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Mutated Intramolecular Chaperones Generate High-Activity Isomers of Mature Enzymes

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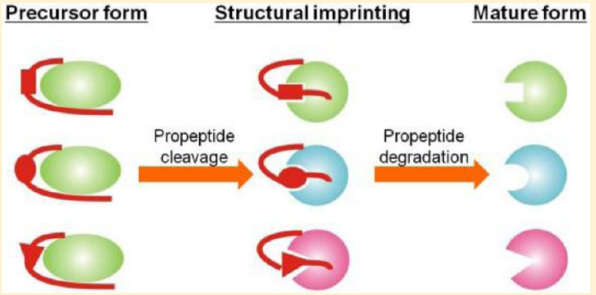
ABSTRACT: The propeptide of carboxypeptidase Y precursor (proCPY) acts as an intramolecular chaperone that ensures the correct folding of the mature CPY (mCPY). Here, to further characterize the folding mechanism mediated by the propeptide, folding analysis was performed using a yeast molecular display system. CPYs with mutated propeptides were successfully displayed on yeast cell surface, and the mature enzymes were purified by the selective cleavage of mutated propeptides. Measurement of the activity and kinetics of the displayed CPYs indicated that the propeptide mutation altered the catalytic efficiency of mCPY. Although the mature region of the wild-type and mutant CPYs had identical amino acid sequences, the mCPYs from the mutant proCPYs had higher catalytic efficiency than the wild-type. These results indicate that proteins with identical amino acid sequence can fold into isomeric proteins with conformational microchanges through mutated intramolecular chaperones.



To carry out a specific biological function, individual proteins must fold into a specific three-dimensional structure, which is determined by the amino acid sequence of the protein (Anfinsen's dogma, Nobel Prize in Chemistry).[1] Protein folding proceeds from the high-energy unfolded state to the more thermodynamically stable folded state, which is known as the native structure.[2] In the free energy landscape, the native state is at the bottom of the folding funnels. Free energy barriers exist between the funnels, and if a molecule is trapped in a misfolded funnel and cannot climb out of the barrier, misfoldings can result.[2] To prevent misfolding, molecular chaperones are used in living organisms.[3,4]

Another type of chaperone, the intramolecular chaperone, also plays a crucial role in protein folding by mediating correct folding.[5,6] Unlike molecular chaperones, intramolecular chaperones are encoded in the primary sequence of proteins and are typically found at the N-terminus.[2,5] Intramolecular chaperones directly assist in folding by binding to other sequence regions, thereby lowering the barrier of the misfolded conformation. After folding, the intramolecular chaperone is removed through an autolytic or exogenous proteolytic activity. Intramolecular chaperons are found in many proteases[7−9] and are essential for protein folding.[7,10,11] The intramolecular chaperone region is usually termed as a propeptide and also functions as an inhibitor. Interestingly, a single-point mutant in the intramolecular chaperone has been shown to alter the folding of the protein, although the mature domain sequence remains unchanged.[12] This phenomenon was termed "protein memory" because the protein still maintained memory of folding.

Carboxypeptidase Y (CPY) is the vacuolar serine carboxypeptidase of the yeast *Saccharomyces cerevisiae* and is produced as an inactive precursor (proCPY) with an N-terminal propeptide.[13] It is reported that proCPY maturation to mature CPY (mCPY) is accompanied by four cleavages of the propeptide by yeast proteinase A (PrA) and proteinase B.[14] The propeptide of CPY functions as an intramolecular chaperone and an inhibitor toward mCPY. mCPY is also inhibited by a CPY inhibitor (I^C),[15,16] and the crystal structure of the mCPY-I^C complex has been determined.[17] The crystal structure revealed that I^C forms an equimolecular complex with mCPY through two binding sites, the N-terminal inhibitory reactive site (the primary CPY-binding site), and the secondary CPY-binding site. The N-terminal inhibitory reactive site of I^C interacts with the substrate-binding site of mCPY and is essential for CPY inhibition because an I^C mutant lacking the N-terminal sequence cannot bind or inhibit CPY.[17]

Our previous report[18] indicated that the C-terminal sequence of CPY propeptide was similar to the N-terminal sequence of I^C and that the C-terminal sequence of the propeptide bound to the substrate-binding site of mCPY and inhibited CPY activity. In this study, to further characterize the propeptide function, we analyzed proCPY mutants in which the propeptide sequence was replaced with I^C sequence using yeast molecular display method. To study propeptide functions, the production system of precursor proteins is required. The molecular display method is available for analysis of propeptide function because molecular display enables easy and stable purification of inactive precursors.[19] proCPY can be produced and purified using host cells lacking PrA, which cleaves the propeptide of CPY, and can be converted to mCPY using proteinase K (PrK), which also cleaves the propeptide.[19] Thus, propeptide function can be directly analyzed by measuring the activity of mCPY

Received: January 31, 2012
Revised: April 6, 2012
Published: April 6, 2012

   dx.doi.org/10.1021/bi3001159 | *Biochemistry* 2012, 51, 3547−3553

after digestion of purified proCPY with PrK. The molecular display method revealed that the mature forms of CPYs with mutated propeptides showed higher activities than the wild-type. This indicates that mutations in the propeptide can cause mature domains with identical amino acid sequences to fold into isomeric proteins with conformational microchanges that may not be visible in the circular dichroism (CD) spectra.

MATERIALS AND METHODS

Strains and Materials. *Escherichia coli* strain DH5α [F^-, *endA1*, *hsdR17*(r_K^-/m_K^+), *supE44*, *thi-1*, λ^-, *recA1*, *gyr96*, Δ*lacU169*(Φ*80lacZ*Δ*M15*)] (Toyobo, Osaka, Japan) was used as the host for the recombinant DNA manipulation. *Saccharomyces cerevisiae* strain BJ2168 (*MATa, leu2, trp1, ura3-52, prb1-1122, pep4-3, prc1-407*) and *S. cerevisiae* strain MT8-1/ΔCPY (*MATa, ade, leu2, trp1, ura3, prc1*) were used as the hosts for protein production. *E. coli* transformants were grown in Luria-Bertani (LB) medium [1% (w/v) tryptone, 0.5% yeast extract, and 1% sodium chloride] containing 50 μg/mL ampicillin. Yeast transformants were cultured in synthetic dextrose (SD) medium [0.67% (w/v) yeast nitrogen base without amino acids and 2% glucose] containing 1% (w/v) casamino acids (Difco) and supplemented with appropriate amino acids.

Construction of Plasmids. The expression vectors for the CPY mutants were constructed on the basis of pCAS:proCPY-FLAG[19] by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The expression vector for proCPY-ICN5, whose propeptide sequence (N80−E84) was replaced with N-terminal of I^C sequences (N2−D6), was constructed on the basis of pCAS:proCPY-FLAG. The constructed vector was named as pCAS:proCPYICN5FG. The expression vector for proCPY-ICN8, whose propeptide sequence (N80−Q87) was replaced with the N-terminal of I^C sequences (N2−Q9), was constructed on the basis of pCAS:proCPYICN5FG. The constructed vector was named as pCAS:proCPYICN8FG. The expression vector for proCPY-ICN12, whose propeptide sequence (N80−N91) was replaced with the N-terminal of I^C sequences (N2−D13), was constructed on the basis of pCAS:proCPYICN8FG. The constructed vector was named as pCAS:proCPYICN12FG. The sequences of constructed plasmids were confirmed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The expression vectors for proCPYN80A, proCPYD81A, proCPYI83A, and proCPYE84A with the substitutions N80A, D81A, I83A, and E84A, respectively, were constructed on the basis of pCAS:proCPYFLAG by site-directed mutagenesis.

Immunofluorescence Labeling and Activity Assay of CPY Displayed on the Yeast Cell Surface. *S. cerevisiae* strains MT8-1/ΔCPY were transformed with the above-constructed plasmids by using a Frozen EZ Yeast Transformation Kit (Zymo Rearch, Irvine, CA). The transformants were isolated on SD medium containing the appropriate amino acids by incubation at 30 °C for 48 h. Immunostaining was performed as follows: an anti-CPY antibody (Rockland, PA), which was preincubated with control yeast cell to remove nonspecific binding, was used as the primary antibody. The cells and the antibody were incubated at room temperature for 1.5 h. The cells were washed with PBS and exposed to the secondary antibody Alexa Fluor-488 goat anti-rabbit IgG (Invitrogen) for 1.5 h at room temperature. After washing, the cells were observed by fluorescent microscopy, and the fluorescence at the excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 485 and 527 nm, respectively, was measured by Floroskan Ascent FL (Labsystems, Helsinki, Finland).

Yeast cells were cultured in the SD medium containing casamino acids and other appropriate amino acids at 30 °C for 24 h, and the cells were collected by centrifugation at 1000*g* and 4 °C for 5 min. After washing the cells with 20 mL of 0.1 M Tris/HCl (pH 7.4) twice, the suspension was adjusted to $OD_{600\,nm}$ of 5. Cells were mixed with 980 μL of 0.1 M Tris/HCl (pH 8.0) and preincubated at 37 °C for 5 min. After addition of 20 μL of 10 mM Suc-Ile-Ile-Trp-MCA, the mixture was incubated with gentle shaking at 30 °C. At appropriate intervals, an aliquot of the mixture was removed, centrifuged at 20000*g* for 5 min, and then the fluorescence of 7-amino-4-methylcoumarin (AMC) released from Suc-Ile-Ile-Trp-MCA in the resulting supernatant was measured using Floroskan Ascent FL. The λ_{ex} and λ_{em} were 355 and 460 nm, respectively.

Protein Purification. *S. cerevisiae* strain BJ2168 was transformed with the constructed plasmids by using a Frozen EZ Transformation Kit (Zymo Research). The transformants were isolated by plating the cells on selective SD medium [0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, and appropriate amino acids]. Purification of proCPY, proCPY-ICN5, and proCPY-ICN8 proteins was performed by selective cleavage method coupled with molecular display, as described previously.[19] The purified proCPY, proCPY-ICN5, and proCPY-ICN8 were completely converted into mature forms by treatment with proteinase K.[20] The mCPY solutions were loaded onto a SOURCE 15Q 4.6/100 PE column (GE Healthcare, Buckinghamshire, UK) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). mCPYs were eluted with the equilibrated buffer containing 2 M NaCl, desalted with 0.1 M sodium phosphate buffer (pH 7.0), and concentrated by ultrafiltration. The fractions containing mCPYs were concentrated by ultrafiltration, and the buffer was exchanged with 0.1 M sodium phosphate buffer (pH 7.0). The activity of purified mCPY was identical to that of the commercially available mCPY.

Determination of Kinetic Parameters. mCPY activity was determined by the hydrolysis of the specific substrate of CPY, i.e. Suc-Ile-Ile-Trp-MCA, in 0.1 M sodium phosphate buffer (pH 7.0). Various concentrations of Suc-Ile-Ile-Trp-MCA were applied to CPY solution (concentration of 1 μg/mL), and the fluorescence from the released AMC was measured using Fluoroskan Ascent FL (Labsystems). The λ_{ex} and λ_{em} were 355 and 460 nm, respectively. Kinetic parameters were calculated by Hanes-Woolf plot.[21] The activity energy was calculated from the Arrhenius equation, i.e. $E_a = -RT\ln(k/A)$, where A is the frequency factor for the reaction, R is the universal gas constant (8.314 J K^{-1} mol^{-1}), and T is the temperature (in kelvin).

Reaction with the Fluorescent SH Probe and CD Spectroscopy. The concentration of ThioGlo 1 fluorescent thiol reagent (Merck, Germany) was maintained at 500 nM, while that of proteins, at 100 nM. The fluorescence of the protein-reagent mixture was measured with Floroskan Ascent FL. The λ_{ex} and λ_{em} were 355 and 510 nm, respectively.

CD spectrum in the 200−260 nm region was measured in a cell with an optical path length of 0.1 cm at 25 °C with a Jasco J-720W spectropolarimeter (Jasco, Tokyo, Japan). The concentration of protein was 100 μg/mL in 0.1 M sodium phosphate buffer (pH 7.0).

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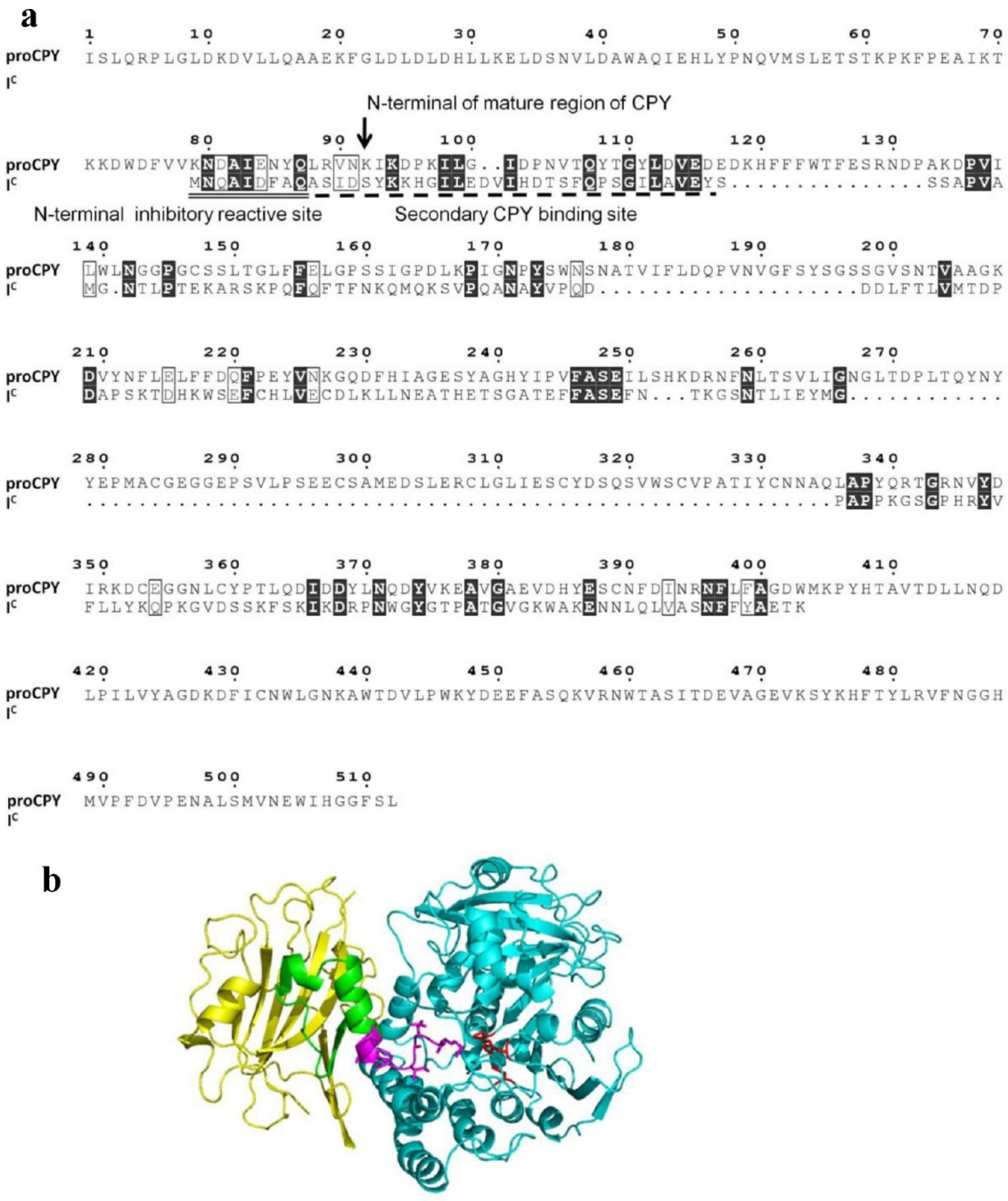

Figure 1. Comparison of CPY and I^C sequences. (a) Amino acid sequence alignment of proCPY and I^C. Full-length amino acid sequence alignment of proCPY (I1−L512) and I^C (M1−K219) is illustrated. The figure was generated using CLUSTAL W[36] and ESPript.[37] The numbers represent the amino acid numbers of proCPY. Similar and identical residues are represented in black letters surrounded by squares and white letters on a black background, respectively. Dots are used to indicate gaps. The double underlining indicates the N-terminal inhibitory reactive site of I^C (the primary CPY-binding site of I^C), and the dashed line indicates a part of the secondary CPY-binding site of I^C. (b) Overall structure of the mCPY-I^C complex. mCPY, the N-terminal inhibitory reactive site of I^C (M1−Q9), a part of the secondary inhibitory reactive site of I^C (A10−E39), and the remaining region of I^C (Y40−K219) are shown in cyan, magenta, green, and yellow, respectively. The catalytic residues of mCPY (Ser146, Asn338, and His397) are shown in red.

RESULTS

Cell Surface Display of CPY Mutants. A previous study[18] indicated similarities between the N-terminus of I^C (N2-E39) and the C-terminus of propeptide and the N-terminus of mCPY (N80-E115) (Figure 1). Furthermore, this high homology sequence contained the N-terminal inhibitory reactive site, which is essential for the I^C inhibitory function toward mCPY through binding to the S1 substrate-binding site of mCPY.[17] This study[18] also revealed that a peptide identical to the N-terminus of I^C (M1−Q9) and the C-terminus of propeptide (K79−Q87) inhibited CPY activity. These results indicate that the high homology sequence of proCPY (N80−E115) may interact with the substrate-binding site of mCPY in a manner similar to the N-terminus of I^C. To examine this possibility, we performed a folding analysis using CPY mutants in which the C-terminal propeptide sequences were replaced

dx.doi.org/10.1021/bi3001159 | *Biochemistry* 2012, 51, 3547−3553

with the I^C N-terminus. If the hypothesis is correct, these mutants should fold correctly and show CPY activity similar to the wild type.

Production of CPY mutants was performed using the cell surface display method.[19] Expression vectors for the proCPY mutants were constructed on the basis of pCAS:proCPY-FLAG, which is the plasmid for the cell surface display of proCPY (Figure 2a).[19] The constructed mutants are shown in Figure 2b. Since the N-terminal inhibitory reactive site is required for inhibitory function,[17] we constructed mutants focusing on the 12 amino acid residues at the C-terminus of the propeptide, similar to the N-terminal inhibitory reactive site of mCPY. These CPY mutants, whose propeptide sequences N80−E84, N80−Q87, and N80−N91, were replaced by portions of the N-terminus of I^C, N2−D6, N2−Q9, and N2−D13, were named proCPY-ICN5, proCPY-ICN8, and proCPY-ICN12, respectively. Constructed plasmids were introduced into *S. cerevisiae* MT8-1/ΔCPY strain to display the mature forms of proCPY mutants on the cell surface. It was reported that the proCPY displayed was present as an inactive form (proCPY) in the proteinase A (PrA)-deficient strain; however, the proCPY displayed could be converted to the active form (mCPY) and had hydrolysis activity in the strain containing the PrA gene.[19,20] Mature forms of proCPY-ICN5, proCPY-ICN8, and proCPY-ICN12 were named as $mCPY^{ICN5}$, $mCPY^{ICN8}$, and $mCPY^{ICN12}$, respectively.

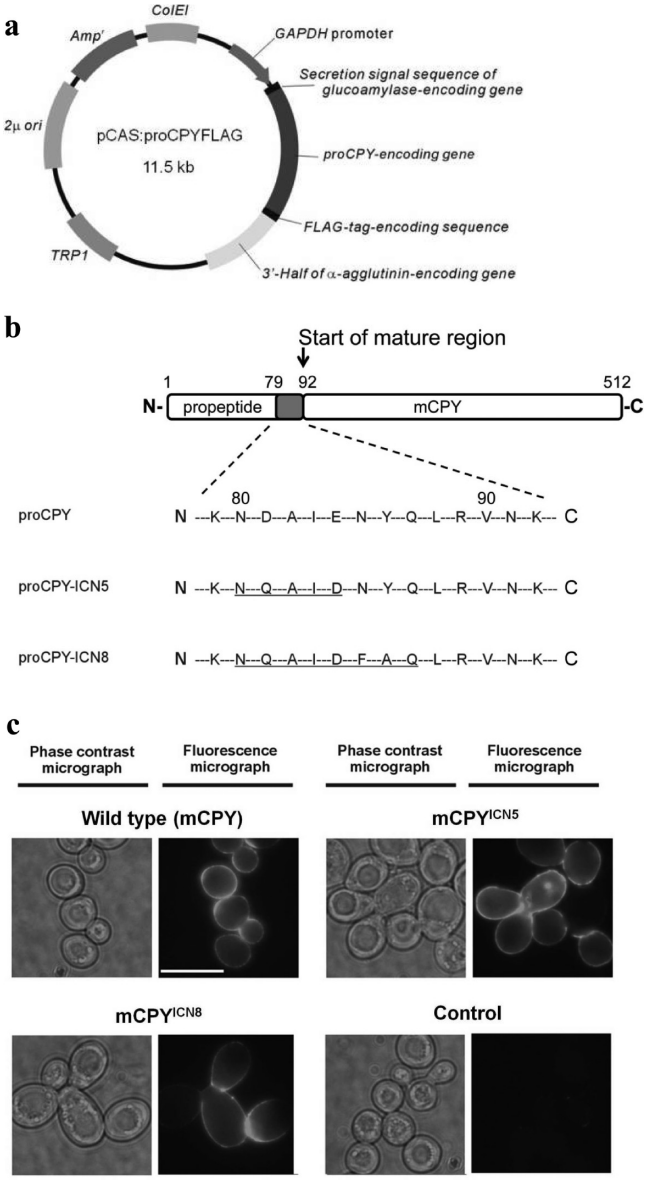

Figure 2. Construction of proCPY mutants. (a) Expression vector for displaying CPY on the cell surface. Produced CPY is displayed with a FLAG tag and the cell wall attachment domain of α-agglutinin. (b) Amino acids sequence of proCPY mutants. A part of the propeptide sequence of CPY is converted to the N-terminal sequences of I^C. Mutated regions are underlined. The numbers above the sequence indicate the residues number of proCPY. The mature region of CPY starts at K92. proCPY-ICN5; propeptide sequence (N80−E84) was replaced with the N-terminal of I^C sequences (N2−D6). proCPY-ICN8; propeptide sequence (N80−Q87) was replaced with the N-terminal of I^C sequences (N2−Q9). (c) Phase contrast and fluorescence micrograph of CPY and its mutants as displayed on yeast cells. The scale bar represents 5 μm.

Figure 2c). Transformants, grown to the stationary phase, were labeled with anti-CPY antibody, followed by Alexa Fluour488-conjugated goat anti-rabbit IgG. Fluorescences were observed on the surface of cells containing the wild-type, $mCPY^{ICN5}$ and $mCPY^{ICN8}$, but not cells containing $mCPY^{ICN12}$ (data not shown) or the control plasmid, indicating that $mCPY^{ICN5}$ and $mCPY^{ICN8}$ were actually displayed on the cell wall. The intensity of immunofluorescence labeling also showed that the $mCPY^{ICN5}$ and $mCPY^{ICN8}$ were displayed on the cell surface, unlike $mCPY^{ICN12}$ (data not shown).

Activity of CPY Mutants on Yeast Cell Surface. The activity of the mCPYs generated from the mutant proCPYs was evaluated by measuring the hydrolysis of Suc-Ile-Ile-Trp-MCA. Replacement of the C-terminal residues of the propeptide with the N-terminal residues of I^C had a great effect on CPY activity (Figure 3a). Compared to the wild type, $mCPY^{ICN5}$ and $mCPY^{ICN8}$ showed 3-fold and 2-fold higher activity, respectively. These results suggest that replacement of the C-terminal residues of the propeptide with the N-terminal residues of the I^C sequence caused the increase in the activity.

The mutated regions were expected to interact with the substrate-binding site of mCPY in a manner similar to the N-terminus of I^C and imprint its structural information onto the substrate-binding site of mCPY. The proCPY bound strongly to thiol reaction reagent (ThioGlo 1 fluorescent thiol reagent), which fluoresces upon interaction with a thiol group, whereas the mCPY bound weakly (Figure 4a). This indicates that the conformation of proCPY was different from that of mCPY and that the conversion into mCPY was largely dependent on the propeptide.

To further confirm the importance of the propeptide amino acids N80−E84, the activity of the alanine-substituted proCPY mutants was measured. The Ala-substituted mutants were named as proCPYN80A, proCPYD81A, proCPYI83A, and proCPYE84A, and the substituted amino acids were Asn80, Asp81, Ile83, and Glu84, respectively. The mature forms of proCPYN80A, proCPYD81A, proCPYI83A, and proCPYE84A were produced on the yeast cell surface, and the activity was measured (Figure 3b). The Ala substitutions dramatically changed the activity, which indicated that the propeptide region (N80−E84) is related to intramolecular chaperone functions.

Measurement of Enzyme Kinetics. To further characterize the mutants, we measured the enzyme kinetics of the

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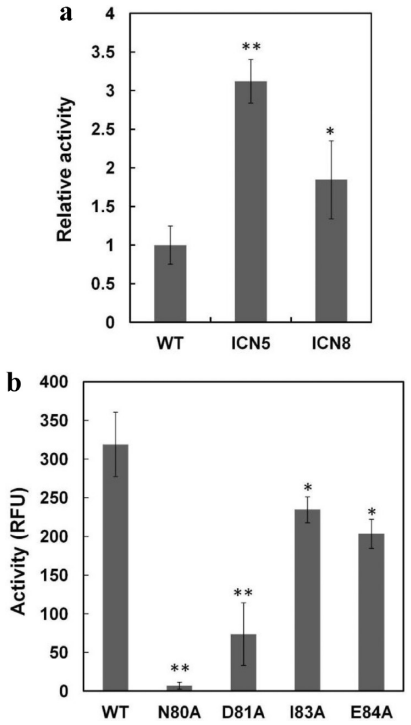

Figure 3. Measurement of activity of mCPY and the mature form of the propeptide mutants displayed on yeast cells. (a) Measurement of activity of mCPY and the mature forms of the propeptide mutants. The relative activities of mCPY (WT), mCPY^{ICN5} (ICN5), and mCPY^{ICN8} (ICN8) were measured using Suc-IIW-MCA as the substrate (n = 3). The activity of WT defined as standard activity (the activity equals 1). (b) Measurement of the activity of Ala-substituted mutants. The activities of the mature forms of proCPYN80A, proCPYD81A, proCPYI83A, and proCPYE84A (N80A, D81A, I83A, and E84A) were measured using Suc-IIW-MCA as the substrate (n = 3). The activities are represented by intensity of fluorescence. In (a) and (b), results are shown as mean ± sem. *P < 0.05, **P < 0.01 compared with wild type.

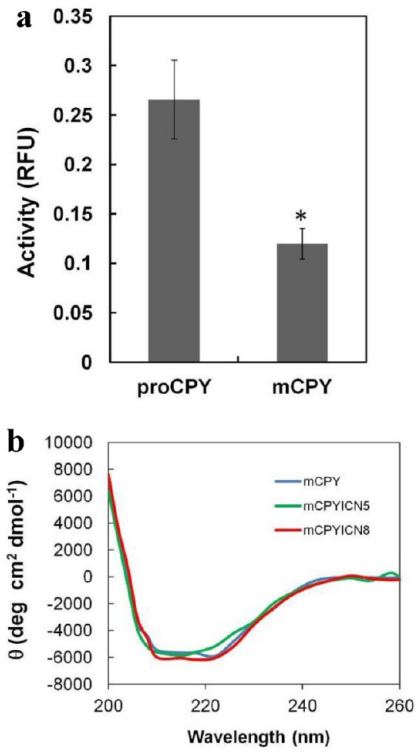

Figure 4. Analysis of CPY structures. (a) Comparison of conformation between proCPY and mCPY. The reaction of proCPY and mCPY to a fluorescent thiol probe was measured (n = 3). Results are shown as mean ± sem. *P < 0.05. (b) Circular dichroism spectra of mCPY (blue), mCPY^{ICN5} (green), and mCPY^{ICN8} (red).

purified mCPY, mCPY^{ICN5}, and mCPY^{ICN8} using Suc-IIW-MCA as a substrate (Table 1). The wild type, mCPY^{ICN5}, and mCPY^{ICN8} exhibited similar K_M; however, the k_{cat}/K_M ratios for mCPY^{ICN5} and mCPY^{ICN8} were greater than that for the wild type. Most notably, the k_{cat}/K_M ratio of mCPY^{ICN8} is 50% greater than that of the wild-type CPY. The activation energies of mCPY^{ICN5} and mCPY^{ICN8} were also lower than that of the wild type (Table 2). The results indicate that CPY mutants with identical amino acid sequences have higher activity than the wild-type CPY. As the mutated region in the propeptide may interact with the substrate-binding site of mCPY, our observations strongly suggest that conformation imprinting may not be the same. The far-UV CD spectrum revealed that the secondary structures of mCPY, mCPY^{ICN5}, and mCPY^{ICN8} were similar (Figure 4b). Therefore, conformation imprinting by the mutated propeptide might accompany the conformational microchanges that cannot be observed in the CD spectra.

Table 1. Kinetic Parameters for the Mature Forms of ProCPY and Its Mutants[a]

	mCPY (WT)	mCPY^{ICN5}	mCPY^{ICN8}
K_M (mM)	7.36 ± 0.28	7.45 ± 1.04	7.41 ± 0.55
k_{cat} (s^{-1})	1.91 ± 0.14	2.28 ± 0.56	2.91 ± 0.50
k_{cat}/K_M (s^{-1}/mM)	0.260 ± 0.028	0.317 ± 0.11	0.391 ± 0.042

[a]Results are shown as mean ± sem.

Table 2. Activity Energy of the Mature Forms of ProCPY and Its Mutants[a]

	mCPY (WT)	mCPY^{ICN5}	mCPY^{ICN8}
E_a (kJ mol^{-1})	44.18 ± 16.70	40.5 ± 9.73	36.67 ± 9.31

[a]Results are shown as mean ± sem.

DISCUSSION

Many proteins have been successfully produced using the yeast cell surface display system[20,22−24] because the system enables easy and rapid protein production,[25] protein analysis,[26] and protein purification.[19] In this study, we constructed an expression system for proCPY mutants (proCPY-ICN5 and proCPY-ICN8) on the yeast cell surface and used this system to analyze the effect of mutations in the propeptide domain on the folding of the mature protein.

This study using a yeast cell surface display system indicated the possibility that mutations in the propeptide of CPY can alter mCPY activity. This phenomenon was called "protein memory" and has previously been reported only in the serine protease subtilisin E (SBE).[12] SBE contains an N-terminal 77-residue propeptide that functions as an intramolecular chaperone and a 275-residue mature domain.[27]

In our study, different propeptide mutations based on the sequence of the protein inhibitor I^C created two functionally distinct enzymes (mCPY^{ICN5} and mCPY^{ICN8}) with identical amino acid sequences and higher activity than the wild-type mCPY (Tables 1 and 2). In SBE, only the single mutation I(−48)V created a functionally distinct protein; however, two

dx.doi.org/10.1021/bi3001159 | Biochemistry 2012, 51, 3547−3553

types of functionally distinct proteins could be created by 3 and 5 amino acid mutations in CPY. This indicates that mutations in propeptide could produce mature enzymes with diverse conformations, despite having identical amino acid sequences (Figure 5a). This suggests that propeptide contains the information controlling the conformation of the mature domain and defines its folding. From these facts, the phenomenon of "protein memory" could be thus termed as "protein folding memory".

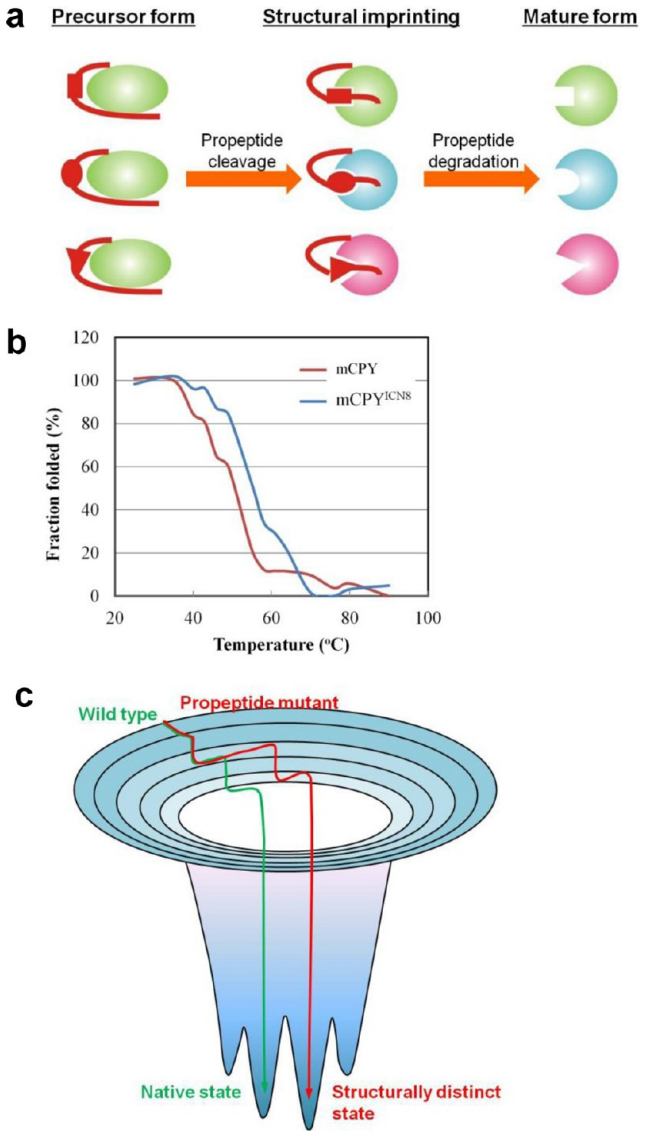

Figure 5. Illustration of folding manners mediated by the mutated propeptide. (a) Model of protein folding memory. (b) Thermal stability of mCPY and mCPY^{ICN8}. The fraction unfolded was monitored using CD and recording the changes in negative ellipticity at 222 nm as a function of temperature. (c) Folding funnel of the wild-type and propeptide mutant. The folding pathways of the wild-type and propeptide mutant were different.

We mutated the C-terminal region of propeptide (N80–Q87), which was expected to bind to the substrate-binding site of mCPY and inhibit CPY activity.[18] It was also reported that another portion of the C-terminus of propeptide (K69–I83) was important for protein folding, because the K68–I83 deletion mutant (proCPYΔ69/83) had completely lost CPY activity.[28] Ala substitution analysis in this study indicated that the C-terminal region of propeptide (N80–E84) is also important for protein folding (Figure 3b). These results showed that the C-terminal region of the propeptide, especially N80–I83, was related to protein folding through binding to the CPY substrate-binding site. Analysis of the crystal structure of mCPY-I^C complex revealed that the conformation of the active site of mCPY in the complex with I^C differed from that of free mCPY.[17] The conformational differences were noted in two locations: inside the loop formed by the residues Cys262–Cys268 and in close proximity to the catalytic residues. The substitution of the N-terminus of the I^C sequence might alter the conformation of the active site of mCPY similar to the active site of the mCPY binding to I^C. The CD spectra of mCPY, mCPY^{INC5}, and mCPY^{ICN8} were similar (Figure 4b), suggesting that the secondary structures of mCPY, mCPY^{INC5}, and mCPY^{ICN8} might not differ largely. In comparison with mCPY^{ICN8} (the highest activity) and mCPY (the original activity), thermal stability of mCPY was less than that of mCPY^{ICN8} (Figure 5b). It seemed that the conformation of mCPY and mCPY^{ICN8} was a little different and mCPY^{ICN8} with high-activity might take more stable structure. These results indicate that conformational changes were observed around the active site of mCPY, rather than the whole structure.

The phenomenon that proteins with identical amino acid sequences can fold into structurally and functionally distinct proteins was also observed in proteins carrying synonymous mutations. Synonymous mutations containing frequent-to-rare codon substitutions in the multidrug resistance 1 (MDR1) gene lead to the formation of proteins with wild-type amino acid sequences, but different structures and functions.[29] Rare codons result in ribosome stalling[30,31] due to lower tRNA populations[32] or alteration of RNA structures.[33,34] RNA stalling supplies ribosomal pause time and enforces alternate folding pathways that lead to distinct minima on the folding free energy landscape.[35] In "protein folding memory" phenomenon, propeptide mutation may lead to an alternate folding pathway and distinct minima on the folding free energy landscape, which results in the production of structurally and functionally distinct enzymes (Figure 5c).

In conclusion, this study strongly suggested that CPY led to the "protein folding memory" phenomenon through propeptide mutation and created proteins with conformational microchanges, despite having the identical amino acid sequence. These findings may provide further insights into the mechanisms underlying protein folding.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CPY, carboxypeptidase Y; proCPY, carboxypeptidase Y precursor; mCPY, mature carboxypeptidase Y; I^C, carboxypeptidase Y inhibitor; CD, circular dichroism; SBE, subtilisin E.

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