NMR Observation of Selected Segments in a Larger Protein: Central-Segment Isotope Labeling through Intein-Mediated Ligation†

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ABSTRACT: Peptide segments in a protein, which can include an active site of interest or be a series of parts constituting the entire structure, are now selectively observed by nuclear magnetic resonance (NMR) spectroscopy using samples prepared by the intein-mediated ligation method. Two separate inteins were used to ligate NMR-transparent segments to both the ends of an NMR-visible segment, producing a partly visible intact protein molecule. The $^{15}N-^{1}H$ correlation spectrum of a 370-residue maltose binding protein labeled with ^{15}N at a continuous segment comprising residues $Gly^{101}-Ser^{238}$ showed the essential elimination of signal overlapping, the signals being at the same positions as for the uniformly labeled sample. This method will allow structural analysis by NMR of over 50-kDa proteins in combination with contemporary NMR techniques suppressing the signal decays of larger proteins.

In the past decade, about 1000 protein and peptide structures have been solved by NMR, but the analysis of larger proteins of over 300 residues is still difficult because of the problem of the signal overlapping due to the thousands of resonating atoms in proteins and the signal broadening caused by the rapid relaxation of the transverse magnetization due to slow molecular tumbling in solution (*1*−*3*). The number of NMR signals in a spectrum can be reduced by selective labeling, including amino acid-specific labeling (*4*) and single residue labeling using an in vitro expression system (*5*, *6*), solving the signal overlapping problem. However, the assignment of signals cannot be achieved because the excellent triple-resonance experiments are not applicable to samples prepared in such ways. If an arbitrary segment along a peptide sequence can be labeled with isotopes, both signal selection and the application of triple-resonance experiments are possible.

We developed a technique for the isotope labeling of the amino or carboxyl terminal half using a splicing element (intein), for example, between residues 1 and 100 in a 500-residue protein (*7*, *8*). Inteins are genes inserted in protein-coding genes of prokaryotes and lower eukaryotes with an in-frame open reading frame (*9*−*11*). They are cleaved off from the host protein after translation. Of particular interest is that the preceding fragment (N-extein) and the following fragment (C-extein) are ligated by a peptide bond, leaving an intact protein (extein) comprising a continuous peptide chain. The gene of the precursor (N-extein + intein + C-extein) was fragmented in the middle of the intein sequence and expressed separately in *Escherichia coli*. The N- and C-terminal precursor fragments were mixed and denatured with a denaturant, and then dialysis for refolding into a splicing active conformation was performed. The splicing reaction was started by heating the mixture solution. Replacement of the preceding and following extein sequences with the N- and C-terminal halves of the target protein sequence on plasmid DNAs and the application of the same splicing protocol as for the natural intein−extein system provided an N- or C-terminal-labeled target protein. We have applied this method to the C-terminal domain of the RNA polymerase α subunit (88 amino acids, αCTD) and maltose binding protein (370 amino acids, MBP[1]), and the usefulness of segmental isotope labeling has been shown by the $^{15}N-^{1}H$ HSQC spectra of segmentally ^{15}N-labeled αCTD (*7*) and MBP (*8*) and the ^{13}C NOESY experiment (*8*) of the segmentally ^{13}C-labeled αCTD.

Central-segment labeling, e.g., labeling of a segment comprising residues 200−300 of a 500-residue protein, which provides samples for the selective observation of any part of interest as a segment of manageable size, is reported in this study (Figure 1). The basic idea is similar to that of terminal-segment isotope labeling. For central-segment labeling, it is required that only the central part is expressed in an isotope-labeled M9 minimal culture; the other parts, i.e., N- and C-terminal parts, being expressed in an unlabeled culture. To satisfy this requirement, the target protein was divided into three parts at two positions. For ligation of the two positions, two inteins were used. The three precursor peptides, expressed individually, were mixed with denaturant

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[1] Abbreviations: MBP, maltose binding protein; CBD, cellulose binding domain; GdnHCl, guanidine hydrochloride; HSQC, heteronuclear single-quantum coherence spectroscopy.

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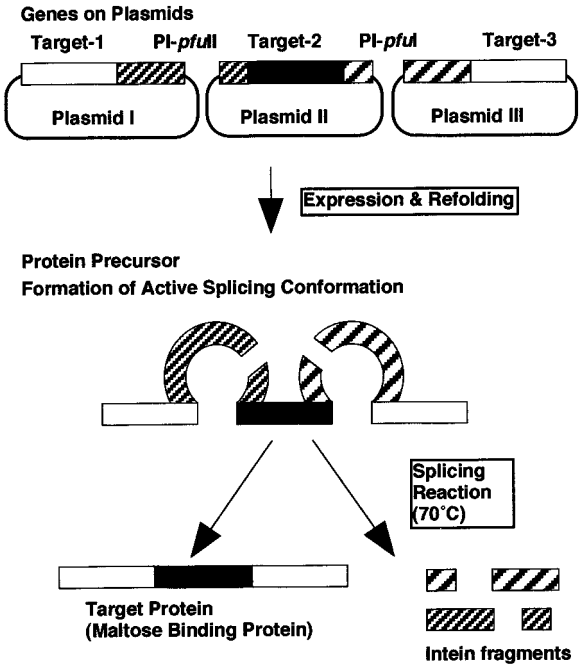

FIGURE 1: Schematic representation of the segmental isotope labeling in the case of MBP. Plasmid I contains the N-terminal part of the target gene (designated as Target-1) ($Lys^{1}-Asn^{100}$) followed by an extra three amino acids (Thr-Asn-Pro) and the N-terminal part of PI-*pfu*II ($Cys^{1}-Tyr^{295}$). Plasmid II contains the C-terminal part of PI-*pfu*II ($Arg^{296}-Asn^{382}$) followed by an extra three amino acids (Cys-Gly-Gln), the central part of the target gene (Target-2, black box) ($Gly^{101}-Ser^{238}$), an extra three amino acids (Gly-Gly-Gly), and the N-terminal part of PI-*pfu*I ($Cys^{1}-Lys^{160}$). Plasmid III contains the C-terminal part of PI-*pfu*I ($Gly^{161}-Asn^{454}$) followed by an extra two amino acids (Thr-Gly) and the C-terminal part of the target gene (Target-3) ($Ser^{239}-Lys^{370}$). The precursor peptide fragments were expressed in *E. coli* BL21(λDE3) transformed with the plasmids. Refolding was performed for formation of the splicing active conformations of inteins as described in the text. For the splicing reaction, heating of the solution to 70 °C was required. For central-segment labeling (Target-2, black box), the central fragment was produced in stable isotope-labeled M9 medium, and the other fragments were produced in the unlabeled medium.

and then refolded into the splicing active conformation. The refolded inteins acted simultaneously, and the three extein pieces were ligated into a continuous peptide chain.

MATERIALS AND METHODS

Preparation of Expression Vectors. A plasmid for the N-terminal fragment of the MBP-intein precursor was constructed as follows. The genes encoding the N-terminal part of MBP (residues 1−100) and the N-terminal part of the PI-*pfu*II intein (residues 1−295) were separately amplified by PCR from the pMAL-p plasmid (New England Biolabs) and the PI-*pfu*II intein gene using PCR primers containing the overlapped sequences of MBP and PI-*pfu*II. Each PCR product was annealed and amplified by PCR again and then cloned into pET35b (Novagen) at the LIC (ligation independent cloning) site (plasmid I). Plasmid I produces an N-terminal precursor fused with cellulose binding domain (CBD) followed by a thrombin site at its N-terminus and a histidine tag at its C-terminus; a connector sequence, Thr-Asn-Pro, is inserted between MBP and PI-*pfu*II by including the corresponding nucleotide sequences in the PCR primers.

A plasmid for the central fragment of the MBP-intein precursor was also constructed by PCR. The gene encoding the C-terminal part of PI-*pfu*II intein (residues 296−382) was amplified from the PI-*pfu*II intein gene, and the chimeric gene constituting the central part of MBP (residues 101−238) and the N-terminal part of PI-*pfu*I intein (residues 1−160) was amplified from pCBDM238IN (*8*), joined by PCR, and then cloned into pET15b (Novagen) at the *Nde*I and *Bam*HI restriction sites (plasmid II). Plasmid II produces a central precursor with a histidine tag at its N-terminus. A connector sequence, Cys-Gly-Glu, between PI-*pfu*II and MBP and a Gly-Gly-Gly sequence between MBP and PI-*pfu*I were inserted by including the corresponding nucleotide sequences in the PCR primers.

Plasmid III was pICM239, and a connector sequence, Thr-Gly, was inserted between PI-*pfu*I (161−454) and MBP (239−370) as described previously (*8*).

Preparation of Precursor Peptides. E. coli BL21(λDE3) was transformed with each plasmid and then grown at 37 °C in 500 mL of LB medium (plasmids I and III) or 500 mL of M9 minimal medium containing $^{15}NH_4Cl$ as a sole nitrogen source (plasmid II). Cells were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at an OD_{620} of 0.5 and then incubated for 3 h. After cell disruption and centrifugation, all precursor fragments were present in the insoluble fraction. They were solubilized with a 20 mM Tris-HCl, pH 7.2, buffer containing 6 M guanidine hydrochloride (GdnHCl). The N-terminal and central precursor fragments were purified on an Ni-chelating column (Pharmacia) and then used for the refolding step.

Refolding of the Precursor and the Splicing Reaction. The N-terminal, C-terminal, and central precursor fragments were mixed in 20 mM Tris-HCl, pH 7.2, buffer containing 6 M GdnHCl and 300 mM NaCl. The mixture solution (240 mL) was dialyzed against 2 L of a buffer (20 mM Tris-HCl, pH7.2, 0.5 mM EDTA, 300 mM NaCl, 2.5 M urea, 10% glycerol) at 4 °C for 12 h twice, and then the dialysis buffer was exchanged to 5 L of another buffer (20 mM Tris-HCl, pH7.2, 0.5 mM EDTA, 300 mM NaCl, 10% glycerol) for 5 h to remove the denaturant. Next, 1,4-dithiothreitol (DTT) was added to 5 mM, and the solution was heated to 70 °C for 2 h for the splicing reaction. Since the product, CBD−MBP, was precipitated after the splicing reaction, the precipitate was collected by centrifugation and dissolved in a buffer containing 6 M GdnHCl, and then the product was refolded again. The refolded product was treated with thrombin to remove the N-terminal CBD, and then the produced MBP was purified to homogeneity on an amylose affinity column (New England Biolabs). Finally, a 0.2 mM × 300 μL protein solution was obtained.

NMR Experiment. The sample conditions were 0.2 mM protein, 1.8 mM maltose, 20 mM phosphate, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% D_2O, pH 6.8. A $^{15}N-^{1}H$ HSQC spectrum was acquired on a 500 MHz spectrometer (Bruker DMX500) at 303 K.

RESULTS

Ribonucleotide reductase from *Pyrococcus furiosus* contains two inteins, PI-*pfu*I and PI-*pfu*II, in its gene (*12*). We used these two inteins to ligate segments at two positions in a protein. PI-*pfu*I is 454 amino acids long and is located at the N-terminal of the host gene. PI-*pfu*II is the C-terminal

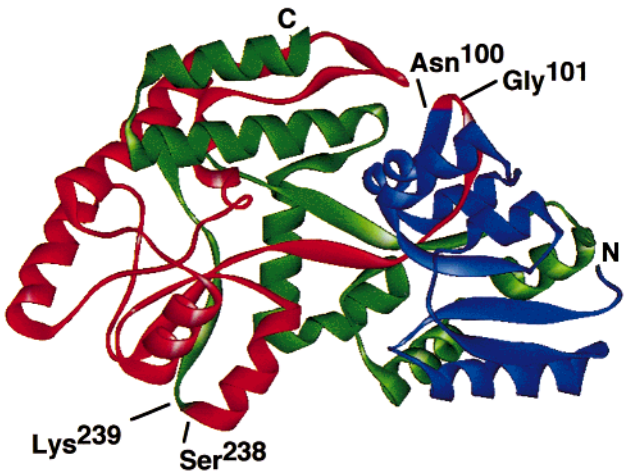

FIGURE 2: Selected segments of the structure of MBP (*15*) (PDB accession code, 1ANF). The N-terminal, central, and C-terminal segments are shown in blue, red, and green, respectively. The ligation positions are indicated by the residue numbers. These residues are located in the exposed loop structures.

intein of the host gene and is 382 amino acids long. PI-*pfu*I was fragmented at Lys^{160}−Gly^{161} as described previously (*7*), and PI-*pfu*II was fragmented at Tyr^{295}−Arg^{296} (residue numbers were counted from the N-terminal residue of each intein), which were susceptible sites to trypsin (PI-*pfu*I) and chymotrypsin (PI-*pfu*II), respectively. If one intein is used at two joints, refolding into an appropriate active conformation would be difficult because a precursor fragment may get entangled with a wrong partner, e.g., the N- and C-terminal fragments will be refolded without the central fragment leading to the production of byproducts. Since there is sequential homology among all inteins, even if different two inteins are used as in this study, the possibility of entanglement cannot be ruled out. Therefore, PI-*pfu*I and PI-*pfu*II are divided at different positions on the sequences to prevent entanglement, i.e., at after motif C for PI-*pfu*I and at motif H for PI-*pfu*II. As judged on sequence alignment (*13*, *14*), these positions seem to be in their endonuclease domains.

We applied this method to maltose binding protein (MBP), which consists of 370 amino acids and has a single structural domain and an intricate chain topology. MBP was divided into three segments, at positions Asn^{100}−Gly^{101} and Ser^{238}−Lys^{239}, which are in exposed loops (Figure 2). They were two of the ligation positions used in the previous study (*8*) to demonstrate segmental-half labeling of MBP, and the ligation by PI-*pfu*I at each of these positions was successful. Our preliminary data indicated that joints have to be flexible and exposed loops (*7*, *8*).

As shown in Figure 1, a fragment of the target protein and fragments of the inteins were joined on plasmids with two or three extra amino acids from the natural exteins. Since the first amino acid residue of the C-terminal extein (Thr for PI-*pfu*I and Cys for PI-*pfu*II) is directly involved in the splicing reaction (*16*, *17*), it is required as the first residue of the C-terminal fragment of the target protein. The last amino acid residue of the N-terminal extein seems to be involved in the intein−extein interaction (Gly for PI-*pfu*I and Pro for PI-*pfu*II). The other inserted amino acids are expected to increase the efficiency of the splicing reaction by bringing about the flexibility at the joints (*7*, *8*). As shown in Figure 3, all the inserted sequences are the same as those of the original exteins. Mutational analysis of the last connector sequence of the N-terminal extein of PI-*pfu*II (Thr-Asn-Pro was substituted by Gly-Gly-Gly or Gly-Gly-Pro) was performed. As a result, the splicing of PI-*pfu*II failed in the case of Gly-Gly-Gly but was successful in that of Gly-Gly-Pro, and the efficiency was equivalent to that in the case of the native sequence (data not shown). This indicates that Pro is involved in the splicing reaction but that the others are not important. The N-terminal fragment was fused with the cellulose binding domain at its N-terminus to increase the expression level in *E. coli* (*8*).

All the precursor peptides were produced by *E. coli* as inclusion bodies. The N-terminal and central segments were partially purified on a Ni column with 6 M GdnHCl and then used for the refolding. The refolding was performed in the reported manner (*8*) with a modification as to the reductant. The three denatured precursor fragments in 6 M GdnHCl were dialyzed against 20 mM Tris-HCl buffer (pH 7.2) containing 2.5 M urea and no reductant, incubated at 4 °C for 24 h, and then dialyzed against a buffer containing

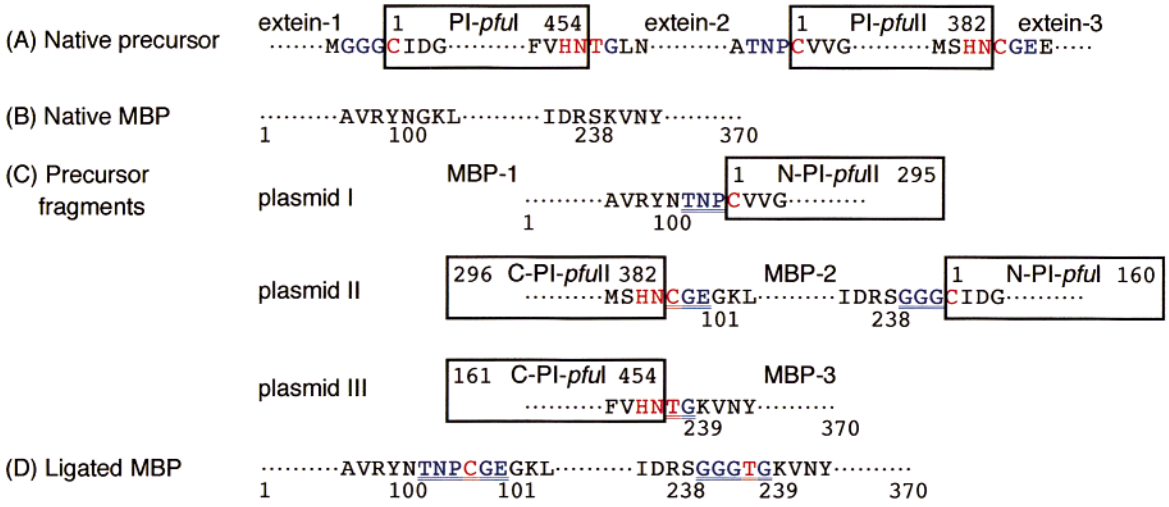

FIGURE 3: Amino acid sequences at the joints of the native and engineered precursor proteins: (A) native precursor protein of ribonucleotide reductase of *Pyrococcus furiosus*, (B) native MBP, (C) precursor fragments described in Figure 1, and (D) ligated MBP (product). Inteins are indicated by boxes. Red and blue show the crucial residues for the splicing activity and the inserted extra residues for flexibility at the joints, respectively. The double underlines indicate the total inserted residues.

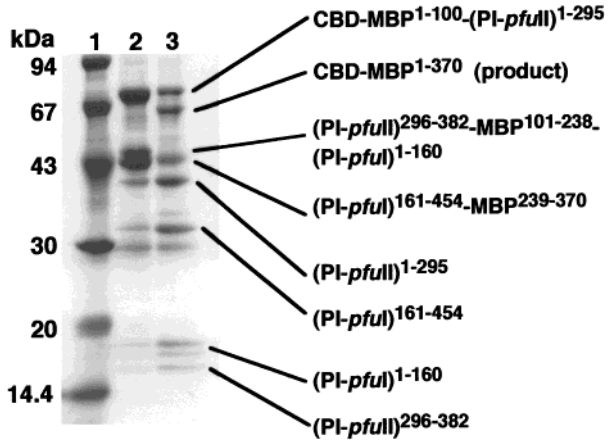

FIGURE 4: SDS−PAGE analysis of the segmental isotope labeling of MBP. The gel was stained with Coomassie brilliant blue. Lane 1, molecular weight markers; lane 2, mixture of the three precursor fragments after refolding and before the splicing reaction; and lane 3, after the splicing reaction. Bands of intein fragments and the product, CBD−MBP, were observed after the splicing reaction. Because the amounts of the unlabeled precursor fragments (N- and C-terminal fragments) were excessive, their bands were visible after the splicing reaction (lane 3), but the labeled central precursor fragment is completely absent in lane 3.

no denaturant or reductant. The splicing reaction was started by adding DTT to the mixture to 5 mM and heating to 70 °C, and then the sample was incubated for 2 h. Our preliminary experiments indicated that the splicing reaction of both PI-*pfu*I and PI-*pfu*II without DTT failed, but it was not required during the refolding of the precursor. The reason DTT was added after the refolding was that the refolding with DTT produced some byproducts and the reaction efficiency was less than 20%, as judged on SDS−PAGE. In such a case, the cleavage reaction occurred partially, but the ligation reaction seemed not to proceed. A protocol without DTT during the refolding and the addition of DTT just before the splicing reaction was the most successful, which provided an almost perfect reaction efficiency. The reason the refolding with DTT at 4 °C produced byproducts has not been determined yet. The splicing reaction was confirmed by the decreases in the amounts of the three precursor fragments and the appearance of the ligated protein and excised intein fragments on SDS−PAGE (Figure 4). The 0.2 mM × 300 μL central-segment ^{15}N-labeled MBP was obtained from 0.5 L of ^{15}N-labeled M9 minimal culture for the central fragment and 1 L of unlabeled LB culture for the N- and C-terminal fragments. Because the unlabeled precursor fragments were in excess, some remained after the splicing reaction. The labeled precursor fragment was almost completely converted to the product, indicating the high efficiency of the reaction.

The ^{15}N−^{1}H correlation spectrum (HSQC) of MBP labeled with ^{15}N at the residues between Gly^{101} and Ser^{238} is shown in Figure 5. Most signals of the segmentally labeled MBP show perfect agreement with those of the uniformly labeled sample; the exceptions being the new signals from the inserted extra residues and the shifted signals around the joints. It is clear that the number of signals was reduced and that overlapping was essentially avoided. The chemical shifts of the obtained signals correspond to the recently reported assignments (*18*).

DISCUSSION

We have used two inteins, PI-*pfu*I and PI-*pfu*II, from *Pyrococcus furiosus*, but many other inteins have been identified and can be used for this purpose. Each intein has a slightly different property from the others, and the sequence homology of exteins at joints is low. For this technique, the C-terminal residue of the N-extein and the N-terminal residue of the C-extein are important. An insertion or mutation of the joint is necessary to obtain a splicing active precursor. Appropriate inteins can be chosen to minimize insertion/mutation at the ligation positions of target proteins. The reaction conditions should also be considered. The reason inteins from a hyperthermophilic bacterium were used was that the refolding of the precursor fragments was expected to be easier due to their high thermal stability. Several refolding and splicing reaction conditions and their efficiencies were investigated in the previous study (*8*). We performed the splicing reaction at 70 °C, because a lower temperature caused a lower splicing efficiency, but it made the target protein, MBP, precipitate. Because of the easiness of the refolding of MBP, it was recovered without loss. If a high temperature during the splicing reaction causes irrevers-

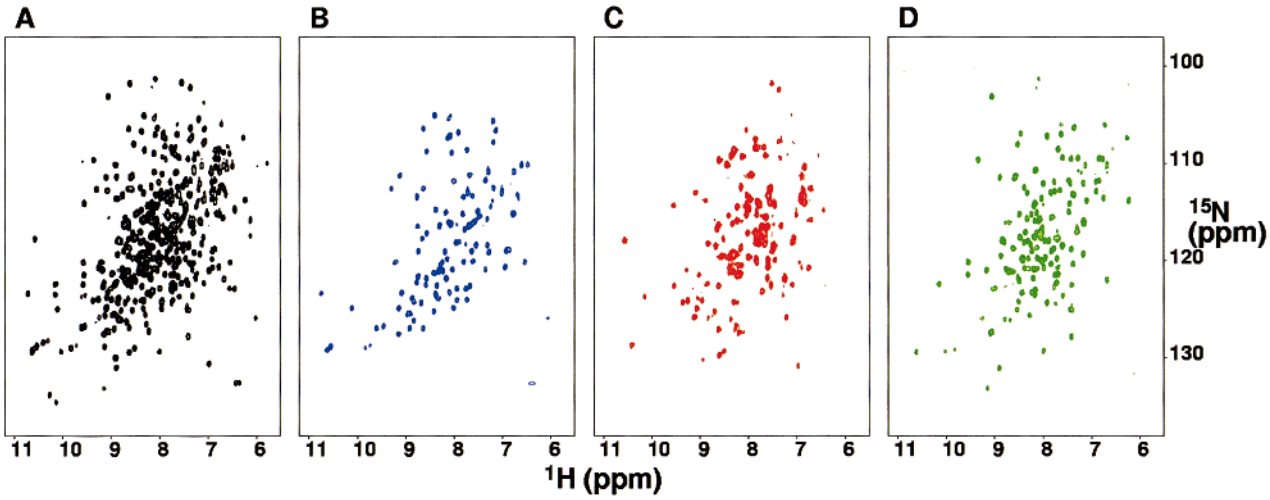

FIGURE 5: 2D ^{15}N−^{1}H HSQC spectra of (A) the uniformly ^{15}N-labeled wild-type MBP/maltose complex and those of MBP segmentally labeled with ^{15}N; (B) for the N-terminal segment, Lys^{1}−Tyr^{99} (*8*); (C) for the central segment, Gly^{101}−Ser^{238}; and (D) for the C-terminal segment, Lys^{239}−Lys^{370} (*8*).

ible damage, other inteins can be examined as well as in the case that an insertion or mutation causes activity loss of the target protein. [The Perler's New England Biolabs intein database (*19*) (InBase) constitutes a comprehensive archive.]

There are two related techniques: trans-protein splicing (*20*−*24*) and expressed protein ligation (*25*−*27*) methods. For expressed protein ligation, a target protein is expressed in *E. coli* as a fusion with a modified intein at its C-terminus. The fused intein generates an α-thioester derivative that can react with the N-terminal cysteine residue of an individually prepared peptide to form a peptide bond. Ligation of two independently folded domains (*27*) (one domain being isotopically labeled) using the expressed protein ligation method has been reported. This method does not require the step of denaturation of the precursor peptides, although our method includes a denaturation step. However, the isotope labeling of any segment between structurally flexible residues in a single domain can be achieved with our method.

We performed ^{15}N labeling in this study, but any type of labeling is applicable, e.g., ^{13}C, ^{2}H, and methyl ^{1}H labeling (*28*, *29*). For the purpose of structure determination, the importance of segmental labeling for sequential assignment through triple-resonance experiments and for extracting unambiguous interproton distances from NOESY spectra should be emphasized (*8*). Recently invented techniques, TROSY (transverse relaxation optimized spectroscopy) experiments, and angle determination experiments involving the measurement of residual dipolar coupling prevent sensitivity losses caused by relaxation and increase the feasibility of NMR for larger proteins (*30*−*34*). The combination of isotope labeling and NMR experimental techniques will certainly facilitate an increase in the size limit to over 50 kDa. Segmental labeling will be useful for any NMR study on larger proteins. Extracting information from a selected region of interest in a protein molecule, e.g., for studies of ligand−protein interactions and drug designing using SAR (structure−activity relationships) by NMR (*35*), is extremely beneficial.

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