N-terminal acetyl group is essential for the inhibitory function of carboxypeptidase Y inhibitor (I^C)

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Abstract Carboxypeptidase Y (CPY) inhibitor, I^C, a yeast cytoplasmic inhibitor in which the N-terminal amino acid is acetylated, was expressed in *Escherichia coli* and produced as an unacetylated form of I^C (unaI^C). Circular dichroism and fluorescence measurements showed that unaI^C and I^C were structurally identical and produce identical complexes with CPY. However, the K_i values for unaI^C for anilidase and peptidase activity of CPY were much larger, by 700- and 60-fold, respectively, than those of I^C. The reactivities of phenylmethylsulfonyl fluoride and *p*-chloromercuribenzoic acid toward the CPY–unaI^C complex were considerably higher than those toward the CPY–I^C complex. Thus, the N-terminal acetyl group of I^C is essential for achieving a tight interaction with CPY and for its complete inactivation.

Key words: Carboxypeptidase inhibitor; Carboxypeptidase Y; N-terminal acetylation; Inhibition mechanism

1. Introduction

Carboxypeptidase Y (CPY) inhibitor, I^C, a cytoplasmic inhibitor of vacuolar proteinases in the yeast *Saccharomyces cerevisiae*, represents the only proteinous inhibitor that is specific for a serine carboxypeptidase reported to date [1–3]. Recently, I^C was identified as a *TFS1* gene product, which is composed of 219 amino acid residues and is possibly involved in nutrient-signaling pathways in starved yeast cells [3–5]. However, the relationship between the regulation of proteolytic activity in vacuoles and the nutrient-signaling pathway has not yet been elucidated.

I^C is a competitive inhibitor of CPY, and forms a complex with it [3,6]. The N-terminal amino acid is acetylated and details of the involvement of the acetyl group in the inhibitory function were recently reported, in which the inhibitor constant, K_i, of the inhibitor, modified with an N-terminal His_6 tag, was greatly increased [3,7].

In the present study, we report on the production of the unacetylated form of I^C (unaI^C) using the *Escherichia coli* expression system and an analysis of its structural and functional properties to clarify the participation of the N-terminal acetyl group in the inhibition of CPY.

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Abbreviations: Bz-Tyr-*p*NA, *N*-benzoyl-L-tyrosine-*p*-nitroanilide; CBZ, benzyloxycarbonyl; CPY, carboxypeptidase Y; I^C, carboxypeptidase Y inhibitor; PCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride; unaI^C, unacetylated form of carboxypeptidase Y inhibitor

2. Materials and methods

2.1. Materials

CPY was purified as previously described [8]. Synthetic oligonucleotides were obtained from Japan Bio Services, Saitama, Japan. Restriction endonucleases and DNA modification enzymes were obtained from Toyobo, Tokyo, Japan. Butyl-Toyopearl 650S was purchased from Tosoh, Tokyo, Japan. Superdex 75 and Superdex 200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. *N*-Benzoyl-L-tyrosine-*p*-nitroanilide (Bz-Tyr-*p*NA), phenylmethylsulfonyl fluoride (PMSF), and *p*-chloromercuribenzoic acid (PCMB) were products of Nacalai Tesque, Kyoto, Japan, in a purity of more than 99%. 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM dithiothreitol (DTT) used throughout the experiment is referred to as buffer A.

2.2. Preparation of I^C from S. cerevisiae

Strain BJ2168 of *S. cerevisiae* was used as the host for the expression of I^C [9] and was transformed by the expression vector pYTF1, which was constructed by insertion of the *TFS1* gene into the *Kpn*I site of pYES2 (Invitrogen, San Diego, CA, USA). Cells grown in the presence of 2% galactose for 42 h were harvested and disrupted by freeze-thawing [3] in buffer A. Ammonium sulfate was added to the homogenate to a concentration of 1.0 M. The solution was applied to a butyl-Toyopearl column (30×60 mm) equilibrated with 1.0 M ammonium sulfate in buffer A. I^C, which eluted at 0.5 M ammonium sulfate, was further purified by gel filtration chromatographies successively using Superdex 200 and Superdex 75 columns (10×300 mm) that had previously been equilibrated with buffer A containing 0.15 M NaCl.

2.3. Preparation of unaI^C from E. coli

E. coli BL21(DE3) was transformed by the expression vector pETF1, produced by ligation of the *TFS1* gene fragment into the pET vector (Novagen, Madison, WI, USA). After the transformed cells were grown in LB medium containing 50 μg/ml ampicillin at 37°C until the OD_{600} reached 0.6, protein expression was induced by the addition of 1 mM isopropyl-1-thio-β-galactoside at 37°C for 4 h. The collected cells were disrupted by ultrasonication in buffer A containing 0.1 M NaCl. The precipitate collected by centrifugation of the cell lysate was washed twice with 4% Triton X-100 in buffer A and suspended in 50 mM Tris–HCl, pH 8.5, containing 6 M guanidine hydrochloride, 10 mM DTT, and 1 mM EDTA, followed by incubation with shaking at 37°C for 1 h. The soluble portion was dialyzed overnight against buffer A containing 0.15 M NaCl. The purified inhibitor was obtained by gel filtration chromatography using a Superdex 75 column (10×300 mm) equilibrated with the same buffer as used in the dialysis.

2.4. Structural analysis

The N-terminal amino acid sequencing of unaI^C was performed on an Applied Biosystems model 476A protein sequencer, according to the manufacturer's instructions.

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The molecular masses of unaI^C and I^C were determined by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry using a Voyager RP mass spectrometer (Applied Biosystems, Foster City, CA, USA). Samples were prepared as follows. The inhibitors, after desalting by reverse-phase chromatography, were added to a matrix solution, 7 mg/ml 3,5-dimethoxy-4-hydroxycinnamic acid in 33% acetonitrile (v/v) containing 0.2% trifluoroacetic acid, to give a final concentration of 3 μM. The system was calibrated with standard proteins that included bovine serum albumin, bovine trypsinogen, and horse apomyoglobin.

Circular dichroism (CD) spectra in the region 200–260 nm were measured in a 0.1 cm sample cell with an optical path length at 25°C with a Jasco J-720W spectropolarimeter. Protein concentrations were adjusted to 8.2 μM in 10 mM sodium phosphate, pH 7.0, containing 1 mM EDTA and 1 mM DTT. Baseline-corrected CD spectra were deconvoluted with the CDFIT software program [10].

Intrinsic fluorescence was measured at 25°C with a Shimadzu RF-5300PC spectrofluorometer by exciting at 295 nm. The emission spectra were recorded between 310 and 440 nm and quantified by specifying the center of the spectral mass, $\langle \nu \rangle$ [11]. Protein concentrations in 50 mM sodium phosphate, pH 7.0, containing 1 mM EDTA, 1 mM DTT, and 0.1 M NaCl were adjusted to 8.2 μM for inhibitors and 3.3 μM for proteinase–inhibitor complexes.

2.5. Determination of inhibitory constants

CPY (32.8 nM) was mixed with various concentrations of unaI^C or I^C (0–656 nM) in 50 mM bis-Tris, pH 6.5, containing 1 mM EDTA, 10% *N*,*N*-dimethylformamide (v/v), and 0.3 mM Bz-Tyr-*p*NA or 1.0 mM benzyloxycarbonyl (CBZ)-Ala-Phe-OH. The initial hydrolytic rates were determined at 25°C as previously described [8,12]. The apparent inhibitor constants, K_i(app), were derived from Henderson plots [13]. The intrinsic inhibitor constants, K_i, were calculated assuming competitive inhibition [6], using the following equation:

$$K_i = K_i(\text{app})/(1 + [S]/K_m) \quad (1)$$

where the K_m values were taken as 0.57 mM and 5.5 mM for Bz-Tyr-*p*NA and CBZ-Ala-Phe-OH, respectively.

2.6. Analysis of the inhibitor and CPY complex

Complex formation between unaI^C or I^C and CPY was analyzed by gel filtration with a Superdex 75 column (10×300 mm). The column was calibrated with the following reference proteins: IgG, bovine serum albumin, ovalbumin, carbonic anhydrase, and RNase A (M_r 160 000, 67 000, 43 000, 31 000, and 13 700, respectively). Mixtures composed of CPY (11 μM) and unaI^C or I^C (0–22 μM) were applied and eluted with buffer A containing 0.15 M NaCl at a flow rate of 0.5 ml/min.

2.7. Reaction with PMSF and PCMB

Free CPY and complexes with unaI^C and I^C (2 μM) were incubated with a 20-fold molar excess of PMSF or PCMB in 50 mM bis-Tris, pH 6.5 at 25°C for 30 min. Residual anilidase activities after the reaction were determined in 0.1 M sodium phosphate solution, pH 7.0, containing 0.3 mM Bz-Tyr-*p*NA, 1 mM EDTA, 10% *N*,*N*-dimethylformamide (v/v), and 1.2 M guanidine hydrochloride. Under such conditions, PMSF and PCMB were extremely diluted and CPY–inhibitor complexes exhibit an activity equivalent to that of free CPY [3].

3. Results and discussion

3.1. Expression, purification, and chemical properties of unaI^C

UnaI^C was expressed as an inclusion body in *E. coli* strain BL21(DE3) with the expression vector pETF1, which was constructed in this study. In the final gel filtration chromatography, unaI^C eluted as a single peak at the same position as native I^C. Approximately 45 mg of homogeneous unaI^C was obtained from 1 l of *E. coli* culture, as confirmed by SDS–PAGE analysis.

Amino acid sequencing showed that the N-terminal amino acid of unaI^C was a methionine residue. MALDI-TOF mass spectrometry indicated that the molecular mass of unaI^C was 24 358.2 as predicted from its amino acid sequence, while the molecular mass of native I^C was larger by 42.3, which corresponds to the mass of an acetyl group, 42.01 (Fig. 1A).

Mass spectrometric analyses of the tryptic fragments of unaI^C and I^C revealed that masses of N-terminal peptides (MNQAIDFAQASIDSYKK) were 1930.01 and 1971.96 for unaI^C and I^C, respectively (data not shown). Thus, it can be concluded that the recombinant protein purified herein (unaI^C) was the unacetylated form of I^C, the primary structure of which was identical to that of native I^C, except for the N-terminal acetyl group.

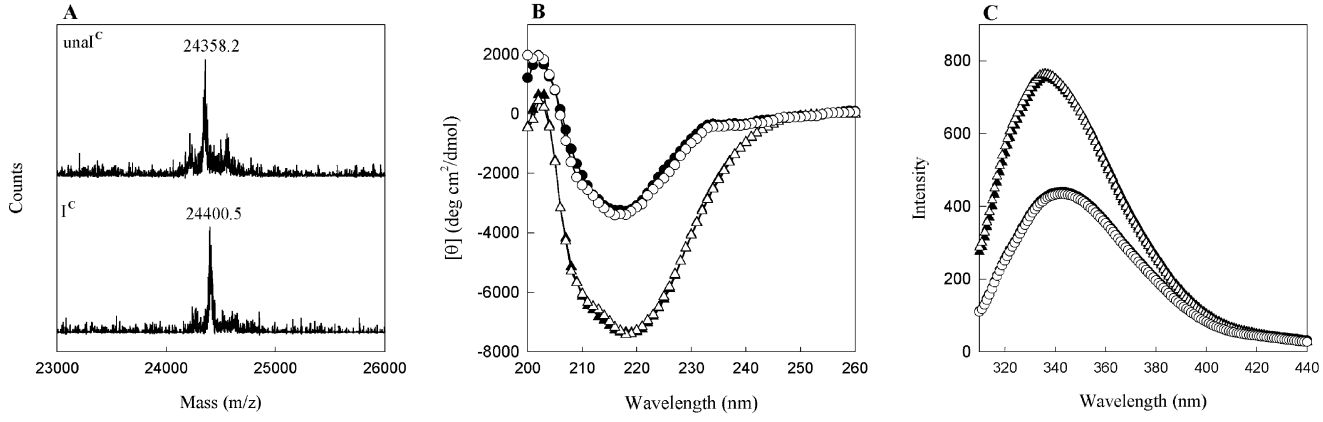

Fig. 1. Structural properties of unaI^C and its complex with CPY. A: MALDI-TOF mass spectra. A mixture of unaI^C or I^C (3 μM), 3,5-dimethoxy-4-hydroxycinnamic acid (7 mg/ml), 33% acetonitrile, and 0.2% trifluoroacetic acid was loaded on a sample plate and the calibration was done using bovine serum albumin, bovine trypsinogen, and horse apomyoglobin. The spectra and molecular masses of unaI^C and I^C are shown in the upper and lower half of the figure, respectively. B: CD spectra. Protein concentrations of unaI^C, I^C, CPY–unaI^C, and CPY–I^C complexes were 8.2 μM in 10 mM sodium phosphate, pH 7.0, containing 1 mM EDTA and 1 mM DTT. Open circles, unaI^C; closed circles, I^C; open triangles, CPY–unaI^C complex; closed triangles, CPY–I^C complex. C: Fluorescence spectra. Intrinsic fluorescence was measured by exciting at 295 nm and the emission spectra were recorded between 310 and 440 nm. Protein concentrations of unaI^C, I^C, CPY–unaI^C, and CPY–I^C complexes were 8.2, 8.2, 3.3, and 3.3 μM, respectively, in 50 mM sodium phosphate, pH 7.0, containing 1 mM EDTA, 1 mM DTT, and 0.1 M NaCl. The symbols are the same as those in B.

3.2. Comparison of structural and inhibitory properties of I^C and unaI^C

The far-UV CD spectra of unaI^C and I^C were nearly identical (Fig. 1B): the α-helix, β-sheet, and random structure contents of the inhibitors were calculated to be 0.8, 78, and 22% for the unacetylated form and 1.9, 80, and 18% for the acetylated form, respectively, indicating that both inhibitors are similar β-type proteins in which the β-structure is the predominant structural element. The intrinsic fluorescence spectra of unaI^C and I^C were nearly identical (Fig. 1C) and the maximum wavelength, λ_{max}, and center of the spectral mass, $\langle\nu\rangle$, of both proteins were 342.5 nm and 28 340 cm^{-1}, respectively. CD and fluorescence spectra indicate that unaI^C was correctly folded, forming the same gross structure as native I^C.

The inhibitory effects of unaI^C and I^C on anilidase activity for Bz-Tyr-*p*NA and peptidase activity for CBZ-Ala-Phe-OH of CPY were significantly different from each other (Fig. 2): an equivalent molar concentration of unaI^C inhibited only 7 and 13% of anilidase and peptidase activities for CPY, respectively, and even a five-fold molar excess of unaI^C inhibited the activities by 30 and 35%, respectively, while an equivalent molar concentration of I^C inhibited 70–80% of both activities. Based on these data, the intrinsic inhibitor constants, K_i, of unaI^C were calculated from Eq. 1 to be 400 nM and 330 nM for Bz-Tyr-*p*NA and CBZ-Ala-Phe-OH hydrolysates, respectively, while the K_i values of I^C were 0.55 nM and 5.4 nM, respectively. These results show that the inhibitory efficiency of unaI^C was much smaller than that of I^C, and the dependence of unaI^C on the substrate for CPY was largely different from that of I^C. The dependence of I^C suggests that the binding affinity of I^C to the C-terminal binding site of CPY may be different from it to the other subsites.

3.3. Complex formation of unaI^C with CPY

Gel filtration chromatography of the unaI^C and CPY mixture showed a peak corresponding to the unaI^C and CPY complex of M_r 89 000, which increased proportionally with the addition of increasing amounts of unaI^C (Fig. 3). When equimolar mixtures of unaI^C (M_r 25 000) and CPY (M_r 61 000) were applied to a column, a single peak having M_r 89 000 was obtained (chromatogram no. 4 in Fig. 3). These elution profiles were the same with those for a mixture of I^C and CPY (data not shown), indicating that unaI^C and I^C form a stable equimolecular complex of M_r 89 000 with CPY. Furthermore, the far-UV CD and intrinsic fluorescence spectra of the CPY–unaI^C and CPY–I^C complexes were nearly identical (Fig. 1B,C), showing that the overall structure of the complex was nearly unchanged as the result of N-terminal acetylation.

PMSF and PMCB, specific reagents for the catalytic center Ser146 and the S1 subsite residue Cys341 of CPY, respectively [14–16], reacted more rapidly toward the CPY–unaI^C complex than toward the CPY–I^C complex (Fig. 4), where the second order reaction rates, k_{2nd}, of PMSF and PMCB were 1.15 and 0.24 min^{-1} mM^{-1} toward the CPY–unaI^C complex, respec-

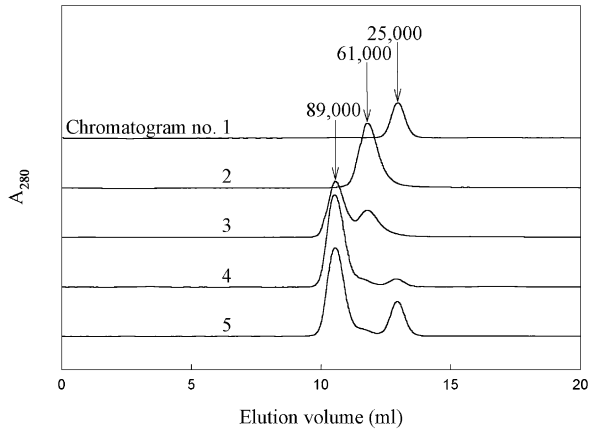

Fig. 3. Gel filtration analysis of unaI^C and CPY mixture. A mixture of CPY and unaI^C was applied to a Superdex 75 column (10×300 mm) and eluted with buffer A containing 0.15 M NaCl at a flow rate of 0.5 ml/min, by monitoring the absorbance at 280 nm. The estimated molecular mass of each peak is shown in the figure. Chromatogram no. 1, 11 μM unaI^C; no. 2, 11 μM CPY; no. 3, 5.5 μM unaI^C and 11 μM CPY mixture; no. 4, 11 μM unaI^C and 11 μM CPY mixture; no. 5, 22 μM unaI^C and 11 μM CPY mixture.

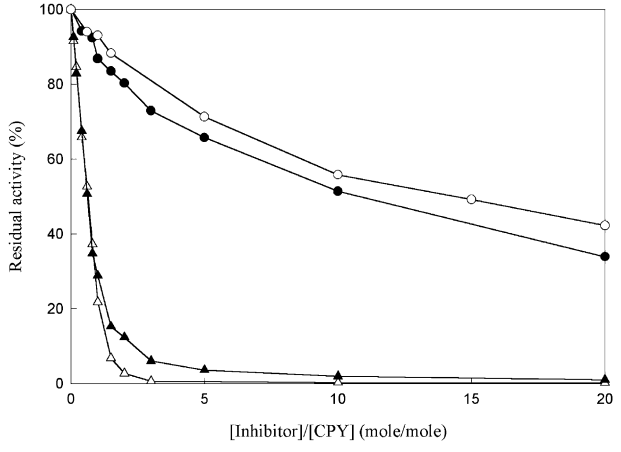

Fig. 2. Stoichiometry of CPY inhibition by unaI^C. CPY (32.8 nM) was mixed with various amounts of unaI^C or I^C (0–656 nM) and the residual activity of CPY was assayed using Bz-Tyr-*p*NA or CBZ-Ala-Phe-OH as the substrate. Circles, unaI^C; triangles, I^C; open symbols, Bz-Tyr-*p*NA activity; closed symbols, CBZ-Ala-Phe-OH activity.

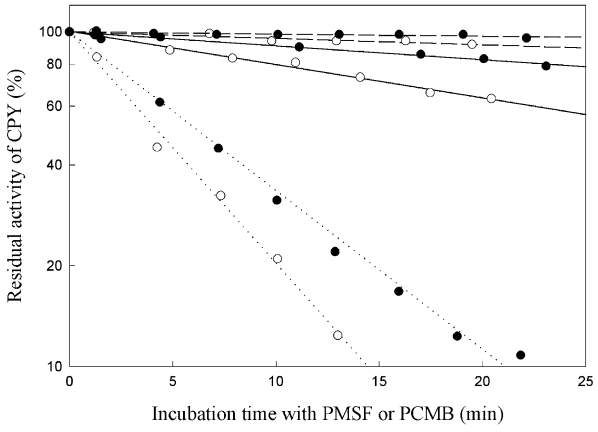

Fig. 4. Effect of PMSF and PCMB on the CPY–unaI^C complex. CPY–unaI^C, CPY–I^C complexes, and CPY were allowed to react with a 20-fold molar excess of PMSF or PCMB. Residual CPY activity of the reaction mixture was assayed with respect to Bz-Tyr-*p*NA in the presence of 1.2 M guanidine hydrochloride. The activity before incubation was taken as 100%. Solid lines, CPY–unaI^C complex; dashed lines, CPY–I^C complex; dotted lines, free CPY; open circles, PMSF reaction; closed circles, PCMB reaction.

tively, and 0.22 and 0.037 min^{-1} mM^{-1} toward the CPY–I^C complex, respectively, although these values were much smaller than those found for free CPY (k_{2nd} of PMSF and PCMB: 8.0 and 2.7 min^{-1} mM^{-1}, respectively). These results indicate that both I^C and unaI^C form a complex with CPY and mask the catalytic Ser146 and Cys341 but these two residues were more loosely covered in the CPY–unaI^C complex than in the CPY–I^C complex, in terms of their ability to accept the very strong inhibitors, PMSF and PCMB.

3.4. Participation of the N-terminal acetyl group of I^C in the inhibition of CPY

The present experiments using unaI^C and its complex with CPY demonstrates that the secondary and tertiary structures of I^C and the complex and its ability to form a complex were negligibly affected by the elimination of the N-terminal acetyl group of I^C. As shown in Fig. 2, however, the inhibitory efficiency of unaI^C toward CPY was greatly decreased, compared with that of I^C: the K_i values of unaI^C were increased by about 700- and 60-fold, respectively, for anilidase and peptidase activities. These results indicate that the interaction of unaI^C with CPY is weaker than that of I^C at least in the local region around the active site of CPY. In other words, the N-terminal acetyl group is important for forming a tight complex.

Considering that CBZ-Ala-Phe-OH contains the C-terminal carboxyl group, the remarkable decrease in the K_i value of unaI^C with respect to CBZ-Ala-Phe-OH hydrolysis suggests that the N-terminal α-amino group of unaI^C disturbs the binding of the carboxyl group to the C-terminal binding site of CPY, in which a hydrogen bond network is formed [16–18]. Hence, the acetyl group of I^C, when it is complexed with CPY, would be situated adjacent to the substrate-binding sites (S1 and S1′ subsites and C-terminal binding site) and would be involved in the masking of the active site of CPY. This hypothesis is supported by the increased reactivity of PMSF and PCMB with the CPY–unaI^C complex.

The present study clearly demonstrates the functional involvement of the N-terminal acetylation in I^C and indicates a novel mechanism of carboxypeptidase inhibition which differs from the stopper-like inhibition of carboxypeptidase A by the C-terminal region of potato carboxypeptidase inhibitor [19]. Although no definitive information is available at present regarding the nature of the binding of the acetyl group to CPY, further studies on mutational analyses of the N-terminal region and X-ray crystallography of CPY–I^C complex would clarify the specific role of the N-terminal acetyl group in the inhibition of I^C.

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