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A Back Hydrogen Exchange Procedure via the Acid-Unfolded State for a Large Protein

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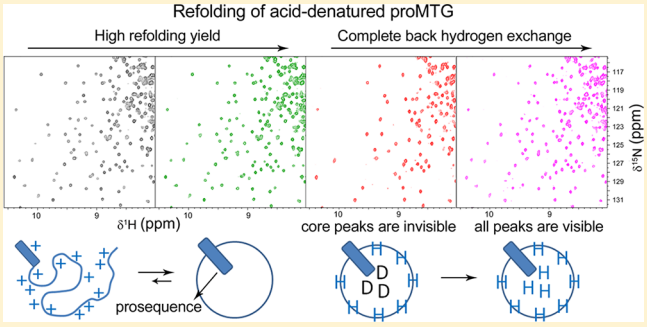
Supporting Information

ABSTRACT: A deuterated protein sample is required for nuclear magnetic resonance (NMR) measurements of a large protein because severe signal broadenings occur because of the high molecular weight. The deuterated sample expressed in ^2H_2O should subsequently be subjected to a back hydrogen exchange at amide groups. To perform the back exchange, the protein molecule is unfolded or destabilized so that internal residues become accessible to the solvent. However, the refolding yield from the destabilized or unfolded state of a large protein is usually low, leading to a dilemma in NMR measurements of large proteins. In our previous paper [Suzuki, M., et al. (2011) *Biochemistry 50*, 10390−10398], we suggested that an acid-denatured microbial transglutaminase (MTG) consisting of 331 amino acid residues can be recovered effectively under low-salt conditions, escaping from the aggregation-prone intermediate. Here, we demonstrate that proMTG, the pro form of MTG consisting of 376 amino acid residues, can be refolded perfectly from the acid-unfolded state under low-salt conditions, as confirmed by circular dichroism and NMR spectroscopies. By performing the same procedure with a deuterated proMTG expressed in ^2H_2O, we observed complete back exchanges for internal residues by NMR spectroscopy. Our procedure has potential applications to the back hydrogen exchange of large proteins for NMR measurements.



A deuterated protein sample is required for the NMR measurements of a large protein with more than 200 amino acid residues because normal, protonated samples lead to severe signal broadenings because of the high molecular weight.[1−7] The deuterated sample expressed in a medium prepared using ^2H_2O should subsequently be subjected to a back hydrogen exchange at amide groups for various protein NMR measurements, such as HSQC.[6,7] In the back exchange treatment with H_2O, the protein molecule should be unfolded or destabilized so that internal residues become accessible to the solvent. However, once unfolded or destabilized, large proteins are often difficult to refold to the native structure. This is one of the obstacles to NMR measurements of large proteins.

Transglutaminases (TGases,[8] protein-glutamine γ-glutamyltransferases, EC 2.3.2.13) with 331 amino acid residues and no disulfide bonds make up a family of enzymes that catalyze the transfer of an acyl group between the γ-carboxyamide group of glutamine residues within peptides and the ε-amino group of lysine residues, resulting in the formation of ε-(γ-glutamyl)-lysine cross-linkages. TGases are widely distributed in various cells and tissues of mammals, and their physiological properties have been studied.[9] The biochemical function of a microbial transglutaminase (MTG) from *Streptomyces mobaraensis*, whose activities were independent of calcium, has been studied extensively.[10,11] MTG is of interest with regard to not only its structure−function relationship but also its application to food processing.[12]

We previously reported that MTG can be refolded with a relatively effective yield of 50% from its acid-unfolded state (U_A) by a stepwise pH-jump procedure under low-salt conditions.[13] Effective refolding was achieved by avoiding the aggregation-prone molten globule-like intermediate (A state).[14,15] Although the yield was smaller than that (80%) of stepwise refolding from the urea-denatured MTG extracted from inclusion bodies in *Escherichia coli*,[16] an important advantage of our procedure is simplicity: the acid unfolding and refolding reactions can be performed in a single test tube by adding acid and alkaline consecutively. In addition, there is no need to worry about an increase in the total volume of the solution by dilution or a large volume of dialysis buffer to remove denaturants.

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MTG is known to be expressed as a preproenzyme and processed into the mature form by two kinds of proteases.[17,18] Its pro region was thought to be essential for efficient protein folding, secretion, and suppression of its enzymatic activity. The crystal structure of the MTG zymogen (proMTG), which contains a prosequence of 45 amino acid residues at the N-terminus, has been determined[19] based on the crystal structure of the mature form of MTG (Figure 1A).[20] The overall

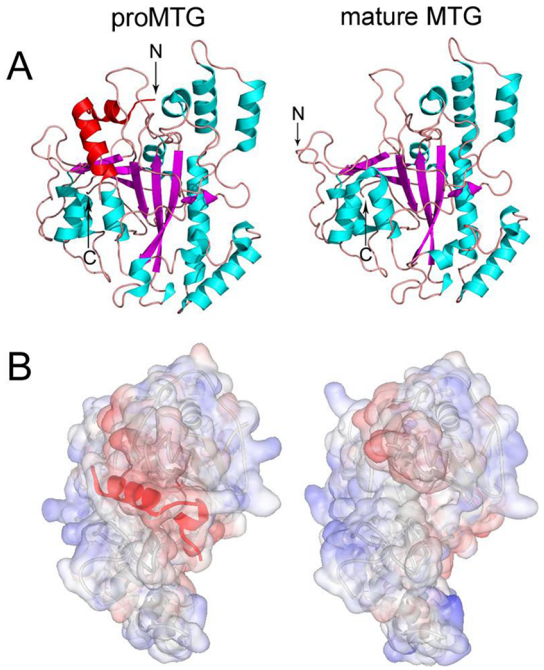

Figure 1. Crystal structure of proMTG and mature MTG. (A) Structures of proMTG[19] [Protein Data Bank (PDB) entry 3iu0] (left) and mature MTG[20] (PDB entry 1iu4) (right). The region corresponding to the N-terminal prosequence of proMTG is colored red. The α-helices, β-strands, and loops are colored cyan, magenta, and orange, respectively. The N- and C-termini are denoted with arrows and labeled. The structures were drawn by using PyMol (The PyMol Molecular Graphics System, version 1.2r3pre, Schrödinger, LLC, New York). (B) Electrostatic potential of proMTG and mature MTG. The red and blue areas are negatively and positively charged, respectively.

structure of proMTG is similar to that of mature MTG, revealing a single compact domain. A major portion of the prosequence assumes an L-shaped structure, consisting of an extended N-terminal segment with short and long α-helices and blocking the access of the substrates.[19]

In this work, we examined the acid unfolding and refolding of proMTG under low-salt conditions. Remarkably, a complete recovery from the U_A state was confirmed by circular dichroism (CD) and NMR spectroscopies. The reactivation efficiency of refolded proMTG reached 84% after proteolysis, which was much higher than that of mature MTG refolded from the acid-unfolded state. The consecutive acid unfolding and refolding led to the complete back hydrogen exchange of [^{2}H,^{13}C,^{15}N]-proMTG, suggesting our procedure can be applied to the back hydrogen exchange of large proteins for NMR measurements.

EXPERIMENTAL PROCEDURES

Materials. High-performance liquid chromatography-grade solvents were purchased from Nacalai Tesque. Trifluoroacetic acid (analytical grade) was from Wako Life Science. All other chemicals were of reagent grade.

Protein Expression and Purification. Mature MTG was secreted using *St. mobaraensis* and subsequently purified and stored in 20 mM sodium phosphate buffer (pH 6.0) at 4 °C as described previously.[10] Details of protein expression and purification are described in Supporting Information.

Circular Dichroism. CD measurements were made with a Jasco spectropolarimeter, J-820, at 4 or 25 °C. The instruments were calibrated with *d*-camphorsulfonic acid. The far- and near-UV CD spectra were measured at protein concentrations of 0.03−0.4 mg mL^{-1} with a 1 mm path-length cell and 0.2−0.4 mg mL^{-1} with a 10 mm path-length cell, respectively. The protein concentrations of proMTG or mature MTG were determined spectrophotometrically by using a molar absorption coefficient of 74205 or 76965 M^{-1} cm^{-1} [21] at 280 nm, respectively.

Unfolding and Refolding Experiments with a pH Jump. The pH-dependent conformational change was measured by CD at 25 °C as described previously.[13] For unfolding experiments, we prepared the proMTG solution (~0.7 mg mL^{-1}) in 1 mM sodium acetate (pH 5.0). The protein solution was diluted with a buffer solution corresponding to the final pH. The final buffer concentration was 20 mM, and sodium phosphate for pH 2.0−3.5 and sodium acetate for pH 3.5−5.0 were used as buffers. For kinetic experiments, the unfolding was started by changing the pH from 5.0 to 2.0 using a 10-fold dilution of the proMTG solution in 1 mM sodium acetate (pH 5.0) with 10 mM HCl.

The salt-dependent conformational transitions of acid-unfolded proMTG were measured by CD in the presence of NaCl at 25 °C as described previously.[13] We prepared the unfolded proMTG solution (~0.1 mg mL^{-1}) in 10 mM HCl (pH 2.0). The concentrations of NaCl were adjusted to 50−400 mM after dilution. The protein concentration was 1.5 μM. The measurements were done immediately and 12 h after preparation of the solution. The dead time of immediate measurements was ~10 min.

For refolding experiments, we prepared the unfolded proMTG (0.33 mg mL^{-1}) solution in 10 mM HCl (pH 2.0). The protein solution was diluted with a buffer solution corresponding to the final pH. The final buffer concentration was 20 mM, and sodium phosphate for pH 2.0−3.5 and 6.0−7.0 and sodium acetate for pH 3.5−5.0 were used as buffers. The solutions of acid-denatured proMTG in 200 and 300 mM NaCl were incubated for 12 h and adjusted to pH 4.0 via addition of 1 M sodium acetate (pH 4.5). After the jump in pH, far-UV CD was measured at 25 °C

For the reactivation experiment, the first jump from pH 2.0 in 10 mM HCl to pH 4.0 was done by 2-fold dilution with 100 mM sodium acetate (pH 4.2). After the first jump, the solution was incubated for 4 h at 4 °C. Then, the pH was adjusted to 6.5 via addition of 1.0 N NaOH. The protein concentrations were determined spectrophotometrically as described above. The yields of protein recovery and reactivation of enzymatic activity were determined by comparing the resulting amount of protein and enzymatic activity with the initial values.

Maturation and Activity Measurements. Refolded proMTG was proteolytically activated by subtilisin Carlsberg from *Bacillus licheniformis* (P4860, Sigma-Aldrich) as described previously.[22] A 1/1000 molar ratio of subtilisin was added to the proMTG solution, and the solution was incubated for 14 h at 30 °C. The enzymatic activity of mature MTG and activated

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proMTG was measured by the colorimetric hydroxamate procedure with CBZ-Gln-Gly as reported previously.[16]

NMR Measurements. NMR spectra were recorded at 40 °C on an 800 MHz spectrometer (AVANCE I spectrometer, Bruker) equipped with a CryoProbe. The protein was dissolved in a 10 mM sodium acetate (pH 5.0) buffer containing 10% (v/v) ^2H_2O.

RESULTS AND DISCUSSION

Acid Unfolding of ProMTG. CD spectra of proMTG were compared with those of mature MTG (Figure 2). The far-UV CD spectrum of proMTG (black line) showed two minima at 222 and 208 nm, typical of highly α-helical proteins (Figure 2A). This spectrum was almost identical to that of mature MTG at pH 5.0 (dashed line).[13] The near-UV CD spectrum of proMTG was also identical to that of mature MTG (Figure 2B). Thus, the secondary and tertiary structures of proMTG were found to be almost the same as those of mature MTG in the native states.

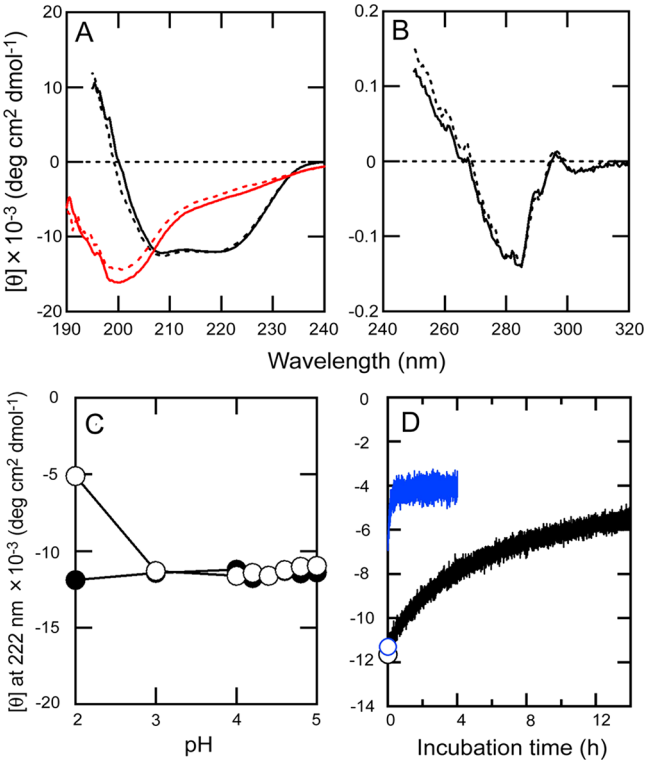

Figure 2. Acid unfolding of proMTG and mature MTG monitored by CD. The far-UV (A) and near-UV (B) CD spectra of native proMTG and mature MTG at pH 5.0 are shown by solid and dashed black lines, respectively. The far-UV CD spectra of acid-unfolded proMTG and mature MTG at pH 2.0 are shown by solid and dashed red lines, respectively, in panel A. (C) Unfolding of proMTG by pH jump from 5.0 to various values monitored 10 min (●) and 18 h (○) after the jump. (D) Time course of acid-induced unfolding of proMTG at pH 2.0 and 25 °C (black) and mature MTG at pH 2.0 and 4 °C (blue). The circles on the vertical axes indicate the ellipticities before the jump in pH.

We investigated the conformational change of proMTG when the pH was lowered from 5.0 to 2.0−4.8. To maintain the low-salt conditions, we used a final buffer concentration of 20 mM. The far-UV CD spectra were measured 10 min and 24 h after the change in pH. ProMTG did not show acid unfolding within 10 min of the change in pH [Figure 2C (●)]. After an 18 h incubation, the unfolding of proMTG was observed at pH 2.0, whereas no conformational change was observed at other pH values [Figure 2C (○)], indicating that proMTG is more stable than mature MTG under acidic conditions.[13] The far-UV CD spectrum of acid-unfolded proMTG (U_A, solid red line) at pH 2.0 showed a large negative trough at 200 nm, the same as the U_A state of mature MTG (dashed red line) (Figure 2A).

To obtain more detailed kinetics of the acid unfolding of proMTG and mature MTG, we measured the time-dependent molar ellipticity at 222 nm. The time-dependent ellipticity of proMTG after the pH jump from 5.0 to 2.0 at 25 °C showed that unfolding was relatively slow and complete unfolding took more than 14 h (Figure 2D). On the other hand, the unfolding of mature MTG was too fast to follow a time course under the same conditions. Thus, we repeated the same measurement at 4 °C. Even at this temperature, the unfolding of mature MTG was fast and completed within a few minutes (Figure 2D).

Salt-Induced Formation of the A State. In the case of the acid denaturation of mature MTG, a molten globule-like intermediate (A) with a native-like secondary structure but with substantially disordered side chains accumulated at pH ∼3.5 under the low-salt conditions and at pH 2.0 under the high-salt conditions.[13] The A state, common to various acid-denatured proteins,[14,15] has been considered to prevent the efficient refolding of mature MTG from acid denaturation. To address the formation of the A state of proMTG, we examined the NaCl-dependent conformational transition at pH 2.0 (Figure 3).

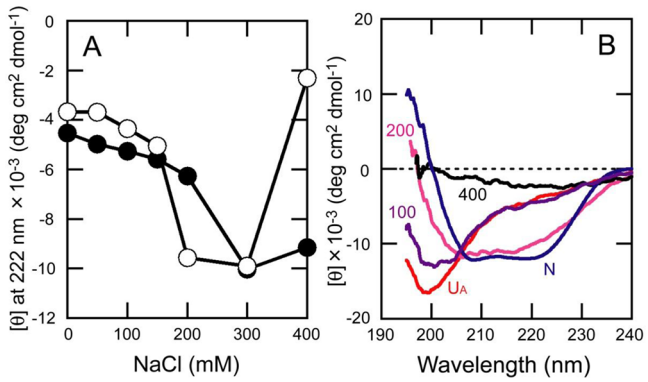

Figure 3. Salt-induced conformational transition of proMTG at pH 2.0. (A) NaCl-dependent transition of proMTG at pH 2.0 measured via the ellipticity at 222 nm 10 min (●) and 12 h (○) after the addition of NaCl. (B) Far-UV CD spectra of acid-unfolded proMTG (U_A) at pH 2.0 at various NaCl concentrations. The NaCl concentrations were 0, 100, 200, and 400 mM. The spectrum of native proMTG (N) at pH 5.0 is shown for comparison. The measurements were performed at 12 h after preparation of the solution.

In the presence of 200 and 300 mM NaCl at pH 2.0, the far-UV CD spectrum was similar to that of native proMTG at pH 5.0 (Figure 3B), indicating the formation of the A state as is the case for mature MTG.[13] In the presence of 400 mM NaCl at pH 2.0 10 min after the preparation, the proMTG solution was apparently soluble and showed the spectrum of the A state. However, 12 h after the preparation, the solution became turbid with a decreased CD intensity (Figure 3A). As for the proMTG samples in 200 mM NaCl at pH 2.0, the solutions were apparently soluble after 12 h. However, the subsequent pH

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jump to 4.0 immediately caused marked aggregation. Thus, the A state of proMTG was also prone to aggregation, so that direct refolding from the A state cannot be achieved.

Efficient Refolding of Acid-Unfolded ProMTG. We then performed refolding experiments with the acid-unfolded protein. To minimize the possible effects of the A state, we used low-salt conditions by keeping the buffer concentration at 20 mM. The refolding experiments were performed with a pH jump from 2.0 to various values (Figure 4A) and monitored by

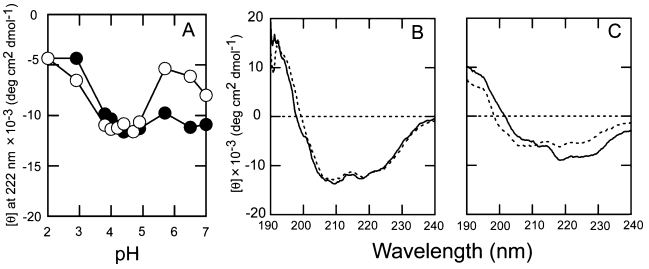

Figure 4. Refolding of acid-unfolded proMTG monitored by CD. (A) Refolding from pH 2.0 to other pH values monitored by the ellipticity at 222 nm 10 min (●) and 12 h (○) after the pH jump. The dead time of the measurements was ~10 min. (B and C) Far-UV CD spectra of refolded proMTG from pH 2.0 to 4.0 (B) or 6.0 (C). Solid and dashed lines indicate the data obtained 10 min and 18 h after the pH jump, respectively.

CD. Up to pH 3.0, no recovery of the secondary structure was observed, indicating that proMTG remains unfolded. At pH 3.8−5.0, the CD spectrum changed to that of native proMTG immediately after the pH jump (Figure 4B). Thus, acid-unfolded proMTG was found to refold to the native state via a simple increase in the pH to >4.0 under the low-salt conditions. However, at pH 5.0−7.0, subsequent aggregation occurred upon further incubation at 25 °C, causing a decrease in ellipticity (Figure 4C). A similar aggregation propensity at pH 5.0−7.0 has been found even for native proMTG without acid unfolding. Considering that the pI value of proMTG is 6.6,[17,18] instability at pH 5.0−7.0 is an intrinsic property independent of the refolding via acid denaturation.

We quantitatively assessed the refolding yield based on the amount of soluble proteins recovered and the recovery of enzymatic activity. After the refolding from the acid-unfolded state, almost no reduction in the amount of protein was observed (~98%) (Table 1), indicating that proMTG remained soluble at each refolding step. This high value is comparable

Table 1. Refolding Yields of Mature MTG and ProMTG from the Acid Unfolding

sample	protein recovery (%)	reactivation (%)	specific activity (units/mg)
mature MTG	–	–	27.0
proMTG refolded from U_A	97.7	83.9[a]	23.6
mature MTG refolded from U_A[b]	85.3	45.3	14.1

[a]Reactivation of proMTG was measured after the maturation process induced by protease.[22] [b]Values from ref 13. Reactivation was calculated from the enzymatic activity with purified mature MTG. Protein recovery of proMTG was slightly decreased after maturation (from ~95 to ~98%) via removal of the prosequence from mature MTG.

with that of mature MTG (86%) (Table 1).[13] The recovery of enzymatic activity was calculated from the specific activity of refolded proMTG after maturation (Table 1). The reactivation of proMTG after maturation was found to be ~84% of native mature MTG activity. In contrast, the relative specific activity of mature MTG refolded from the acid unfolding was 45.3% (Table 1),[13] showing that the recovery of the enzymatic activity of proMTG was much higher than that of mature MTG. Taken together, proMTG can be refolded completely via acid denaturation by a simple refolding procedure in terms of protein secondary structure and enzymatic activity.

The completeness of the refolding was further assessed with a two-dimensional NMR spectrum, $^{1}H-^{15}N$ HSQC. The number of expected cross-peaks is 351 because proMTG consists of 376 (331 + 45) amino acid residues, there is one N-terminal residue, and there are 24 proline residues. The HSQC spectrum of ^{15}N-labeled native proMTG showed well-dispersed amide proton peaks representing the rigid and unique native conformation. The number of detected cross-peaks (348) in Figure 5A is consistent with the expected number of peaks (351). Peak assignments are under way in which the complete assignments require the deuterated proMTG as described below.

Native proMTG with ^{15}N labeling was unfolded and subsequently refolded according to the procedures described above. The HSQC spectrum of refolded proMTG (Figure 5B) agreed well with the spectrum before unfolding (Figure 5A), showing almost the same number of observed peaks (349). This indicates that proMTG refolded completely from the acid-unfolded state at the atomic conformation level.

Preparation of the Deuterated NMR Samples. For the high-resolution NMR measurements of proMTG, we prepared [^{2}H,^{15}N,^{13}C]proMTG expressed in $^{2}H_2O$. Its $^{1}H-^{15}N$ HSQC spectrum of [^{2}H,^{15}N,^{13}C]proMTG in 90% (v/v) H_2O (Figure 5C) exhibited 225 cross-peaks, many fewer than [^{15}N]proMTG (Figure 5A). The missing peaks are assigned to the residues located in the core regions. Their amide protons were prevented from back exchange even in 90% (v/v) H_2O, remained deuterated, and, therefore, were invisible on the spectrum.

Then, we examined the acid unfolding and refolding procedure to enhance the back hydrogen exchange of the deuterated proMTG molecule. The spectrum of refolded [^{2}H,^{15}N,^{13}C]proMTG with 347 cross-peaks (Figure 5E) was indistinguishable from that of [^{15}N]proMTG (Figure 5A), indicating that a complete back hydrogen exchange was accomplished for all residues.

As a reference, we examined the back hydrogen exchange of native [^{2}H,^{15}N,^{13}C]proMTG at pH 8.0. To enhance the exchange reaction, we added 2 or 8 M urea to the solution at pH 8.0. After a 3 day incubation at room temperature, we exchanged the buffer, using a desalting column, with 10 mM sodium acetate (pH 5.0) containing no urea, optimal conditions for NMR measurements. We successfully recovered the native proMTG molecule from 2 M urea at pH 8.0 and measured the NMR spectrum at pH 5.0. However, the back exchange was insufficient. The HSQC spectrum exhibited 297 cross-peaks, and more than 50 amide peaks remained unchanged (Figure 5D). Upon complete unfolding of proMTG in 8 M urea at pH 8.0, no refolding was achieved because proMTG aggregated during the buffer exchange step. Thus, for proMTG, the back hydrogen exchange at low pH was more efficient than that at pH ~8.0.

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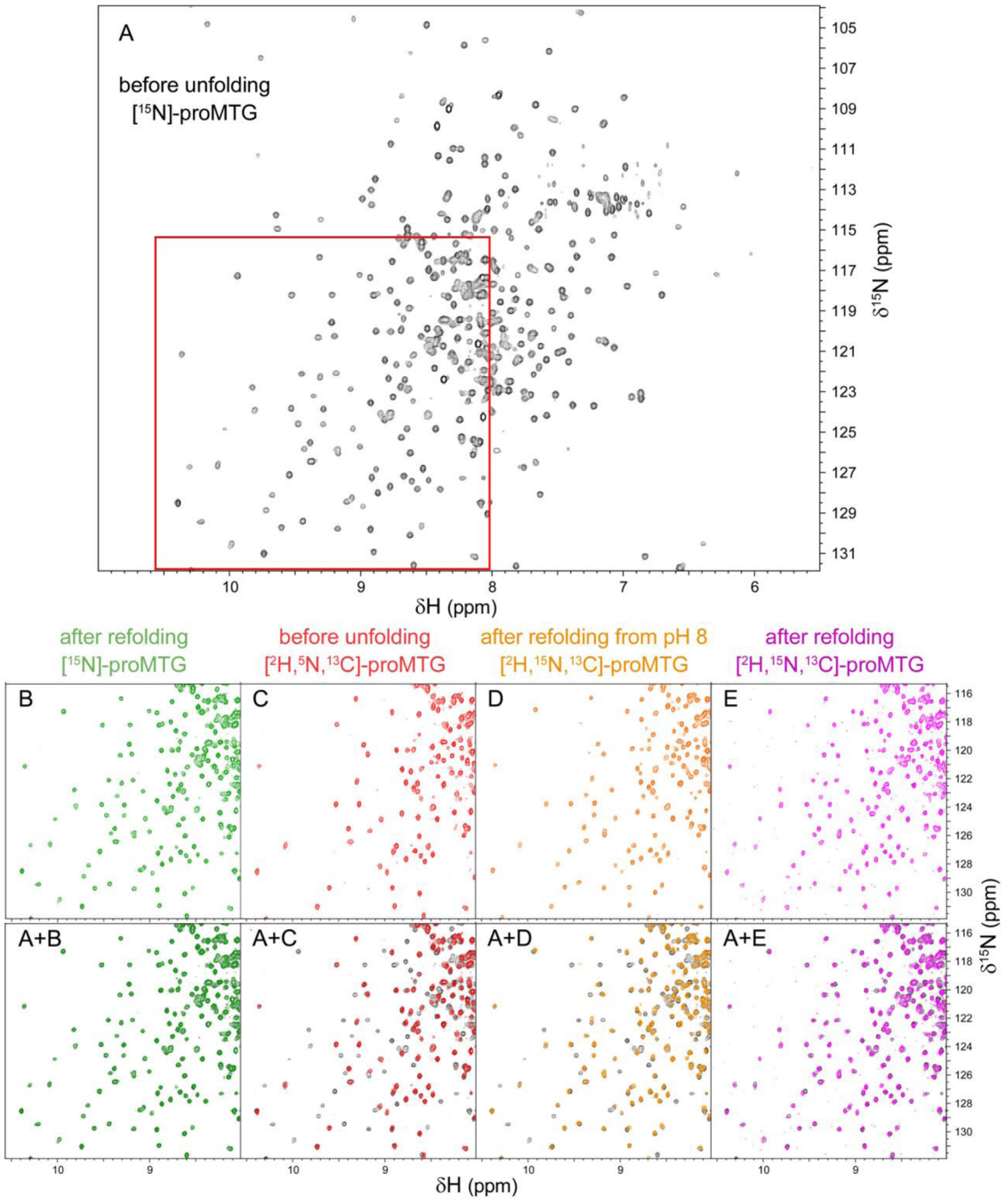

Figure 5. Results of the back hydrogen exchange of internal residues of proMTG. Spectra of [^{15}N]proMTG before unfolding (A, black) and after refolding (B, green) and [^{2}H,^{13}C,^{15}N]proMTG before unfolding (C, red) and after refolding from pH 8 (D, orange) and pH 2 (E, magenta). Spectra B–E are superimposed on spectrum A in the bottom row. (A+B) Spectrum B is the same as spectrum A, indicating complete refolding from the acid-denatured state. (A+C and A+D) Some signals are missing in spectrum C or D, indicating the incomplete back hydrogen exchange of the amide proton of the internal residues. (A+E) Spectrum E is the same as spectrum A, indicating complete back exchange and complete refolding.

Application of the Back Hydrogen Exchange to NMR Measurements. It is well reported that high levels of deuteration of proteins are indispensable for solution-state NMR studies of large proteins, especially with masses of >40–50 kDa.[1−5] By replacing protons with deuterium atoms, we obtained significantly sharpened NMR peaks of large proteins, otherwise broadened because of the slow rotational motion and the dipolar coupling between protons. In light of this, our acid unfolding and refolding procedure will be potentially useful for the complete back hydrogen exchange of large proteins for high-resolution NMR studies. Protein molecules in an acid-unfolded state under low-salt conditions are generally more soluble than other denatured states because of the intramolecular and intermolecular repulsive forces between positive charges.[13−15] When the protein solution is kept under the low-salt conditions to maximize the repulsive forces, the pH jump procedure is expected to allow effective refolding, preventing the formation of aggregates. Furthermore, the great advantage of our procedure is simplicity: a complete back hydrogen exchange might be possible even in a single test tube via

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addition of acid and alkaline consecutively. We demonstrated this to be true with proMTG that consisted of 376 amino acid residues.

In many cases, the back hydrogen exchange of deuterated samples has been examined at pH ~8.0 because the intrinsic rate of hydrogen exchange is much faster at that pH than that at low pH.[23] For proteins that do not unfold at high pH, low concentrations of denaturants are usually added to cause mild denaturation or destabilization to enhance the back hydrogen exchange for the internal residues. We used this method for ferredoxin-$NADP^+$ reductase, a protein with 314 residues, achieving a nearly complete assignment of the backbone resonances.[6,7] Although this procedure works well for some proteins, it was not applicable to proMTG: Incomplete back exchanges were observed in 2 M urea at pH 8.0 (Figure 5D). Thus, a complete back hydrogen exchange was achieved by the simple acid unfolding and refolding procedure, producing a high-quality sample for NMR measurements.

At the same time, to evaluate the balance between the incubation time for exchanges and the time for unfolding, hydrogen exchange rates and time constants of each residue of proMTG and mature MTG at 4 and 25 °C were calculated, respectively, based on the intrinsic exchange rate constants.[23,24] Then, we summarized the representative values of the time constants in Table S2 of the Supporting Information. As shown in Figure 2 and Table S2 of the Supporting Information, proMTG is expected to complete the back exchange within the incubation time (14 h) at pH 2.0 and 25 °C because the largest time constant of a residue of proMTG is 17.8 min, which is sufficiently shorter than the time constant of unfolding (5.2 h). On the other hand, if we conduct the back exchange of mature MTG at pH 2.0 and 4 °C, several hours would be required for the complete back exchange on the basis of the largest time constant of 103 min. We can reduce largely the incubation time at 25 °C under the same conditions.

Furthermore, to establish the generality of our approach, it will be important to examine its applicability to other large proteins. In this context, it would be interesting to apply this methodology to mature MTG. However, we already reported that the refolding yield of mature MTG from the acid-unfolded state is not very high: 85.3% in protein yield and 45.3% in reactivation yield (Table 1). This indicates that, even with the same approach, we expect that the results will be ambiguous because of a high proportion of misfolded mature MTG. Probably, the best method for obtaining fully deuterated mature MTG is the subtilisin-assisted conversion of fully deuterated proMTG to mature MTG. Because the conversion yield of proMTG is high (Table 1), we will be able to produce the fully deutrated mature MTG in high yield. In addition, we consider that the mechanism of the conversion of proMTG to mature MTG probed by NMR will be the next important topic to be addressed.

Contribution of the Prosequence to Refolding. For various proteins, the prosequence is essential for producing the mature form of a protein in vivo as well as in vitro by facilitating appropriate folding.[25,26] The prosequence also functions as an intramolecular chaperone.[27] Typical examples are serine proteases such as α-lytic protease, subtilisin, and carboxypeptidase Y, whose folding yields are very poor without a prosequence. In vitro folding studies of pro-α-lytic protease and prosubtilisin revealed that the prosequences lower the energy barriers between their intermediate and native conformations presumably by stabilizing the folding transition states.[28,29]

In vivo, MTG is secreted in the pro form (proMTG),[17] and the structure of proMTG is similar to that of mature MTG.[19] Therefore, we rationalized that in vivo the MTG prosequence stimulates refolding of the mature part and exerts a function analogous to that of the propeptides of subtilisin BPN′ and other proteases.[30] This is likely to be true for the in vitro refolding of proMTG from the acid-unfolded state.

The thermostability of proMTG is reportedly higher than that of mature MTG.[17] Consistent with this, proMTG was more resistant to acid unfolding than mature MTG:[13] proMTG remained in a native state even after 18 h at pH 3.0, although mature MTG acid-unfolded completely and immediately at the same pH. In addition, proMTG showed slower unfolding kinetics than mature MTG (Figure 2D). The crystal structure of proMTG suggests that the prosequence binds in the active site cleft and fills the large cavity (Figure 1). It is likely that the insertion of the prosequence into the cleft suppresses the local fluctuations around the binding site and fixes the conformation, contributing to the increased stability and moreover a high refolding yield.

The enhanced stability of proMTG might be explained by the electrostatic potential of the MTG molecule. pI values differ between proMTG and mature MTG; the pI of mature MTG is 8.1, whereas that of proMTG is 6.6 based on the results of isoelectric focusing.[17] The prosequence contains seven acidic residues and only one basic residue, causing a highly negative charge (Figure 1B). The negatively charged prosequence interacts with the positively charged area around the binding site to neutralize the electrostatic potential, making proMTG more stable than mature MTG.

■ CONCLUSION

The refolding yield of the acid-unfolded state depends on various factors, including the presence of the prosequence. We showed that the prosequence of proMTG is important for enhancing the refolding yield probably via a chaperoning role upon the refolding of mature MTG. In a manner independent of the properties of proteins, refolding from the acid-unfolded state under low-salt conditions might be a promising way to obtain high yields by escaping from the aggregation-prone molten globule-like intermediate. Furthermore, the great advantage of our procedure is simplicity. As demonstrated with [^{2}H,^{15}N,^{13}C]proMTG, the reversible acid unfolding and refolding under low-salt conditions provide a simple and powerful method for performing a back hydrogen exchange, essential for the high-resolution NMR measurements of large proteins.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures of protein expression and purification, data on yields of isotopically (non)labeled proMTG at each expression and purification step, and the purification of elution profiles of proMTG. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

CBZ-Gln-Gly, *N*-carbobenzoxy-L-glutaminylglycine; CD, circular dichroism; HSQC, heteronuclear single-quantum coherence; MTG, microbial transglutaminase; NMR, nuclear magnetic resonance; TGases, protein-glutamine γ-glutamyltransferases.

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