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A new general method for the biosynthesis of stable isotope-enriched peptides using a decahistidine-tagged ubiquitin fusion system: An application to the production of mastoparan-X uniformly enriched with ^{15}N and ^{15}N/^{13}C

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Abstract

A new strategy is described for the production of peptides enriched with stable isotopes. Peptides of interest are expressed in *Escherichia coli* (*E. coli*) cells as recombinant fusion proteins with *Saccharomyces cerevisiae* ubiquitin. This method yields as much as 30–100 mg/l of isotope-enriched fusion proteins in minimal media. A decahistidine tag attached to the N-terminus of ubiquitin enables a one-step purification of the fusion protein via Ni^{2+}-chelating affinity chromatography. The ubiquitin moiety is then easily and specifically cleaved off by a protease, yeast ubiquitin hydrolase. Since this enzyme is also expressed at a high level in *E. coli* cells and can be purified in one step, the presented strategy has an advantage in view of costs over others that use commercially available proteases. In addition, since ubiquitin fusion proteins easily refold, the fusion protein can be expressed either in a soluble form or as inclusion bodies. This flexibility enables us to prepare peptides that are unstable in a soluble state in *E. coli* cells. As an example, the expression and the uniform stable isotope enrichment with ^{15}N and/or ^{13}C are described for mastoparan-X, a tetradecapeptide known to activate GTP-binding regulatory proteins. An amide group at the C-terminus of this peptide can also be formed by our method. The presented system is considered powerful for the stable isotope enrichment of short peptides with proton resonances that are too severely overlapped to be analyzed solely by proton NMR.

Abbreviations: CD, circular dichroism; *E. coli*, *Escherichia coli*; G protein, GTP-binding regulatory protein; GST, glutathione S-transferase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum coherence; H_{10}-Ub-MPX-G, decahistidine-tagged ubiquitin fusion of MPX-G; IPTG, isopropyl-β-O(−)-thiogalactopyranoside; MalE, maltose-binding protein; MP-X, mastoparan-X; MPX-G, mastoparan-X with a glycine residue added to the C-terminus; NOESY, nuclear Overhauser effect spectroscopy; PCR, polymerase chain reaction; *S. cerevisiae*, *Saccharomyces cerevisiae*; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEV, tobacco etch virus; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; TOF-MS, time-of-flight mass spectroscopy; TPPI, time-proportional phase increment; TRNOE, transferred nuclear Overhauser effect; WATERGATE, water suppression by gradient-tailored excitation; YUH, yeast ubiquitin hydrolase.

Introduction

Multidimensional heteronuclear spectroscopies of proteins enriched with ^{15}N and/or ^{13}C have been shown to greatly improve the proteins' spectral resolutions (Clore and Gronenborn, 1991; Bax and Grzesiek, 1993). Although this strategy has mostly been applied to larger proteins (MW > 5000), it should also be beneficial to resolve the resonances of short peptides with poorly dispersed proton chemical shifts, because short peptides usually do not adopt ordered conformations. The expression systems used thus far for stable isotope enrichment of larger proteins, however, are not

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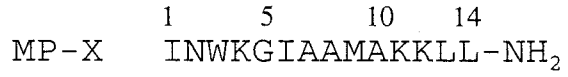

Figure 1. Amino acid sequence of MP-X.

suitable for short peptides for several reasons: (i) Short peptides are difficult to directly express in *E. coli* cells, because they usually do not adopt an ordered conformation and are easily degraded by cellular proteases. (ii) When short peptides are expressed as a fusion protein with maltose-binding protein, glutathione S-transferase, or thioredoxin to increase the stability in the cell, it is difficult to choose a method that specifically cleaves the fusion protein at the desired site. (iii) Fusion carrier proteins commercially available are too large in size to be fused with short peptides; most of the mass of the fusion protein is due to the carrier. A new general method to prepare short peptides enriched with stable isotopes is thus needed.

Here we report the expression of isotope-enriched short peptides by developing a ubiquitin fusion system (Miller et al., 1989). The specificity of the protease in this system, yeast ubiquitin hydrolase (Miller et al., 1989), is sufficiently high and its cost is negligible in comparison to that of the isotope sources, because the protease itself can also be expressed in *E. coli* cells and is easily purified. Moreover, a peptidylglycine α-amidating enzyme can be used to add an amide moiety to the peptide's C-terminus, which is often essential for the activities of many hormonal peptides and neuropeptides. Using this system, we have produced [U-^{15}N] and [U-^{15}N/^{13}C] mastoparan-X (MP-X), a 14 amino acid residue peptide with a C-terminal amide group (Figure 1), which is known to directly activate GTP-binding regulatory proteins (G proteins, reviewed by Gilman (1987)) in a similar manner to those of G protein-coupled receptors (Higashijima et al., 1988). The isotope-enriched peptides thus obtained should be useful for analyzing peptide–protein, peptide–lipid, and many other complex biological interactions by multinuclear multidimensional NMR techniques.

Materials and methods

Materials

The following materials were used: pET vectors (Novagen, WI, U.S.A.), Ni^{2+}-NTA-Agarose (Qiagen, Hilden, Germany), Sep-Pak Plus C_{18} cartridges (Waters, MA, U.S.A.), a Cosmosil 5C18-AR reversed-phase C_{18} column (Nacalai Tesque, Kyoto, Japan), [^{15}N] ammonium chloride [99% ^{15}N] and [$^{13}C_6$] glucose [99% ^{13}C] (ISOTEC Inc., OH, U.S.A.), and peptidylglycine α-amidating enzyme (Wako Pure Chemicals, Osaka, Japan). All other reagents were of analytical grade (Nacalai Tesque, Kyoto, Japan).

Chemical peptide synthesis

In addition to the bacterial expression described later, for comparison purposes, the standard solid-phase methodology was employed to synthesize MP-X using an Applied Biosystems model 431A peptide synthesizer. The peptide was purified by a Shimadzu LC-8A system with a Cosmosil 5C18-AR reversed-phase C_{18} column (20 × 250 mm), and its purity was confirmed by a Shimadzu LC-6A system with a C_{18} column (4.6 × 250 mm). The peptide's identity was confirmed by TOF-MS spectra recorded on a Voyager-DE mass spectrometer (PerSeptive Biosystems).

Construction of the YUH expression vector

The gene encoding YUH was cloned from the total DNA of *S. cerevisiae* by using PCR. The sequences of the PCR primers were designed according to the reported DNA sequence of YUH (Miller et al., 1989) and so as to introduce an *Nde*I site at the N-terminus of the open reading frame and an *Xho*I site just after the C-terminus. To introduce a hexahistidine tag at the C-terminus of the protein, the resultant open reading frame was ligated into the *Nde*I/*Xho*I sites of the pET-20b vector, thus producing pET-20b/YUH (Figure 2). This plasmid was transformed into *E. coli* strain JM109 and was prepared on a mini-scale. The plasmid sequence was verified using a dideoxy-sequencing method.

Construction of an expression vector for the decahistidine-tagged ubiquitin fusion protein

A new vector, pTKK19, was created from the pET-19b vector by respectively replacing its Ori region by that of the pUC vectors to increase the copy number and its β-lactamase gene region by a kanamycin resistance gene to increase the stability of the plasmid (Studier et al., 1990). The yeast ubiquitin gene was cloned from the total DNA of *S. cerevisiae* using PCR with primers designed according to the reported DNA sequence of the protein (Özkaynak et al., 1984). The primers also introduced an *Nde*I site to the N-terminus and an *Eco*T22 I site followed by *Sal*I and *Bam*HI sites to the C-terminus of the gene. The open reading frame was ligated into the *Nde*I/*Bam*HI sites of pTKK19,

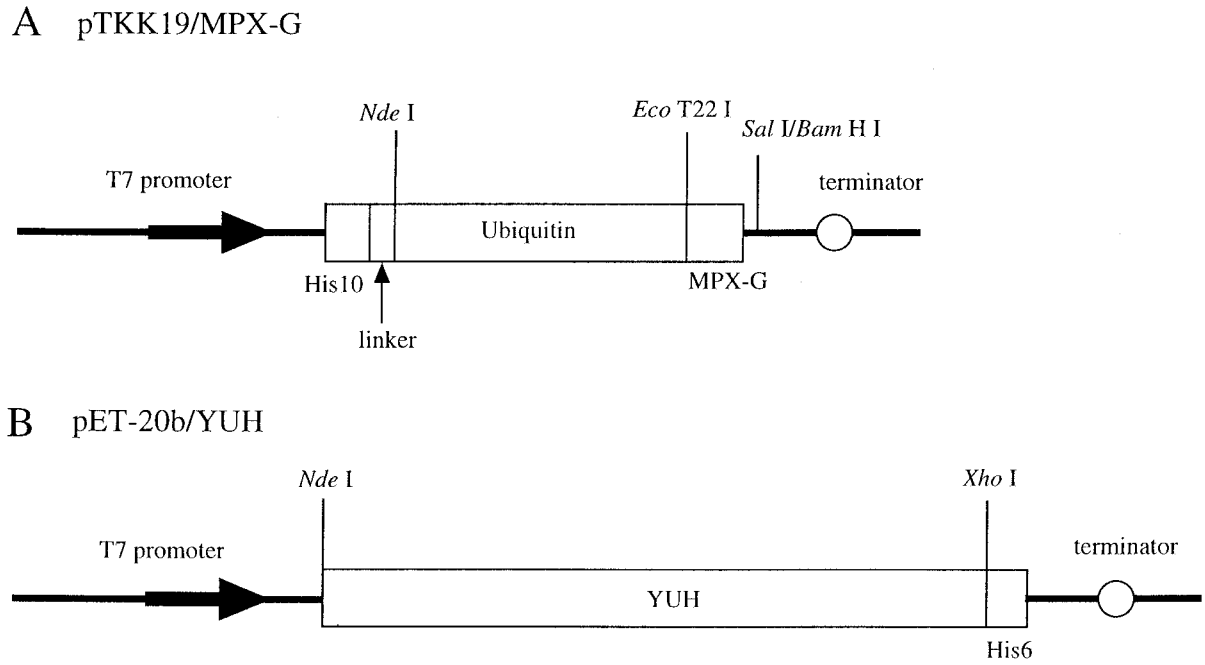

Figure 2. Structural genes of the plasmids used for the production of isotope-enriched recombinant ubiquitin fusion proteins and recombinant YUH under the control of the T7 promoter. Origins and features of the production plasmid vectors are described in the Materials and Methods section. (A) The expression cassette in plasmid pTKK19/MPX-G used for producing H_{10}-Ub-MPX-G. (B) The expression cassette in plasmid pET-20b/YUH used for producing YUH.

to create pUBK19. The pUBK19 plasmid was cut by *Eco*T22 I, blunted using a blunting kit (TAKARA, Osaka, Japan), and cut by *Sal*I again. It was then ligated with the chemically synthesized oligonucleotide encoding MP-X (see Figure 1) followed by a single glycine codon (MPX-G), a stop codon, and a *Sal*I site, annealed, and then cut by *Sal*I, to create pUBK19/MPX-G (Figure 2). The additional glycine residue was introduced because the peptidylglycine α-amidating enzyme used to amidate the peptide's C-terminus catalyzes two reactions: the hydroxylation of the α-carbon at the C-terminal glycine residue and the subsequent N–C bond cleavage in the resultant hydroxyglycine moiety (Katopodis et al., 1990; Suzuki et al., 1990; Takahashi et al., 1990). The resultant plasmid was transformed into *E. coli* strain JM109 and was prepared on a mini-scale. The sequence of the pUBK19/MPX-G plasmid was confirmed using a dideoxy-sequencing method.

Purification of hexahistidine-tagged YUH

E. coli BL21(DE3) cells harboring the plasmid encoding YUH (pET-20b/YUH) were cultured at 37 °C overnight in 5 ml of LB medium containing 50 μg/ml kanamycin, and were then transferred to 3 l of the same medium. When the OD_{600} reached 1.0, IPTG (0.4 mM) was added, and protein expression was induced for 12 h at 37 °C. Cells were harvested, dispersed in 150 ml of buffer A containing 50 mM Tris-HCl (pH 8.0), 100 mM sodium chloride, and 1 mM β-mercaptoethanol, and then disrupted using sonication. After centrifugation, the supernatant was loaded onto a 15 ml column of Ni^{2+}-NTA-Agarose equilibrated with buffer A. Following wash steps with 50 ml of buffer A containing 500 mM sodium chloride and 100 ml of buffer A, the YUH protein was eluted by imidazole gradient from 0 to 200 mM in buffer A (160 ml). The YUH protein was eluted at about 100 mM imidazole and was used without further purification or buffer exchange for a subsequent cleavage reaction. Protein concentrations were determined by amido black staining with BSA as the standard (Schaffner and Weissmann, 1973).

Purification of H_{10}-Ub-MPX-G from soluble and insoluble cellular fractions

The *E. coli* strain BL21(DE3) harboring the plasmid encoding H_{10}-Ub-MPX-G was cultured overnight in 5 ml of LB medium containing 50 μg/ml kanamycin, and was then transferred to 3 l of M9 minimal medium

supplemented with trace elements, as described elsewhere (Mori et al., 1979; Mizutani et al., 1986), and with $^{15}NH_4Cl$ and/or [$^{13}C_6$] glucose (refer to Table 1). When the OD_{600} reached 1.0, IPTG (0.4 mM) was added, and protein expression was induced for 12 h. Cells were harvested, dispersed, disrupted, and centrifuged as above. The resultant supernatant was loaded onto a 15 ml column of Ni^{2+}-NTA-Agarose equilibrated with buffer A. Following wash steps with 50 ml of buffer A containing 500 mM sodium chloride and 100 ml of buffer A, the fusion protein was eluted by an imidazole gradient from 0 to 400 mM in buffer A (120 ml). The protein was eluted at about 200 mM imidazole and was used without further purification or buffer exchange for a subsequent cleavage reaction.

The fusion protein that formed the inclusion bodies and pelleted in the above centrifugation was also purified: the pellet was dispersed in 100 ml of buffer A containing 6 M guanidine hydrochloride, and was then shaken at 200 rpm at 4 °C overnight. After centrifugation, the supernatant was loaded onto a 15 ml column of Ni^{2+}-NTA-Agarose equilibrated with buffer A containing 6 M guanidine hydrochloride. After a wash with 100 ml of the same buffer, the absorbed fusion protein was refolded in situ by a 'reversed' gradient of guanidine hydrochloride from 6 to 0 M (120 ml). Following a wash step with 100 ml of buffer A (without guanidine hydrochloride), the adsorbed protein was eluted as for the soluble fusion protein.

Cleavage of H_{10}-Ub-MPX-G with YUH and purification of MPX-G

The H_{10}-Ub-MPX-G protein (1 mg/ml) was cleaved by YUH (1 mg/ml) in buffer A as described above at 37 °C for about 1 h, and the cleavage was monitored by tricine-SDS-PAGE (Schägger and von Jagow, 1987). To avoid damage to the HPLC column and to roughly purify MPX-G, the mixture was applied to Sep-Pak Plus C_{18} cartridges, and was then eluted with increasing acetonitrile concentrations in 10% steps. HPLC analyses indicated that MPX-G was eluted with approximately 40% acetonitrile. Fractions containing MPX-G were pooled, lyophilized, and redissolved in 0.1% TFA. The MPX-G protein was thoroughly purified by reversed-phase HPLC via a Cosmosil 5C18-AR column with solvent B (0.1% TFA in water) and solvent C (0.1% TFA in acetonitrile). The elution was carried out with a linear gradient from 20 to 50% of solvent C over 30 min at a flow rate of 1 ml/min, with the absorbance monitored at 214 nm. The resultant peptide fractions were pooled and lyophilized.

α-Amidation of MPX-G to produce C-terminally amidated MP-X

The α-amidation of the C-terminus of MPX-G to produce C-terminally amidated MP-X was performed as follows. Briefly, the reaction mixture contained 50 mM HEPES-NaOH (pH 7.0), 10 mM potassium iodide, 3 mM ascorbic acid, 0.25 μM copper sulfate, 7.5% glycerol, 0.05% catalase, 2 mg/ml MPX-G, and 2000 units/ml of peptidylglycine α-amidating enzyme, and was incubated for 2–6 h at 37 °C. This incubation time was optimized by monitoring the HPLC chromatograms (see below). The reaction mixture was filtered using Ultrafree-MC (Millipore), and was then subjected to HPLC. Reversed-phase HPLC was performed via a Cosmosil 5C18-AR column (6×150 mm) with solvents B and C. The elution was carried out with a linear gradient from 20 to 50% of solvent C over 20 min at a flow rate of 1 ml/min, with the absorbance monitored at 214 nm. The resultant MP-X fractions were pooled and lyophilized.

Identification of biologically synthesized MP-X

TOF-MS measurements were performed on a Voyager-DE mass spectrometer to determine the peptide's mass. Amino acid analyses were performed on a Beckman System Gold amino acid analyzer after hydrolysis in 6 M hydrochloric acid at 110 °C for 24 h and derivatization by 4-dimethylaminoazobenzene-4′-sulfonyl chloride. The amino acid sequence was confirmed using a pulse liquid phase protein sequencer (Applied Biosystems model 477A).

NMR analyses

NMR samples contained approximately 2 mM of MP-X in either 99.96% D_2O or 90% H_2O/10% D_2O at pH 6.0 (direct pH meter reading). All NMR measurements were performed on either a Bruker AMX-500 or ARX-400 spectrometer at 20 °C. The experiments are listed in Table 2. All proton 2D NMR spectra were acquired in a phase-sensitive mode using time-proportional phase increments (TPPI) (Marion and Wüthrich, 1983) for quadrature detection in the t_1 dimension. All heteronuclear NMR spectra were acquired in a phase-sensitive mode using States-TPPI (Marion et al., 1989) for quadrature detection in the indirect dimensions.

Non-enriched MP-X was used to record the TOCSY spectra. The water resonance was suppressed by coherent presaturation during the 1 s relaxation delay. TOCSY spectra were recorded using an MLEV-17

Table 1. Components of minimal medium for the production of stable isotope-enriched fusion proteins (for 1 l)

Component	
Solution A	11 − (Sol B + Sol C + Sol D + Sol E)
Solution B	10 ml
Solution C	10 ml
Solution D	1 ml
Solution E	25 ml (U-^{15}N)[a] or 5 ml (U-^{15}N, ^{13}C)[b]

Contents of each solution Component	Amount
Solution A (autoclaved, with the pH adjusted to 7.3 by NAOH)	
KH_2PO_4	4 g/l
K_2HPO_4	4 g/l
$Na_2HPO_4 \cdot 12H_2O$	7 g/l
NaCl	1 g/l
$^{15}NH_4Cl$	0.5 g/l (U-^{15}N)[a] 1 g/l (U-^{15}N,^{13}C)[b]
Solution B (trace elements, autoclaved)	
$FeSO_4 \cdot 7H_2O$	4 g/l
$CaCl_2 \cdot 2H_2O$	4 g/l
$AlCl_3 \cdot 6H_2O$	1 g/l
$MnSO_4 \cdot nH_2O$	1 g/l
$CoCl_2 \cdot 6H_2O$	0.4 g/l
$ZnSO_4 \cdot 7H_2O$	0.2 g/l
$Na_2Mo_4 \cdot 2H_2O$	0.2 g/l
$CuCl_2 \cdot 2H_2O$	0.1 g/l
H_3BO_3	0.1 g/l
Solution C (autoclaved)	
$MgSO_4$	1 mol/l
Solution D (sterile filtered)	
Thiamine hydrochloride	150 mmol/l
Solution E (sterile filtered)	
Glucose	200 g/l

[a]The amount was used for uniformly labeling the ^{15}N-enriched fusion protein.
[b]The amount was used for uniformly labeling the ^{15}N- and ^{13}C-enriched fusion protein.
[c]The amount is the final concentration in the medium for solution A and the concentration in the solution for other solutions.

pulse scheme (Bax and Davis, 1985) with an isotropic mixing time of 80 ms.

Uniformly ^{15}N-enriched MP-X was used to record a 2D ^{1}H-^{15}N HSQC spectrum (Bodenhausen and Ruben, 1980) and a 3D ^{15}N-edited TOCSY-HSQC spectrum with an 80 ms mixing time, using the parameters in Table 2. For the 2D HSQC and 3D TOCSY-HSQC spectra, the water resonance was suppressed by using the WATERGATE scheme (Piotto et al., 1992) and a water flip-back pulse (Grzesiek and Bax, 1993). Decoupling of ^{15}N was achieved by WALTZ-16 modulation (Shaka et al., 1983). Uniformly ^{13}C- and ^{15}N-enriched MP-X was used to record a 2D ^{1}H-^{13}C HSQC spectrum, a 3D HNCO spectrum (Kay et al., 1990; Muhandiram and Kay, 1994), a 3D HN-CACB spectrum (Kay et al., 1990; Wittekind and Mueller, 1993; Muhandiram and Kay, 1994), a 3D CBCA(CO)NH spectrum (Kay et al., 1990; Grzesiek

Table 2. Parameters for NMR experiments

NMR experiments	Nucleus			No. of points			Final matrix size			Spectral width (Hz)		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
Non-enriched MP-X												
2D TOCSY	^{1}H	^{1}H		512	2048		1024	2048		6250	6250	
^{15}N-enriched MP-X												
2D HSQC[c]	^{15}N	^{1}H		256	2048		256	1024[a]		1200	6250	
3D TOCSY-HSQC[c]	^{1}H	^{15}N	^{1}H	128	128	1024	256	256	512[a]	6250	1200	6250
^{13}C- and ^{15}N-enriched MP-X												
2D HSQC[e]	^{13}C	^{1}H		256	2048		256	2048		20000	6250	
3D HNCO[c,h]	^{15}N	^{13}C′	^{1}H	128	48	1024	256	256	512[a]	1200	1000	6250
3D HNCACB[d,f]	^{13}C	^{15}N	^{1}H	96	56	1024	256	256	512[a]	8000	1250	6250
3D CBCA(CO)NNH[d,f]	^{13}C	^{15}N	^{1}H	96	60	1024	256	256	512[a]	8000	1250	6250
3D TOCSY-HSQC[g]	^{1}H	^{13}C	^{1}H	80	32	1024	128	128	512[b]	6250	7400	6250

[a,b]The spectral data in this dimension were extracted only for the amide[a] or aliphatic[b] regions.
[c,d]The ^{15}N carrier was placed at 119 ppm[c] or 118.6 ppm[d].
[e,f,g]The ^{13}C carrier was placed at 86.7 ppm[e], 45.4 ppm[f], or 35.7 ppm[g].
[h]The ^{13}C′ carrier was placed at 176.5 ppm. The ^{1}H carrier was placed on the water resonance at 4.85 ppm.

and Bax, 1992; Muhandiram and Kay, 1994), and a 3D ^{13}C-edited TOCSY-HSQC spectrum with an 80 ms mixing time, using the parameters listed in Table 2. The 3D ^{13}C-edited TOCSY-HSQC experiment was performed using the pulse sequence modified from the 3D ^{15}N-edited TOCSY-HSQC. For all of these measurements, the water resonance was suppressed by using the WATERGATE scheme and water flip-back. Decoupling of ^{15}N and C′ was achieved by WALTZ-16 modulation (Shaka et al., 1983). Decoupling of ^{13}C was achieved by GARP modulation (Shaka et al., 1985).

Data processing was performed on either a Bruker X-32 UNIX workstation with UXNMR software or on a Silicon Graphics INDIGO2 workstation using the program packages NMRDraw and NMRPipe (Delaglio et al., 1995). For proton 2D NMR data sets, a 60° phase-shifted sine-squared window function was used in both dimensions prior to Fourier transformation. Final matrix sizes were usually 1024 × 2048 real points in the F1 and F2 dimensions, respectively. For 3D heteronuclear NMR data sets, linear prediction methods (Zhu and Bax, 1990, 1992) were employed to extend the data in the indirectly acquired dimensions. After linear prediction, a 72° phase-shifted sine-squared window function and a single zero-filling were used in all dimensions prior to Fourier transformation.

In all experiments, the ^{1}H carrier was placed on the H_2O resonance at 4.85 ppm, and both the ^{15}N and ^{13}C carriers were set differentially, respectively, depending on the experiments, as shown in Table 2. All proton chemical shifts were referenced to the methyl resonance of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) used as an internal standard. The ^{13}C and ^{15}N chemical shifts were indirectly referenced using the following ratios of the zero-point frequencies: 0.101329118 for ^{15}N to ^{1}H and 0.25144953 for ^{13}C to ^{1}H (Wishart et al., 1995b).

Circular dichroism

A CD spectrum of MP-X was recorded on a JASCO J-720 spectropolarimeter (Jasco, Japan). MP-X was dissolved to 100 μM in a buffer containing 90% H_2O/10% D_2O, 10 mM sodium acetate-d_4, and 1 mM DTT-d_{10} at pH 6.0, which is the same condition as that used in the NMR measurements. The spectrum was acquired at room temperature (≈293 K) over 195–250 nm using a 0.5 mm cuvette at a sensitivity of 50 mdeg/cm, and a scan speed of 100 nm/min. It was recorded digitally, and each signal was averaged 4 times, followed by subtraction of an equally signal-averaged solvent baseline by feeding through a data processor.

Results

Preparation of YUH

YUH was the major soluble protein found in the lysate of *E. coli* cells expressing YUH (Figure 3B, lane 2), whereas it was not found in the disrupted cell pellet (data not shown). This enzyme was purified to more than 95% by Ni^{2+}-NTA-Agarose chromatography (Figure 3B, lane 3), and its yield was 120–150 mg per liter culture.

Purification of H_{10}-Ub-MPX-G from soluble and insoluble fractions

Since H_{10}-Ub-MPX-G was found in both the soluble and insoluble (inclusion body) fractions of cells grown at 37 °C in M9 medium enriched with stable isotopes (Figure 3A, lanes 2 and 3), purification was performed from both fractions. For the soluble fusion protein, the purification was straightforward. As for the insoluble fusion protein, it could be fully solubilized by unfolding with 6 M guanidine hydrochloride, and then was fully refolded in a Ni^{2+}-NTA-Agarose column using a reverse gradient of guanidine hydrochloride. The correct folding of this refolded fusion protein was confirmed by the observation that the protein was specifically and completely cleaved by YUH (data not shown). The total yield of purified protein was about 60 mg per liter culture: 30 mg from the soluble fraction and 30 mg from the insoluble fraction per liter culture. Note that both the fusion protein fractions derived from the soluble and insoluble fractions were purified to more than 95% with this one-step purification, and were sufficiently pure for subsequent digestion by YUH (Figure 3A, lanes 4 and 5).

Cleavage of H_{10}-Ub-MPX-G by YUH, purification of MPX-G, and amidation of the C-terminus

The cleavage time courses of H_{10}-Ub-MPX-G by YUH were monitored by tricine-SDS-PAGE (Figure 4). The fusion protein was completely cleaved by a 1 h incubation (Figure 4, lane 4), and non-specific digestion was not observed even after 16 h (Figure 4, lane 5). The absence of non-specific digestion was also confirmed by an HPLC analysis of a cleaved peptide (data not shown). Figure 5A shows a reversed-phase HPLC chromatogram of MPX-G that had been roughly purified via Sep-Pak Plus C_{18} cartridges. MPX-G was eluted at about 40% acetonitrile, and rechromatography of this peak showed a single peak (Figure 5B). The yield of this cleavage/purification step was about 50%. The amidation time course of

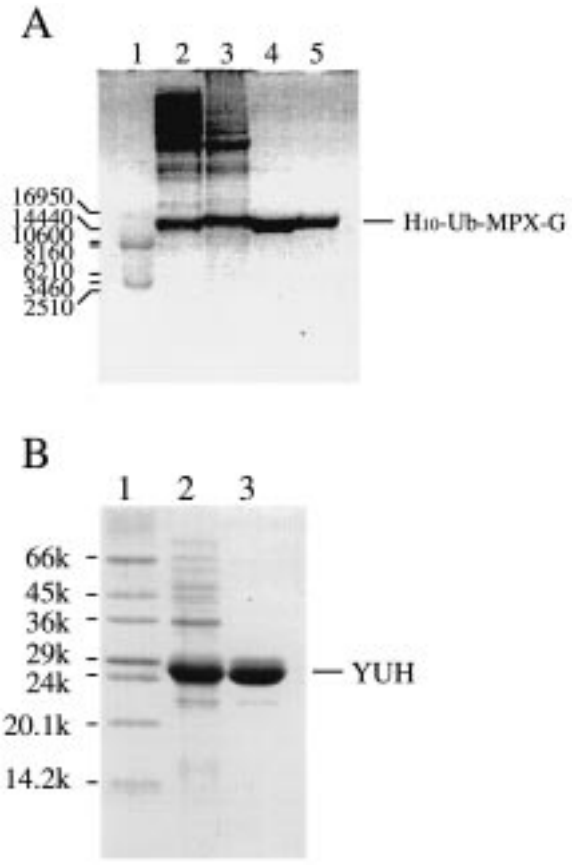

Figure 3. SDS-PAGE analysis of H_{10}-Ub-MPX-G and YUH. (A) Purification of isotope-enriched H_{10}-Ub-MPX-G analyzed by 16.7% tricine-SDS-PAGE. Lane 1, molecular-weight standards, Sigma MW-SDS-17S (myoglobin fragments 16950, 14440, 10600, 8160, 6210, and 2510 Da, and glucagon 3460 Da); lanes 2 and 3, supernatant and pellet fractions of a lysate of cells expressing [U-^{15}N] H_{10}-Ub-MPX-G grown at 37 °C, respectively; lanes 4 and 5, [U-^{15}N] H_{10}-Ub-MPX-G purified via Ni^{2+}-NTA-Agarose from the supernatant and the pellet fractions, respectively. (B) Purification of YUH analyzed by 15% SDS-PAGE. Lane 1, molecular-weight standards, Sigma SDS-7 (bovine serum albumin, 66000; egg albumin, 45000; glyceraldehyde-3-phosphate dehydrogenase, 36000; carbonic anhydrase, 29000; trypsinogen, 24000; soybean trypsin inhibitor, 20100; milk α-lactoalbumin, 14 200); lane 2, crude extract of cells expressing YUH; lane 3, YUH purified via Ni^{2+}-NTA-Agarose.

MPX-G by the peptidylglycine α-amidating enzyme was monitored by reversed-phase HPLC (Figure 5B, C–D). The amidated product, MP-X, was eluted earlier than the substrate MPX-G, and the intermediate of the amidation was eluted later than the substrate MPX-G (Figure 5C). Figure 5D shows that a 2 h reaction was sufficient for the complete amidation of MPX-G. The yield of this amidation/purification step was about 40%.

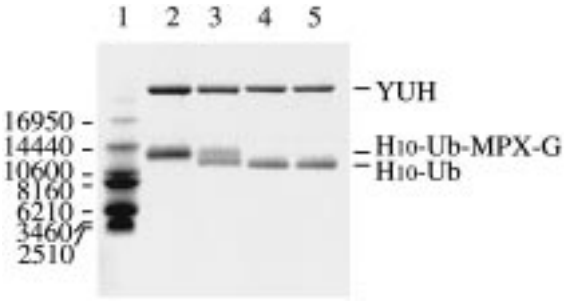

Figure 4. 16.7% tricine-SDS-PAGE analysis of the cleavage time course of H_{10}-Ub-MPX-G by YUH. Lane 1, molecular-weight standards, Sigma MW-SDS-17S (see Figure 3A); lanes 2–5: reaction mixture after an incubation of 0, 0.2, 1, and 16 h, respectively. The product MPX-G cannot be detected in this gel because of its low molecular weight (1.6 kDa).

The final yield of purified MP-X enriched with stable isotopes, as determined by quantitative amino acid analysis, was >0.6 and >0.3 mg per liter culture for ^{15}N- and ^{15}N/^{13}C-enriched MP-X, respectively. When the concentration of [$^{13}C_6$] glucose was doubled, the yield per liter culture increased; nevertheless, the yield per gram glucose decreased. When *E. coli* cells were grown in LB medium, the yield of (non-enriched) MP-X was about 1 mg/l. The obtained MP-X showed >95% homogeneity by HPLC analysis (Figure 5C) and >99% total isotope enrichment as determined by TOF-MS (Figure 6).

Sequential resonance assignments of MP-X

A TOCSY spectrum of non-enriched MP-X in an aqueous solution (Figure 7A) indicates that its amide proton resonances are severely overlapped. This is because the peptide does not adopt an ordered conformation under the conditions used, as revealed by its CD spectrum (Figure 8). Nevertheless, the peptide's amide nitrogen resonances are well dispersed in a ^{1}H-^{15}N HSQC spectrum of ^{15}N-enriched MP-X (Figure 7B). Its ^{13}C resonances are also well dispersed, as demonstrated by the ^{1}H-^{13}C HSQC spectrum of ^{15}N/^{13}C-enriched MP-X (Figure 7C). The assignments of the backbone (C^{α}, N, and HN) and $^{13}C^{\beta}$ resonances were hence carried out based on the CBCA(CO)NH and HNCACB spectra (data not shown). Owing to the well-resolved amide nitrogen resonances, the assignments from the two spectra were straightforward. Carbonyl carbon resonances were assigned by a 3D HNCO spectrum (data not shown). The assignments of the side-chain proton resonances were obtained from a 3D ^{15}N-edited TOCSY-HSQC spectrum of ^{15}N-enriched MP-X (data not shown). The

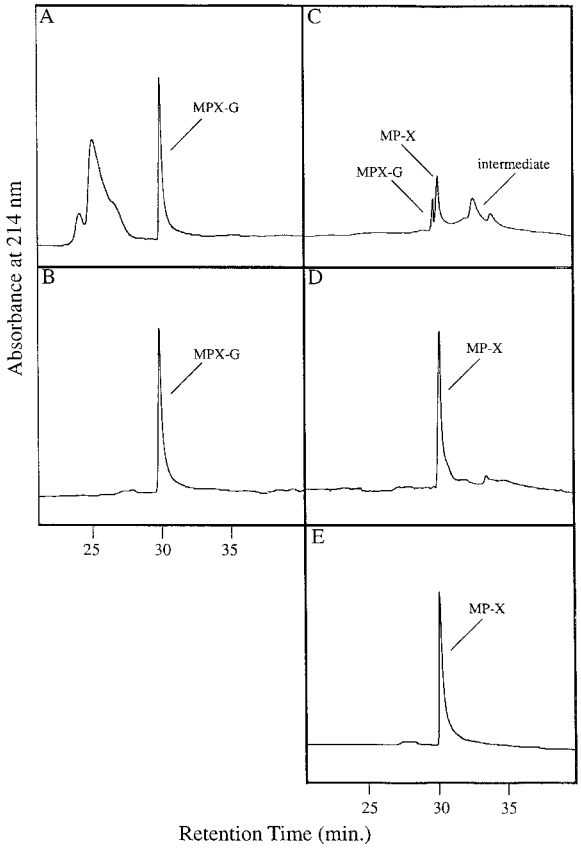

Figure 5. Reversed-phase HPLC chromatograms of MPX-G and MP-X uniformly enriched with ^{15}N. (A) [U-^{15}N] MPX-G partially purified via Sep-Pak Plus C_{18} cartridges. (B) Purified [U-^{15}N] MPX-G. (C) Partially α-amidated [U-^{15}N] MPX-G after a 0.5 h reaction with the peptidylglycine α-amidating enzyme. (D) Fully α-amidated [U-^{15}N] MPX-G after a 2 h reaction. (E) Purified [U-^{15}N] MP-X after full α-amidation.

assignments for the side-chain carbon resonances were obtained from the 3D ^{13}C-edited TOCSY-HSQC and 2D ^{1}H-^{13}C HSQC spectra of ^{13}C- and ^{15}N-enriched MP-X (data not shown). The obtained chemical shifts are listed in Table 3. The details of the ^{13}C and ^{15}N chemical shift resolutions in a random-coil state will be discussed later.

Discussion

Preparation of uniformly ^{15}N- and/or ^{13}C-labeled peptides using the decahistidine-tagged ubiquitin fusion protein system

From the need to record recent multinuclear multidimensional NMR spectra, a variety of systems have been recently developed to produce proteins uniformly

Table 3. Chemical shifts of ^{1}H, ^{15}N, $^{13}C'$, and ^{13}C resonances of MP-X at 20 °C and pH 6.0[a]

Residue	Chemical shift (ppm)							
	NH		$^{13}C'$	$C^{\alpha}H$		$C^{\beta}H$		Others
Ile^{1}	–[b]	–[b]	–[b]	60.3	(3.71)	39.1	(1.74)	C^{γ} 26.7 (1.37, 1.07); $C^{\gamma m}$ 16.5 (0.69); C^{δ} 13.1 (0.81)
Asn^{2}	–[b]	–[b]	174.9	52.6	(4.81)	38.5	(2.76, 2.84)	γNH_2 113.3 (6.98, 7.65)
Trp^{3}	124.3	(8.37)	176.7	57.9	(4.58)	29.5	(3.25, 3.30)	C2 126.4 (7.33); C4 120.3 (7.61); C5 121.5 (7.15) C6 124.4 (7.21); C7 114.6 (7.48); $N^{\varepsilon 1}$ 130.3 (10.21)
Lys^{4}	123.1	(8.17)	177.2	56.9	(4.14)	32.7	(1.62, 1.76)	C^{γ} 24.7 (1.23, 1.23); C^{δ} 29.1 (1.60, 1.69); C^{ε} 42.1 (2.93)
Gly^{5}	109.4	(7.69)	174.5	45.3	(3.78)			
Ile^{6}	121.1	(7.95)	176.9	61.6	(4.08)	38.7	(1.89)	C^{γ} 27.4 (1.48, 1.19); $C^{\gamma m}$ 17.4 (0.94); C^{δ} 13.0 (0.85)
Ala^{7}	128.2	(8.39)	178.3	53.0	(4.24)	18.9	(1.40)	
Ala^{8}	123.5	(8.16)	178.6	52.9	(4.24)	18.9	(1.40)	
Met^{9}	119.7	(8.16)	176.5	55.7	(4.38)	32.8	(2.08)	C^{γ} 32.0 (2.56, 2.62); C^{ε} 16.9 (2.09)
Ala^{10}	125.6	(8.16)	178.3	52.9	(4.24)	18.9	(1.40)	
Lys^{11}	121.0	(8.18)	177.0	56.5	(4.23)	32.9	(1.77)	C^{γ} 24.9 (1.41, 1.46); C^{δ} 29.1 (1.68); C^{ε} 42.1 (2.99)
Lys^{12}	123.2	(8.20)	176.8	56.6	(4.25)	32.9	(1.81)	C^{γ} 24.9 (1.41, 1.46); C^{δ} 29.1 (1.68); C^{ε} 42.1 (2.99)
Leu^{13}	124.2	(8.24)	177.5	55.1	(4.33)	42.2	(1.59, 1.68)	C^{γ} 27.0 (1.65); C^{δ} 24.9 (0.93), 23.3 (0.87)
Leu^{14}	123.8	(8.17)	180.2	55.0	(4.31)	42.2	(1.59, 1.68)	C^{γ} 27.0 (1.65); C^{δ} 24.9 (0.93), 23.3 (0.87)
NH_2	108.1	(7.12, 7.50)						

[a]The ^{1}H chemical shifts are referenced to internal DSS. The ^{13}C and ^{15}N chemical shifts were indirectly referenced using the following ratios of the zero-point frequencies: 0.101329118 for ^{15}N to ^{1}H and 0.25144953 for ^{13}C to ^{1}H (Wishart et al., 1995b).
[b]Not detected.

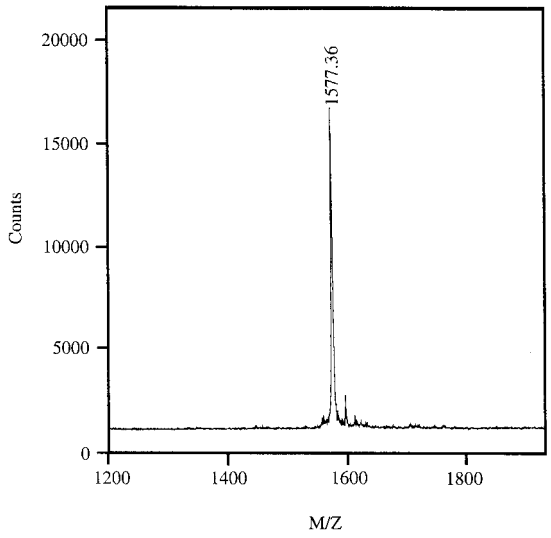

Figure 6. TOF-MS analysis of [U-^{15}N] MP-X. The measured molecular mass (1577) indicates >99% ^{15}N enrichment.

enriched with stable isotopes. However, only a few systems that produce short peptides have been reported. This is because it is more difficult to express short peptides than larger proteins for the following reasons: (i) Short peptides expressed in cells are vulnerable to enzymatic degradation because they usually do not adopt a rigid conformation. (ii) When peptides are expressed as a fusion protein with large carrier proteins, such as glutathione S-transferase (GST), to overcome the above problem, the peptide moiety of interest must be cleaved off from the purified fusion protein by chemical or enzymatic reactions (for a review, refer to Nilsson et al. (1992)). Chemical cleavage methods may be applied such as a cyanogen bromide cleavage; however, these methods are limited by the amino acid sequence of the desired peptide. For example, cyanogen bromide cleaves all of the methionine residues; therefore, peptides that include methionine residues may not be prepared. On the other hand, commercially available proteases are not always appropriate for the preparation of short peptides, because some of them (e.g., TEV protease) leave one or two amino acid residues at the N-terminus of the peptide, which should be avoided when studying short peptides. Some proteases that do not leave extra amino acid residues (e.g., Factor Xa) do not always show strict specificity, and cleavage sometimes occurs at sites unexpected from the enzyme's nominal specificity. Moreover, all of the commercially available sequence-specific proteases are too expensive to prepare the large amount of peptides needed for NMR experiments. (iii) GST and maltose-binding protein (MalE), widely used as fusion carriers, are too large

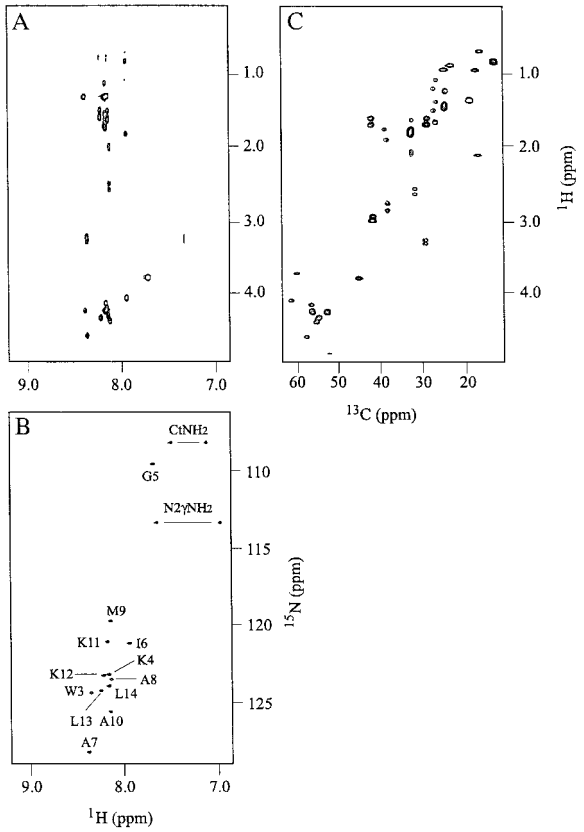

Figure 7. A homonuclear TOCSY spectrum of non-enriched MP-X and heteronuclear HSQC spectra of [U-^{15}N] and [U-^{13}C/^{15}N] MP-X. (A) A TOCSY spectrum of non-enriched MP-X recorded at a 1 mM peptide concentration in a buffer containing 90% H_2O/10% D_2O, 10 mM sodium acetate-d_4, and 1 mM DTT-d_{10} at pH 6.0 and 20 °C. (B) A ^{1}H-^{15}N HSQC spectrum of [U-^{15}N] MP-X. The measurement condition was the same as in (A). Resonances are annotated with the standard one-letter code for amino acids followed by the residue number. (C) A ^{1}H-^{13}C HSQC spectrum of [U-^{13}C/^{15}N] MP-X. The peptide concentration is 1 mm, and the buffer is the same as in (A) except that 99.96% D_2O was used instead of 90% H_2O/10% D_2O.

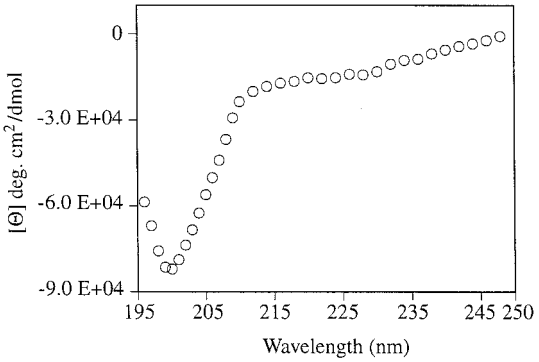

Figure 8. A CD spectrum of MP-X at a 100 μM peptide concentration in a buffer containing 90% H_2O/10% D_2O, 10 mM sodium acetate-d_4, and 1 mM DTT-d_{10} at pH 6.0 and 20 °C. This spectrum shows that MP-X does not adopt an ordered conformation under the NMR measurement conditions.

(27 and 43 kDa, respectively) to be fused with short peptides, such as the 1.6 kDa MP-X peptide. For example, when the yield of the GST fusion of MP-X is 30 mg per liter culture, the final yield of MP-X would be less than 50% of that obtained using this ubiquitin fusion system, if the same yields as those in the present study are assumed for the cleavage, amidation, and purification steps. MalE fusion proteins would give less than 30%.

Our ubiquitin system solves all of these problems found in the currently used expression systems: (i) When the peptide moiety of interest that is fused to ubiquitin in a soluble form is expected to be vulnerable to enzymatic degradation during expression, the cells' growth conditions can be chosen such that the expressed protein forms protease-resistant inclusion bodies, because they can be easily refolded to a soluble form during purification. (ii) YUH is an ideal protease in that it does not leave extra amino acid residues on the peptide of interest, it does not show any non-specific digestion activity against the several kinds of peptides tested thus far (data not shown), and its cost is negligible because it can be easily over-expressed in *E. coli* and purified using the same affinity resin. (iii) Since ubiquitin is a very small protein of only 11 kDa (76 amino acid residues and flanking decahistidine tag with a linker), the weight fraction of the peptide moiety in the fusion protein is large.

Note that the possibility of the ubiquitin system to express fusion proteins in both soluble and inclusion body forms provides other advantages. An often encountered phenomenon that a slight modification in a protein's amino acid sequence results in greatly reduced solubility is presumably due to incorrect polypeptide chain folding. Even when the expressed ubiquitin fusion protein possesses only limited solubility upon extraction, this protein can easily be solubilized by unfolding/refolding. In addition, the expression of fusion proteins at a lower temperature that gives soluble proteins enables in vivo modification of the peptide moiety, such as phosphorylation of the tyrosine side chains. In our preliminary experiment, in which a plasmid encoding a fusion protein of ubiquitin and a peptide containing a tyrosine residue was transformed into an *E. coli* strain TK (Simcox et al., 1994) harboring a plasmid encoding the elk tyrosine kinase (compatible with all ColE1 plasmids), the peptide was

obtained with its tyrosine side chain phosphorylated (data not shown).

Applications of peptides uniformly enriched with stable isotopes

Although the TRNOE method is useful for analyzing the conformations of peptides bound to large proteins (Clore and Gronenborn, 1982,1983; Campbell and Sykes, 1991,1993; Ripoll and Ni, 1992; Scherf et al., 1992; Andrieux et al., 1995; Anglister et al., 1995; Ni et al., 1995; Yao and Mayo, 1996; Campbell et al., 1997) or lipids (Wakamatsu et al., 1987; Milon et al., 1990; Okada et al., 1994; Mayo et al., 1996), this method has the drawback of poor signal dispersion of the peptide proton resonances. In this case, the overlapping problem has often been resolved by using a 3D ^{15}N-edited TRNOE experiment with selectively ^{15}N-enriched peptides (Carpenter and Ni, 1992; Wang et al., 1993). However, the heteronuclear TRNOE strategy with uniformly enriched peptides has not yet been used, presumably for the following reasons: uniformly enriched peptides are too expensive to be chemically synthesized, and the procedures needed to express and to enrich short peptides with stable isotopes in *E. coli* cells are much more complex than those used for proteins. Thus, in this study we employed a new general method to prepare isotope-enriched peptides that uses a ubiquitin fusion system. In fact, we could prepare MP-X uniformly enriched with ^{15}N and/or ^{13}C in 2 weeks.

The enrichment of peptides with stable isotopes may be essential when analyzing fragments of membrane proteins bound to lipid micelles, since most micelle-bound peptides adopt an α-helical conformation which gives only poorly dispersed proton resonances. In our experience, although it was difficult to analyze the spectra of micelle-bound peptides of $>$3 kDa solely by ^{1}H NMR, the amide resonances were dispersed in the ^{1}H-^{15}N HSQC spectra. In fact, several groups reported structural analyses of membrane-bound peptides by using isotope-enriched peptides and multidimensional heteronuclear spectroscopies (Henry and Sykes, 1992; McDonnell et al., 1993; van de Ven et al., 1993; Cordier-Ochsenbein et al., 1996; Williams et al., 1996; Jelinek et al., 1997; MacKenzie et al., 1997).

Chemical shift dispersion of uniformly isotope-enriched short peptides in a random-coil state

MP-X shows poorly dispersed proton NMR resonances, because this peptide is in a 'random-coil' state in an aqueous solution (Figure 8). Unambiguous assignments of the proton resonances are quite difficult, especially for residue pairs that show almost the same chemical shifts for the ^{1}HN, ^{1}H$^{\alpha}$ and ^{1}H$^{\beta}$ resonances: Ala^{8} and Ala^{10}, Lys^{11} and Lys^{12}, and Leu^{13} and Leu^{14} (Table 3). In spite of the poor proton resonance dispersion, the amide nitrogen resonances of these pairs are well resolved, as shown in Figure 7A and Table 3. Several groups have reported that the chemical shifts of the amide nitrogen resonances of proteins are well resolved, even when the proteins are denatured or in a random-coil state (Neri et al., 1992a,b; Schwalbe et al., 1997). Our result supports these previous observations. Wishart et al. (1995a) described the ^{15}N random-coil chemical shifts of the common amino acids using a set of 40 peptides including Gly-Gly-X-Ala-Gly-Gly and Gly-Gly-X-Pro-Gly-Gly, which revealed significant systematic shift differences arising from the presence of proline in the peptide sequence. In our study, there are three lysine residues at positions 4, 11, and 12, three alanine residues at positions 7, 8, and 10, and two leucine residues at positions 13 and 14. Although these residues are not followed by proline, each residue type shows significant chemical shift dispersion: 2.2 ppm for lysine, 4.7 ppm for alanine, and 0.4 ppm for leucine (see Table 3). These observations show that even non-proline amino acid residues can significantly affect the amide nitrogen chemical shifts of the preceding residues. Therefore, our method to analyze short peptides using full isotope enrichment is useful, even when they do not contain proline residues.

Since the CH resonances of MP-X are also well resolved in the ^{13}C dimension (Figure 7C), isotope enrichment is useful for NOE cross-peak assignments as well as for sequence-specific resonance assignments. Assignments of NOEs by multidimensional heteronuclear NOE experiments (Ikura et al., 1990; Jahnke et al., 1995) of MP-X bound to a G protein was reported (H. Kusunoki et al., 1998).

Conclusions

In summary, this paper describes a ubiquitin fusion system for preparing short peptides enriched with stable isotopes. Since this system is efficient and the isotope enrichment is powerful for resolving peptide resonances, the preparation of isotope-enriched proteins by the ubiquitin system should be useful in ana-

lyzing the interactions of small peptides with proteins or lipids.

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