) interactions. Our cell-free system is derived from wheat germ extract from which translation inhibitors, such as ribosome inactiva-

Abbreviations: FMN-BP, FMN-binding protein; azTyr, 3-azido-L-tyrosine.

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tion factors,[17,18] nucleases, and proteases, have been removed: typical protein synthesis continues for more than 120 hours and the concentration of the protein product reaches 1 mg/mL.[4] The aim of this study was to develop a new application of our cell-free system for protein structure chemistry.

Chemical modification is a classical but convenient method in protein chemistry. In particular, site-specific modification based on structural data can be very informative. However, control of the modification efficiency and selectivity of the target site are very difficult. A cell-free protein synthesis system can incorporate new reactivity into the protein by introducing unnatural amino acid residues. Thus, high reactivity and selectivity of the chemical reaction are expected. For this purpose, recently, several unnatural amino acids have been developed and used for chemical probing.[19,20] Furthermore, mutants of aminoacyl tRNA synthetases have been devised for the efficient incorporation of unnatural amino acids.[8,9] Here, we selected 3-azido-L-tyrosine (azTyr) as the unnatural amino acid, because its high reactivity and selectivity have been confirmed: namely, the azido group in the polypeptide reacts specifically with triarylphosphine derivatives.[19]

We considered testing chemical probing in our cell-free system by using cofactor-binding proteins, because various cofactor-binding proteins exist in living cells and cofactors play key roles in the function and/or structure of proteins. The production of cofactor binding protein by a cell-free system is itself a challenging task, because the mechanisms underlying the incorporation of a cofactor into a polypeptide are the subject of ongoing study. In general, apo-forms of cofactor-binding proteins are produced by denaturants, acids, nucleophilic reagents, and/or heat treatments.[21,22] As such, we considered that our cell-free system might provide a mild method for the production of apo-proteins.

We selected flavoprotein as the target, because many kinds of flavoprotein have been reported and their flavin binding sites have structural variations.[21,23–25] Furthermore, because flavins are incorporated into the protein nonenzymatically, the addition of supplementary specific enzymes such as heme lyase[26,27] is not necessary. Therefore, the development of an apo-protein production system has been very important in studies of flavoprotein.[21,25,28] After some preliminary experiments, we chose the FMN-binding protein (FMN-BP) from *Desulfovibrio vulgaris* (Miyazaki F)[29] as a model protein, because this is one of the smallest FMN-containing proteins reported thus far and only one tyrosine residue, which is the chemical modification site, is present in a subunit. FMN-BP was found in *Desulfovibrio vulgaris* (Miyazaki F) as a candidate of the electron-transfer protein in anaerobic respiration.[29] However, the other *Desulfovibrio* species does not code this protein gene. Thus, the function of this protein has not been identified. In 1997, the structure of FMN-BP was solved by NMR analysis.[30] Recently, X-ray crystal structure was also analyzed.[31] FMN-BP is a homodimer [Fig. 1(A)],[31] and its protein fold is reported to be similar to that of chymotrypsin,[30] although there is debate concerning this fold, as described by Murzin.[32] The bound FMN contacts with both subunits and is locked by the Leu122 residue at the C-terminal end [Fig. 1(A)].

Here, we report the production of the apo- and holoforms of the FMN-BP and detection of the structural changes that occur upon FMN binding by using unnatural amino acid chemical probing coupled with a wheat germ cell-free protein synthesis system.

MATERIALS AND METHODS

Materials

Preparation of the wheat germ extract has been reported previously.[4] FMN was purchased from Sigma (St Louis, MO). DE52 was a product of Whatman (Maidstone, England). Sephacryl S-100 was obtained from Amersham Biosciences (Uppsala, Sweden). Safranine T was from Tokyo Kasei Kogyo, Japan. Dithionite was from Wako Pure Chemical Industries, Tokyo, Japan. Other chemical reagents were of analytical grade.

Plasmid Vectors and Cell-Free Protein Synthesis

The plasmid vector pBT100, which contains the gene encoding FMN-BP, has been previously reported.[29] The gene was amplified by PCR using the following primers: FMN-N 5′-GCG CCC ATG GTG CCG GGT ACA TTT TTC-3′; FMN-C 5′-GCG CGG ATC CTC AGT CCA GTC GGC C-3′. The amplified fragment was digested with NcoI and BamHI, and then ligated into the multicloning site of a pEU3 plasmid, which is a custom-made vector for the cell-free translation system.[5] Protein synthesis in the presence or absence of FMN was performed as described previously.[5] In brief, for holo-protein synthesis, FMN was added to give a final concentration of 50 μM in the reaction mixture.

Purification of the Apo-Form of FMN-BP

After protein synthesis, the reaction mixture was dialyzed against 50 mM Tris-HCl (pH 8.0) and then loaded onto a DE52 column. The apo-form was eluted by a linear-gradient of NaCl (0–200 mM). Fractions containing FMN-BP were combined and then subjected to gel-filtration chromatography using a Sephacryl S-100 column. The purified sample was concentrated with an Amicon ultra centrifugal filter device (5000 Mr cut-off, Millipore) and stored at 4°C until use.

Conversion of the Apo-Form to the Holo-Form

The apo-form (20 μM) of FMN-BP was converted to the holo-form by incubation with 60 μM FMN at 4°C for 30 min. Unbound FMN was removed by Sephadex G-25 gel-filtration chromatography.

Spectroscopy

UV-visible absorption spectra were recorded with a Beckman DU 640 spectrophotometer. Fluorescence

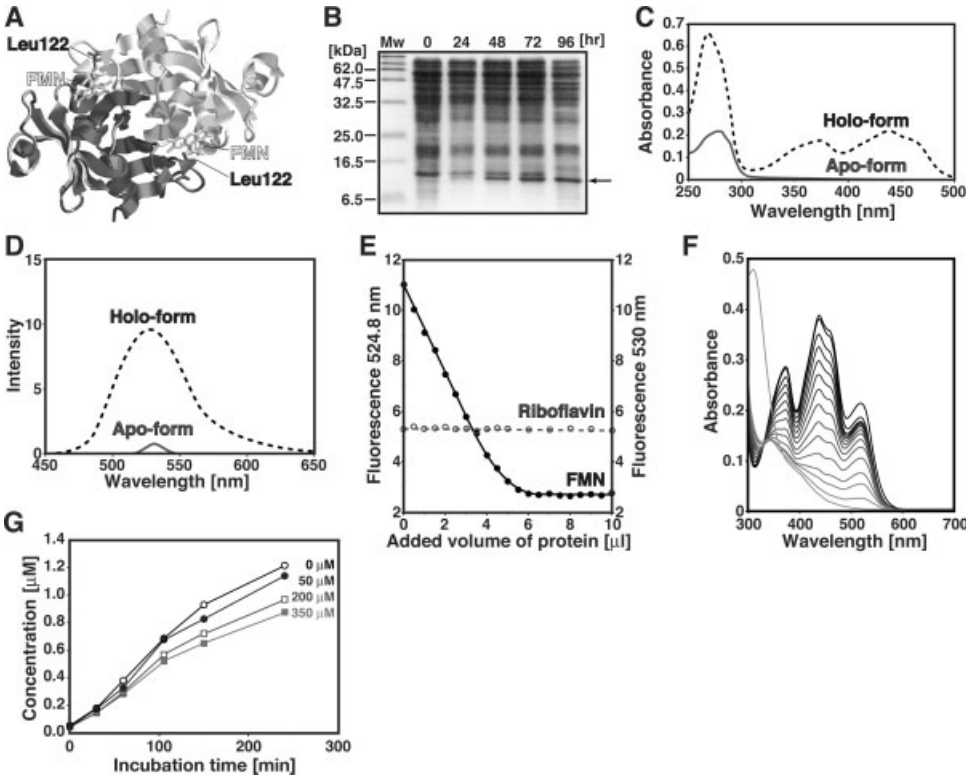

Fig. 1. Production of the apo- and holo-forms of FMN-BP. **A:** Dimer structure of FMN-BP. The two subunits and bound FMN are depicted. The Leu122 residue in the two subunits is highlighted by a stick model. **B:** The time-dependent synthesis of apo-FMN-BP. The synthesized protein was analyzed by 18% SDS-polyacrylamide gel elctrophoresis. The gel was stained with Coomassie brilliant blue. Marker proteins (1 μg each) are the prestained protein markers (New England Bio Labs). Arrow indicates the synthesized protein. **C:** UV-visible spectra of the apo-forms (solid line) and converted holo-forms (broken line) of FMN-BP. **D:** Fluorescence spectra of the apo-forms (solid line) and the converted holo-forms (broken line) of FMN-BP. **E:** Titration curve of FMN (solid line) and riboflavin (broken line) with the apo-form of FMN-BP. **F:** Titration curves of the converted holo-form of FMN-BP with dithionite under anaerobic conditions. The midpoint potential was determined as described in the Materials and Methods. **G:** Time dependent protein synthesis of the FMN-BP with various concentrations of FMN. The concentrations of FMN were shown in the figure.

emission spectra were recorded at 25°C using a JASCO FP-6300DS spectrofluorometer. Circular dichroism (CD) spectra (200–320 nm) were recorded at 25°C using a JASCO J-820 spectropolarimeter. The CD spectra presented in this article are the average of the five scans.

Determination of the K_d Value for FMN and Measurement of the Midpoint Potential of the Converted Holo-Form

The value of the dissociation constant (K_d) for FMN was determined by titration of an FMN solution with the apo-form of FMN-BP at 25°C in the dark as follows. In brief, 3 mL of 0.1 μ*M* FMN in the buffer (50 m*M* potassium phosphate (pH 7.0) and 5 m*M* EDTA) was titrated with aliquots of 50 μ*M* apo-FMN-BP, and emission value at 524.8 nm was recorded. The dissociation constant was calculated as described previously.[33] The midpoint potential of the holo-form was determined spectrophotometrically at 25°C by equilibration with safranine *T* ($E_{m,7} = -289$ mV) in 200 m*M* potassium phosphate (pH 7.0) during anaerobic titration with dithionite as reported previously.[34]

Analytical Gel-Filtration Chromatography

Analytical gel-filtration chromatography was performed with an ÄKTA explorer 10S chromatography system (Amersham Biosciences) equipped with a Superdex 75 HR 10/30 column. The flow rate of the buffer (50 m*M* Tris-HCl (pH 8.0) and 200 m*M* NaCl) was 0.5 mL/min, and 100 μL of the sample (5 μg) was injected. Elution profiles were monitored by the absorption of UV at 280 nm.

Analysis of Subunit Structure by Ultracentrifugation

The structures of the subunits of the apo- and holoforms were analyzed by analytical ultracentrifugation: sedimentation velocity and sedimentation equilibrium experiments were carried out with an Optima XL-I analytical ultracentrifuge (Beckman-Coulter, Fullerton, CA)

in a four-hole An60Ti rotor at 20°C using standard double sector centerpieces and quartz windows. The sedimentation velocity experiments were carried out at an A_{280} of 0.8 for the apo- and holo-forms at a rotor speed of 50,000 rpm Scans were recorded without intervals between successive scans. The number of hydrodynamic species and their sedimentation coefficients were determined by the SEDFIT program.[35] For both proteins, the sedimentation equilibrium experiments were carried out at an A_{280} of 0.15, 0.3, and 0.5 for both proteins at 5000, 7000, and 9000 rpm, respectively, and the data were analyzed by global fitting to a single species model.

Plasmid Vector Construction for Unnatural Amino Acid Probing

A 6xHis tag DNA sequence was introduced at the N-terminal coding region of pEU3-FMN-BP by PCR using the following primers: FMN-N His Tag, 5′-CGC GCC ATG GGT CAC CAC CAC CAC CAC CAC TCT TCT GGT CTG CCG GGT ACA TTT TTC G-3′; FMN-C, 5′-GCG CGG ATC CTC AGT CCA GTC GGC C-3′. The Tyr35 codon (UAC) was changed to the amber stop codon (UAG) by site-directed mutagenesis using the following primers: FMN Y35Amber F, 5′-CAA CAC CTG GAA CAG CTA GCT GAA GGT GCT GGA CGG C-3′; FMN Y35Amber R, 5′-GCC GTC CAG CAC CTT CAG CTA GCT GTT CCA GGT GTT G-3′. The original stop codon (UAG) was changed to the tandem ochre stop codons (UAA-UAA) using following primers: FMN Amb123OchreF, 5′-GGC GGA GCA GAC CCT GTA ATA AGG CCG CGT CTG CCT GAA G-3′; FMN Amb123OchreR, 5′-CTT CAG GCA GAC GCG GCC TTA TTA CAG GGT CTG CTC CGC CG-3′.

Preparation of azTyr-tRNA^{Tyr}

Fluorescein-triarylphosphine derivatives and 3-azTyr were synthesized as described.[36,37] Suppressor tRNA^{Tyr} and mutant Tyr-tRNA synthetase (TyrRS) were purified form *E. coli* expression systems as previously reported.[38] Aminoacylation of the suppressor tRNA^{Tyr} was detected by gel-mobility shift assay. In brief, the aminoacylation mixture (10 μL) contained 100 m*M* Tris-HCl (pH 7.6), 10 m*M* $MgCl_2$, 40 m*M* KCl, 4 m*M* ATP, 200 μ*M* 3-azTyr, 0.2 μg TyrRS, and 8 μ*M* amber suppressor tRNA. The reaction was performed at 30°C for 10 min and stopped by lowering the pH to 4.75 with 6% acetic acid. Aliquots of the reaction mixture were then subjected to electrophoresis using a 10% polyacrylamide gel under acidic conditions (pH 4.75). The RNA bands were visualized by staining with methylene blue.

Incorporation of azTyr into FMN-BP and Chemical Probing

Protein synthesis was performed as described earlier except for the addition of 200 μg/mL TyrRS mutant (Y43G), 170 μ*M* 3-azTyr-tRNA, 500 μ*M* 3-azTyr instead of Tyr, and 6 m*M* 2-mercaptoethanol instead of DTT. For the holo-form, 50 μ*M* FMN was also added. The reactions were carried out at 26°C for 16 hours in the dark. The synthesized FMN-BP was purified by Ni-NTA superflow (Qiagen) column chromatography. The proteins eluted (containing 50 m*M* Hepes-KOH (pH 7.6), 250 m*M* imidazol, and 200 m*M* NaCl) were used immediately for chemical probing with fluorescein-triarylphosphine derivatives. The chemical modification reaction was started by the mixing of 0.1 μ*M* purified FMN-BP with 2 μ*M* fluorescein-triarylphosphine in 10 μL of 50 m*M* Hepes-KOH (pH 7.6) buffer at 37°C in the dark. Aliquots were taken out at appropriate times and analyzed by 18% SDS-polyacrylamide gel electrophoresis. Fluorescence (below 526 nm excited by 532 nm) was detected directly on the gel with a Typhoon 9400 Variable Mode Imager (Amersham Biosciences). After the measurement of the fluorescence, the proteins were visualized by silver staining.

RESULTS

Preparation of the Apo-Form of FMN-BP

Figure 1(B) shows the time course of synthesis of the apo-form of the FMN-BP. The synthesis continued for at least 96 hours. The synthesized protein was purified by previously reported method[29] (data not shown). The freshly prepared apo-form bound FMN and was converted to the holo-form. To estimate the content of FMN in the converted holo-form, the UV-visible absorption spectra were measured after Sephadex G-25 gel filtration chromatography [Fig. 1(C)]. The spectrum clearly showed that one subunit of the apo-FMN-BP captures 1.02 molecules of FMN, suggesting that almost all of the apo-form was converted to the holo-form. Although the apo-form did not contain the FMN apparently, we measured the fluorescent spectra to detect the trace FMN [Fig. 1(D)]. The spectra showed that the apo-form contains 1.49% of FMN [Fig. 1(D)], suggesting that intrinsic FMN in the wheat germ was incorporated into the protein in the translation process. In this article, we used this protein as an apo-form, although it contains 1.49% FMN per one subunit. The K_d value for FMN was determined as 0.68 n*M* by fluorescence quenching of the FMN titration [Fig. 1(E)]. This result is in good agreement with the fact that almost FMN-BP in the living cells exists in the holo-form.[29] In contrast, riboflavin did not bind to the apo-form even in the presence of phosphate [Fig. 1(E) open circle dotted line]. Furthermore, we measured the redox potential of the converted holo-form by using safranine T under anaerobic conditions [Fig. 1(F)]. The midpoint redox potential value was calculated as −277 mV, which coincided with that of the recombinant FMN-BP expressed in *E. coli*,[29] suggesting that FMN was correctly bound to the apo-FMN-BP. Furthermore, we tested the effect of FMN on the productivity of the FMN-BP [Fig. 1(G)]. According to the increase of the FMN concentration, the velocity of the protein synthesis was decreased. We confirmed that the holo-form could be

synthesized by supplementing the reaction mixture with 50 μM FMN (data not shown). These results clearly demonstrate that the wheat germ cell-free protein synthesis system successfully synthesized the active apo-form of the FMN-BP.

Open and Closed Structures of FMN-BP

The apo-form of FMN-BP is relatively unstable as compared with the holo-form: the purified apo-form was aggregated by a freeze-saw cycle even in the presence of 50% glycerol. Therefore, we stored the sample at 4°C until needed. Furthermore, some of the protein dissociated to the monomer during storage at 4°C (described in the next section). These results suggest that there are considerable structural differences between two forms.

In the holo-form of FMN-BP, the bound FMN contacts with both subunits and is locked by the C-terminal region of one of the subunits [Fig. 1(A)]. Because FMN is missing in the apo-form, the stabilization that it provides is lost. Furthermore, we recently found that the Leu122 residue at the C-terminal end significantly affects the redox potential of the bound FMN (Kitamura et al. Unpublished results). Therefore, we suspected that the apo-form of FMN-BP might be a monomer. To address this problem, we carried out the analytical gel-filtration chromatography (data not shown). Unexpectedly, however, the elution profile of the apo-form was similar to that of the holo-form: the apo-FMN-BP eluted only slightly faster than holo-FMN-BP, strongly suggesting that the apo-form behaves as a dimer.

To confirm the subunit structure, we carried out the analytical ultracentrifugation with both sedimentation velocity and sedimentation equilibrium experiments were performed (data not shown). The sigmoidal sedimentation curves showed that both the apo- and holo-forms of FMN-BP were rheologically homogeneous. Although a few of the subunits of the apo-form had dissociated to the monomer, the main portion retained the dimer structure. It should be mentioned that apo-FMN-BP acquired a monomeric form during storage for 14 days at 4°C (data not shown).

To address the equilibrium between the monomeric and dimeric forms of FMN-BP, we carried out the ultracentrifugation analysis at various concentrations of the apo-form and found that the dissociation of the dimer to monomer caused irreversible structural changes (i.e., the correct K_d could not be calculated). On the basis of these experimental results, we conclude that the apo-form of FMN-BP is a dimer.

These findings prompted us to measure the CD-spectra of the two forms as the difference in their molecular shapes may be reflected in the content of α-helices and β-sheets. Figure 2(A) shows the CD-spectra of the both forms of FMN-BP. Beyond our expectations, the spectra of the apo- and holo-forms differed completely from one another. In general, the CD spectrum at a wavelength of around 208 nm reflects the state of α-helices and β-sheets in the protein.[39–41] As shown in Figure 2(A), the trough of the apo-form at 208 nm was deeper and shifted as compared with that of the holo-form, demonstrating that considerable structural differences exist between the two forms. Furthermore, the CD spectrum at 250–300 nm reflects the environment of Tyr and Trp residues.[39–41] Although the spectrum of the holo-form had a clear peak in this region, the spectrum of the apo-form lacked this peak [Fig. 2(B)]. FMN-BP has only one Tyr and two Trp residues (Tyr35, Trp32, and Trp106), which assemble around FMN by means of hydrophobic interactions [Fig. 2(C)]. Therefore, we expect that movements of the Tyr and Trp residues cause the loss of the peak at 250–300 nm in the apo-form spectrum. Thus, together with experimental results and structural information, we predict that the Tyr35 residue in the apo-form is open to the solvent.

Optimization of the Wheat Germ Cell-Free Protein System for Chemical Probing

If the Tyr35 residue is open in the apo-form as compared with the holo-form, then the reactivity of this residue toward chemical modification reagents will be increased in apo-FMN-BP owing to reduced steric hindrance. To confirm this idea, we carried out the chemical

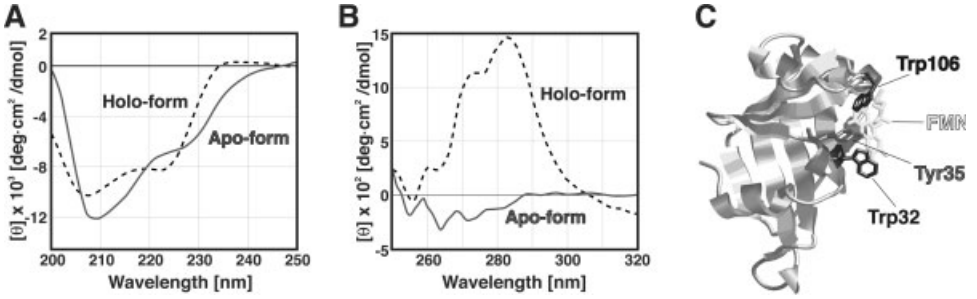

Fig. 2. Circular dichroism (CD) spectra of the holo- and apo-FMN-BP. **A:** CD spectra (200–250 nm) of the apo-form (solid line) and the holo-forms (broken line). **B:** CD spectra (250–320 nm) of the apo-form (solid line) and the holo-forms (broken line). **C:** Aromatic amino acid residues and bound FMN in a monomer subunit of the FMN-BP. The Trp, Tyr, and FMN are highlighted by stick models.

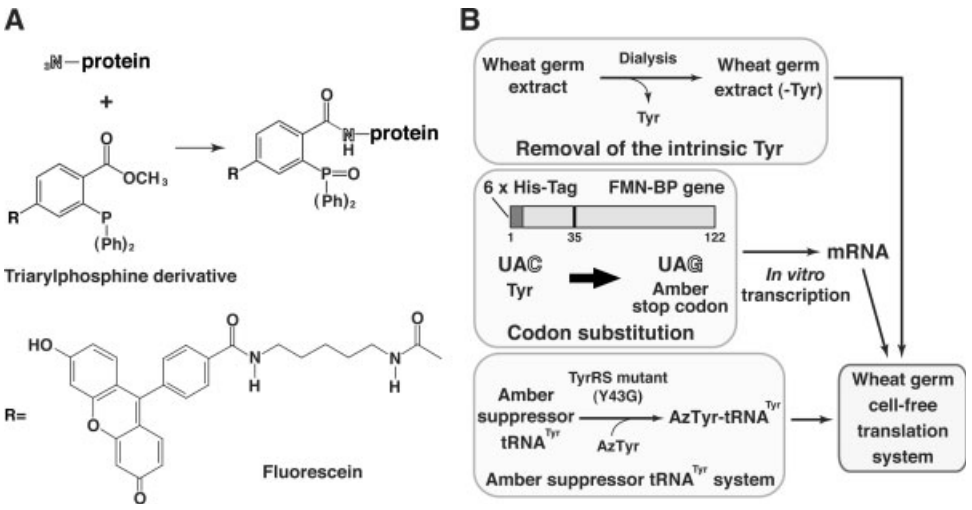

Fig. 3. Optimization of the cell-free system for chemical probing. **A:** Reaction between the azido group and the triarylphosphine derivative (modified Staudinger ligation). The azido group incorporated in the protein reacts specifically with the ester group of the triarylphosphine derivative. R indicates the fluorescein in the reagent. **B:** Optimization of the wheat germ cell-free synthesis system for the site-specific incorporation of azTyr. The apo-form synthesis system was altered as described in the text.

probing of the Tyr35 residue. We selected fluorescein-triarylphosphine as the modification reagent, because fluorescein is detectable spectroscopically and its bulky structure is expected to lead to steric hindrance with amino acid residues around the Tyr35 residue [Fig. 3(A)].

For the purpose of the chemical probing, we optimized our cell-free system as shown in Figure 3(B). First, intrinsic free Tyr was removed from the wheat germ extract by dialysis. Second, the Tyr35 codon (UAC) in the gene encoding FMN-BP gene was changed to an amber stop codon (UAG) and a 6xHis tag was attached at the N-terminal region for rapid purification. Third, a yeast amber suppressor $tRNA^{Tyr}$ system[38] was prepared to incorporate azTyr specifically into the protein at position 35. In brief, the yeast amber suppressor $tRNA^{Tyr}$ [Fig. 4(A)] and the yeast TyrRS Y34G mutant, which specifically activates azTyr instead of Tyr, were purified from an *E. coli* expression system. The purity and azTyr charging activity of the prepared suppressor $tRNA^{Tyr}$ were confirmed by the gel-mobility shift assay [Fig. 4(B)]. The azTyr-$tRNA^{Tyr}$ was recovered by ethanol precipitation under acidic conditions. The protein synthesis activity of the azTyr-$tRNA^{Tyr}$ was tested by a conventional batch synthesis system [Fig. 4(C)]. In the absence of azTyr-$tRNA^{Tyr}$, synthesis of apo-FMN-BP stopped at residue 35 [Fig. 4(C)]. We confirmed that the full-length FMN-BP was not produced by the autoradiography of the SDS-polyacrylamide gel (data not shown). At the 60-min period, protein synthesis was markedly accelerated by the addition of the azTyr-$tRNA^{Tyr}$ [Fig. 4(C)] and the full-length FMN-BP was then synthesized (data not shown). After these pilot experiments, we carried out the synthesis of the azTyr labeled FMN-BP by the dialysis synthesis system.

Comparison of the Reactivity of azTyr35 Residue in the Apo- and Holo-FMN-BP and Proposed Mechanism for the Structural Change

Synthesis of the holo- or apo-form of FMN-BP with azTyr was carried out with or without 50 μM FMN, respectively (data not shown). Both forms were successfully recovered by a Ni-NTA affinity column via their 6xHis tag. Quantities of the synthesized protein showed that there was no difference in the yields of the both forms, irrespective of the absence or presence of FMN. The purified proteins were used immediately for chemical probing by the fluorescein-triarylphosphine derivative because the half-life of the azido group in the protein was around 72 hours at 4°C in the stock buffer (data not shown). The azTyr35 residue was labeled by fluorescein faster in the apo-form than in the holo-form. The modification rates of both reactions could be calculated from the fluorescence intensities (Fig. 5). As shown in Figure 5, the time dependent increase in fluorescence showed that for the first 10 min the reactions could be approximated by a linear function. The initial velocity was 20-fold faster for the holo-form reaction than for the apo-form reaction. The extent of modification of the apo-form reached 90% in 30 min. Furthermore, it should be mentioned that FMN-BP synthesized with normal Tyr was not modified by the fluorescein-triarylphosphine derivative at all (data not shown).

On the basis of these experimental results, we can propose a model that take place in FMN-BP upon FMN binding (Fig. 6). In the absence of FMN, the apo-form of the FMN-BP is produced by the wheat germ cell-free protein synthesis system. The purified apo-form behaves as a dimer and its molecular shape is relatively open as compared with that of the holo-form. The addition of FMN induces the conversion of apo-FMN-BP to the holo-form within a few sec-

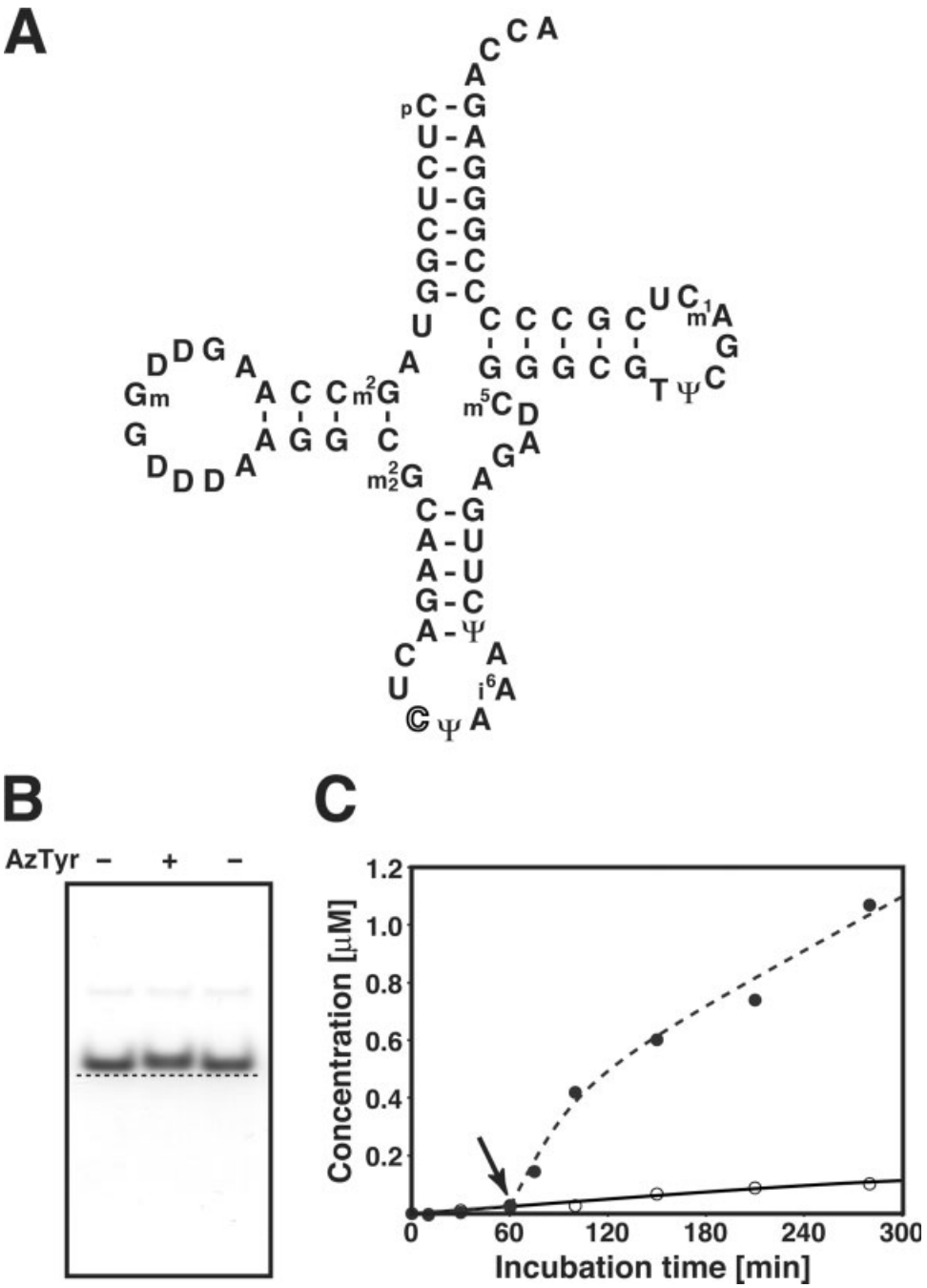

Fig. 4. The yeast amber suppressor $tRNA^{Tyr}$ system. **A:** Cloverleaf structure of the yeast amber suppressor $tRNA^{Tyr}$. The anticodon (CΨA) forms base pairs with the amber stop codon (UAG) in mRNA. **B:** Detection of aminoacylation by the gel-mobility shift assay. The azTyr-$tRNA^{Tyr}$ (center lane) migrated more slowly through the gel than the uncharged suppressor $tRNA^{Tyr}$ (left and right lanes). The broken line indicates the front of the bands of the uncharged suppressor $tRNA^{Tyr}$. **C:** The azTyr-$tRNA^{Tyr}$ dependent synthesis of the apo-FMN-BP. Time-dependent incorporation of ^{14}C-Leu was monitored by the batch synthesis. Addition of the azTyr-$tRNA^{Tyr}$ at 60 min brought about synthesis of the full-length FMN-BP (broken line). Without the addition of azTyr-$tRNA^{Tyr}$, synthesis stopped at position 35 (solid line), as described in the text.

onds. In the holo-form, the Tyr35 residue is attracted to the isoalloxazine ring of the bound FMN and the binding site closes. Finally, the Leu122 residue at the C-terminal end of a second subunit locks in the isoalloxazine ring with hydrophobic interactions, thereby forming the stable mature dimer structure that is observed in the X-ray crystal study.[30] The extended molecular shape seen in the apo-form changes to a globular shape in the holo-form.

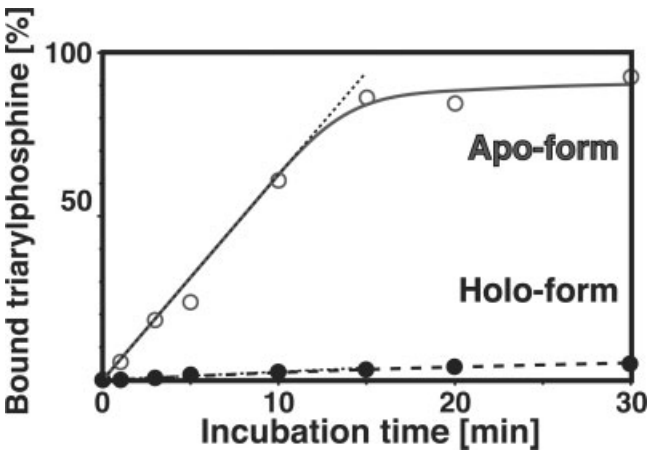

Fig. 5. Comparison of the initial velocity of the chemical modification by the fluorescein-triarylphosphine derivative. The fluorescence intensity of each band was measured. Initial velocities were calculated by the dotted lines.

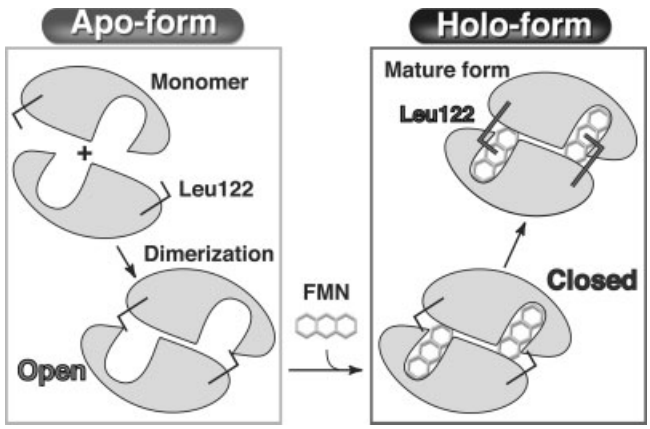

Fig. 6. Model of the structural changes that occur in FMN-BP upon FMN binding. The synthesized apo-form of FMN-BP behaves as a dimer. The FMN-binding site in the apo-form is open as compared with that of the holo-form. The purified apo-form is converted to the holo-form within a few seconds by the addition of FMN. Upon FMN binding, the FMN-binding site closes and is finally locked by the Leu122 residue, as observed in the crystal structure.

DISCUSSION

Here, we have successfully produced the apo-form of a cofactor-binding protein by using *Desulfovibrio vulgaris* (Miyazaki F) FMN-BP as a model protein. For more than 60 years, the production of the apo-forms of flavoproteins has been an important agenda in the field, because reconstruction of a holoflavoprotein from its apoprotein and flavin cofactor yields valuable insight into in protein folding, function and mechanism.[21,42,43] There are many methods for preparing reconstitutable apoflavoproteins, but they are all based on either weakening of binding of flavin or stabilizing the apoprotein formed by means of lowering the pH, increasing the salt concentration, changing the solvent or increasing the temperature.[21] Furthermore, several affinity column chromatography techniques have been developed to separate the apo-form.[44] Moreover, the apo-form of the monomeric sarcosine oxidase was recently produced in a controlled riboflavin-dependent *E. coli* strain.[45] Because apo-forms of proteins are generally unstable as compared with holo-forms, irreversible structural changes in the protein should be avoided. Thus, a mild system for producing apoproteins would be desirable. For a long time, the cell-free protein synthesis system has been anticipated as a novel system for producing apoproteins. However, the protein productivity of the initial cell-free protein synthesis systems was very poor: the synthesized protein was usually detected only by radioisotope labeling. We have achieved high productivity in our wheat germ cell-free system by removing the translation inhibitors.[17,18] In addition, here we have demonstrated efficient production of the apo-form of FMN-BP and have verified that the synthesized apo-form is correctly converted to the holo-form by UV-visible absorbance spectra, fluorescence spectra, determination of the K_d value for FMN, and measurement of the midpoint potential (Fig. 1). Thus, the apoprotein synthesized by this system is of sufficient quantity and quality for use in spectroscopic analyses in the flavoprotein study.

Among the apoflavoproteins reported, apoflavodoxin is one of the best-studied flavoproteins.[42,43,46] It has been reported that *Desulfovibrio vulgaris* (Hildenborough) apoflavodoxin is less specific for FMN as compared with the other flavodoxins, and that the protein binds riboflavin.[46] Furthermore, a two-dimensional NMR study has clarified the interaction of *D. vulgaris* apoflavodoxin with the phosphate,[47] and an X-ray crystal structure of the protein–riboflavin complex has been also studied.[48,49] Our experiments indicated that the apo-form of FMN-BP does not bind riboflavin under the conditions tested [Fig. 1(E)], suggesting that the FMN binding site of apo-FMN-BP differs considerably from that of *D. vulgaris* apoflavodoxin. Structural differences in the FMN binding site between the apo-form of flavodoxin and that of FMN-BP were also observed in the CD-spectra. It has been reported that *Anabaena* apoflavodoxin has a relatively closed structure owing to hydrophobic interactions of the aromatic amino acid residues around the FMN-binding site and that its CD-spectrum at 250–300 nm shows a clear peak,[50] which was missing in the CD spectrum of apo-FMN-BP [Fig. 2(B)]. These results prompted us to carry out the unnatural amino acid probing around the FMN binding site.

To develop the chemical probing system, we altered our cell-free translation system as shown in Figures 3 and 4. For the unnatural amino acid probing, preventing misincorporation of the intrinsic amino acid is very important. Here, we employed the yeast amber suppressor $tRNA^{Tyr}$ system for site-specific incorporation. Although the TyrRS Y34G mutant ligates azTyr to the CCA termini of the amber suppressor $tRNA^{Tyr}$ more than 400-fold faster as compared with the wild-type Tyr-RS,[9] we predicted that wheat TyrRS in the extract would attach intrinsic Tyr to the amber suppressor $tRNA^{Tyr}$. Therefore, we removed the intrinsic Tyr and added the aminoacylated form of tRNA (azTyr-amber suppressor

$tRNA^{Tyr}$) to the reaction mixture. The conventional batch synthesis clearly showed that the synthesis was dependent on this input of azTyr-amber suppressor $tRNA^{Tyr}$ [Fig. 4(C)]. To prepare the labeled protein, we synthesized the apo- and holo-forms of the FMN-BP by a dialysis system, because this system could remove more intrinsic Tyr. In fact, the extent of chemical modification in the apo-form reached 90% in 30 min (Fig. 5). Until now, several expanded codon systems have been developed, such as new suppressor tRNA species,[51] four codon systems,[52,53] and novel base pairs by artificial nucleotides.[54,55] We believe that these systems will be adaptable to our cell-free system, although misincorporation of the intrinsic amino acid must be prevented.

Here, we modified the protein by a fluorescein-triarylphosphine derivative. This technique is directly applicable to studies of protein–protein, protein–nucleic acid, and/or protein–small molecule interactions by fluorescence probing analyses using fluorescence resonance energy transfer, photo-induced electron transfer, and intramolecular charge transfer. Furthermore, because functional groups and/or isotopes can be introduced at the target site, the methods described in this article can be utilized in various fields, for example, site-directed stable isotope labeling for NMR studies, heavy atom introduction for X-ray crystal studies, spin-labeling for EPR analysis, isotope labeling for mass spectrometry, and the generation of the ligand binding site for the immobilization of the protein. Moreover, the new functional groups derived from unnatural amino acids may bring novel functions to the protein. We hope that our cell-free translation system will contribute to the study of the cofactor binding proteins.

CONCLUSIONS

In this study, we have demonstrated efficient production of the apo-form of FMN-BP and have verified that the synthesized apo-form is correctly converted to the holo-form by UV-visible absorbance spectra, fluorescence spectra, determination of the K_d value for FMN, and measurement of the midpoint potential. During the course of the study, we found that the apo-form of the FMN-BP behaves as a dimer. Furthermore, the local structural change induced by FMN binding was also suggested. To verify the idea, we developed the chemical probing system by the site-directed introduction of unnatural amino acid using wheat germ cell-free translation system. The azTyr35 residue in the apo-form was rapidly modified by a fluorescein-triarylphosphine derivative as compared with the holo-form, suggesting that the Tyr residue in the apo-form is accessible to the solvent.

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