In Vitro Disulfide-Coupled Folding of Guanylyl Cyclase-Activating Peptide and Its Precursor Protein

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ABSTRACT: Guanylyl cyclase-activating peptide II (GCAP-II), an endogenous ligand of particulate guanylyl cyclase C (GC-C), is processed from the precursor protein and circulates in human blood. GCAP-II consists of 24 amino acid residues and contains two disulfide bridges. The correct disulfide paring of GCAP-II is an absolute requirement for its biological activity. This study shows that the folding of the peptide from the reduced form yields a peptide with the native disulfide paring as a minor product and with non-native ones as major products, regardless of the presence or absence of reduced and oxidized glutathione. The results suggest that GCAP-II does not possess sufficient information to permit the adoption of the native conformation and to effectively form the correct disulfide pairing and, as a result, that GCAP-II is correctly folded by assistance of a factor(s) such as an intra- or intermolecular chaperone. We studied whether a peptide in the pro-leader sequence of the precursor protein (proGCAP-II) contains sufficient information to facilitate the folding of GCAP-II. For this purpose, we prepared proGCAP-II in *Escherichia coli* by a recombinant technique and examined the disulfide-coupled folding of proGCAP-II from the reduced form. proGCAP-II was quantitatively recovered with the correctly folded structure from the reduced form both in the presence and in the absence of reduced and oxidized glutathione. The protein contains only disulfide linkages at the same positions as the mature form of proGCAP-II, GCAP-II, and the biologically active isomer of GCAP-II in the molecule. These results provide evidence that the propeptide of proGCAP-II is a critical factor in the formation of the correct disulfide paring in the folding of the protein.

Guanylin and uroguanylin serve as endogenous ligands (*1*, *2*) of particulate guanylyl cyclase C (GC-C)[1] (*3*). The enzyme is localized on the intestinal brush border cell membranes and has previously been shown to be a receptor protein for heat-stable enterotoxins (STa) produced by enteric bacteria (*4*, *5*). This constitutes an important signaling system, which functions in the regulation of the level of cGMP as a second messenger in intestinal or kidney cells, that is, the regulation of chloride transport in electrolytes by means of a paracrine interaction (*6*). The accumulation of epithelial cGMP induces the activation of a cystic fibrosis transmembrane conductance regulator via cross-talk with protein kinase A in the apical membranes, which, in turn, results in chloride and water secretion from the inside of a cell to the outside (*7*). Guanylin and uroguanylin were originally isolated from intestinal mucosa and urine, respectively (*1*, *2*, *8*). Northern blot analysis indicates that guanylin occurs not only in intestinal tissue but also in a variety of other tissues, such as kidney, airway epithelia, pancreas, and liver, while uroguanylin occurs in the intestine, atrium, and ventricle (*9*). Recently, another endogenous ligand of GC-C has been isolated from human blood and identified as a form of uroguanylin which is extended at the N terminus (Figure 1). This ligand has been referred to as guanylyl cyclase-activating peptide II (GCAP-II) (*10*). cDNA cloning of the peptides showed that uroguanylin and GCAP-II are produced from the same precursor protein (prepro form, Figure 1a) and that a peptide in the presequence (amino acid residues 1−26) of the protein functions as a signal peptide for the secretion of these peptides (*11*, *12*). After secretion, prouroguanylin and/or proGCAP-II (amino acid residues 27−112) are further processed to produce the mature forms of

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[1] Abbreviations: GC, guanylyl cyclase; STa, heat-stable enterotoxin of enterotoxigenic *E. coli*; STp, heat-stable enterotoxin produced by a porcine strain of *E. coli*; STp(4−17), derivative containing the amino acid sequence of residues 4−17 of STp; STII, heat-stable enterotoxin II; cGMP, cyclic guanosine monophosphate; GCAP-II, guanylyl cyclase-activating peptide II (the plasma form of uroguanylin); pro-GCAP-II, precursor protein of GCAP-II with amino acid residues 27−112 of pre-proGCAP-II; Fmoc, 9-fluoromethoxycarbonyl; Acm, acetamidomethyl; Trt, trityl; DMF, dimethylformamide; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; Tris/HCl, tris(hydroxymethyl)aminomethane hydrochloride; PBS, phosphate-buffered saline; CD, circular dichroism; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; Gu/HCl, guanidine hydrochloride; GSSG and GSH, disulfide and thiol forms of glutathione, respectively; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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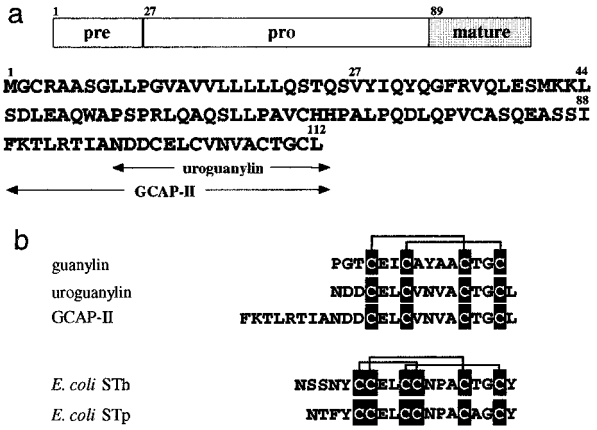

FIGURE 1: (a) Primary structure of pre-proGCAP-II (or pre-prouroguanylin), a precursor of GCAP-II and uroguanylin. (b) The disulfide linkages in guanylin, uroguanylin, GCAP-II, and heat-stable enterotoxins are shown by solid lines.

uroguanylin or GCAP-II. It is noteworthy that both pro-GCAP-II and GCAP-II are found in opossum plasma (*12*). Thus, uroguanylin in opossum urine is thought to be derived from GCAP-II in the plasma via glomerular filtration, since no uroguanylin mRNA is observed in kidney (*12*).

Guanylin and GCAP-II consist of 15−24 amino acid residues and are homologous to STa in primary structure, as shown in Figure 1b. These peptides contain four cysteine residues, which are localized at the same relative positions and are linked by disulfide bridges at the same positions as those in STa, as shown in Figure 1b. The disulfide parings of guanylin and GCAP-II play a critical role in the expression of biological activity (*1*, *2*, *13*). However, little is known concerning the folding pathway of the peptides, more specifically, the process by which the correct disulfide paring is achieved. In general, in eukaryotes, peptides having multiple disulfide linkages undergo a disulfide-coupled folding during and/or after the elongation of peptide chains (*14*, *15*). Moreover, noncovalent interactions and a redox voltage thermodynamically or kinetically control folding and disulfide-bond formation in vivo, resulting in the correct formation of the tertiary structures of peptides. Recently, the disulfide-coupled folding of several peptides and proteins has been examined, and the results suggest that, in some cases, mature peptides do not possess sufficient information to allow for correct folding, and that a peptide in the pro-leader sequence assists in the folding of the domain of the mature peptide or protein (*14*, *16*−*20*). Preliminary experiments in this laboratory regarding the folding mechanism of GCAP-II showed that the mature form of proGCAP-II, GCAP-II, cannot correctly fold spontaneously, suggesting the possibility that folding and disulfide bond formation in GCAP-II are controlled by a peptide in the pro-leader sequence (amino acid residues 27−88) of proGCAP-II.

To further elucidate the folding mechanism of GCAP-II, we report in this paper an investigation of the disulfide-coupled folding of GCAP-II in the presence or absence of reduced and oxidized glutathione. In addition, we examined the in vitro folding of proGCAP-II produced in *Escherichia coli* by a recombinant technique and determined the disulfide parings of proGCAP-II to elucidate the role of a peptide in the pro-leader sequence of GCAP-II in the folding mechanism. The data obtained shed light on our understanding of the folding mechanism of small peptides, such as GCAP-II and/or uroguanylin.

MATERIALS AND METHODS

T4 DNA ligase and restriction enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan) and New England Biolabs, Inc. (Bervely, MA), respectively. Amino acid derivatives for peptide syntheses were obtained from Japan PerSeptive Biosystems, Inc. (Tokyo, Japan). Reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were reagent grade. Continuous flow solid-phase peptide synthesis was carried out using a MilliGen 9050 peptide synthesizer (Bedford, MA).

Peptide Synthesis. The protected peptide (GCAP-II), corresponding to the 24 C-terminal amino acid residues of proGCAP-II, was synthesized by the Fmoc solid-phase method on a MilliGen peptide synthesizer. Two different types of protecting groups, the Acm group (residues 103 and 111) and the Trt group (residues 100 and 108), were employed for blocking cysteine residues, to selectively form the disulfide paring of GCAP-II essentially as described in an earlier report (*21*). Coupling reactions were carried out with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and diisopropylethylamine in DMF. The peptide resin was treated with 20% piperidine/DMF containing 2% diazabicycloundecene to remove the Fmoc group at the N terminus and then with TFA in the presence of ethanedithiol, thioanisole, and *m*-cresol to release the peptide from the resin. The resulting peptide, carrying Acm groups on Cys residues (103 and 111), was air-oxidized to form the first disulfide bond between Cys^{100} and Cys^{108} and purified by HPLC. The Acm group of the peptide was removed by iodine, which then permitted the formation of the second disulfide bond. Finally, the resulting peptide was purified by HPLC and characterized by mass spectrometry and amino acid analysis. STp(4−17) was synthesized essentially as described earlier (*21*).

Reversed-Phase High-Performance Liquid Chromatography. The HPLC apparatus was comprised of a Waters 600 multisolvent delivery system (Bedford, MA) equipped with a Hitachi L-3000 photodiode array detector and a D-2000 chromato-integrator (Tokyo, Japan). Synthesized peptides were purified by HPLC using a Develosil UG-5 column (ODS, 4.6 × 150 mm; Nomura Chemicals, Aichi, Japan). The peptides were eluted using a linear gradient of CH_3CN in 0.05% TFA at a flow rate of 1 mL/min increasing at a rate of 1%/min from solvent A (0.05% TFA/H_2O) to solvent B (0.05% TFA/CH_3CN). The separated peptides were rechromatographed using 10 mM $AcONH_4$ (pH 5.7) in place of 0.05% TFA.

Binding Activity of GCAP-II to GC-C Expressed on 293T Mammalian Cells. 293T mammalian cells, which express porcine GC-C, were prepared as reported (*22*). The cells (3×10^6) were suspended in 50 mM Tris/HCl (pH 8.0, 200 μL) containing 0.5 M NaCl and 1 mM phenylmethanesulfonyl fluoride and sonicated. STp(4−17) was radioiodinated with chloramine T and $Na^{125}I$ and purified by HPLC to approximately 2000 Ci/mmol as described previously (*22*). 293T cell membranes, which expressed the recombinant GC-C, and [^{125}I]STp(4−17) (6×10^4 cpm) were incubated at 37 °C for 1 h in a final volume of 60 μL of PBS(−) in the

presence or absence of 10^{-6} M synthetic GCAP-II. [^{125}I]-STp(4−17) which bound to the membranes was separated from free [^{125}I]STp(4−17) by filtration on GF/C glass filters (Whatman) which had been pretreated with poly(vinylpyrrolidone) as described previously (*23*). The radioactivities of the membranes were measured using a γ-well counter. The binding assays were performed in duplicate.

cGMP Assay. The cyclic GMP assay was carried out using 293T cells expressing porcine GC-C, as reported previously (*22*). 293T cells (10^5 cells) were incubated with synthetic GCAP-II (10^{-5} M) at 37 °C for 15 min in 20 mM Tris/HCl (pH 7.6, 300 μL) containing 0.1 mM 3-isobutyl-1-methylxanthine. The reaction was stopped by the addition of 3 M sodium acetate (pH 4.6), followed by boiling. The amounts of cGMP in the samples were assayed in duplicate using a cGMP radioimmunoassay kit according to the specifications provided by the manufacturer (Yamasa Shoyu Inc., Tokyo, Japan).

Circular Dichroism Measurement. Far-UV CD spectra (190−260 nm) of each of the synthetic peptides (10 nmol) in PBS(−) (0.5 mL) were measured at 25 °C in a cuvette with a 0.2 cm path length using a model J-700 spectropolarimeter (Jasco, Tokyo, Japan).

Reduction and Reoxidation of GCAP-II. Fully reduced GCAP-II was prepared by incubating GCAP-II (100 nmol) with 20 equiv of DTT in 50 mM Tris/HCl (pH 8.0, 500 μL) under a N_2 atmosphere at 50 °C for 1 h. Reduced GCAP-II was purified by HPLC and lyophilized. Reduced GCAP-II (10 nmol) was dissolved in 0.05% TFA (20 μL) and treated with 9 volumes each of the buffers described in Figure 6 at 25 °C for 2 days. The reaction mixture was subjected to HPLC, and the recovery of the reoxidized peptide was estimated from the HPLC peak area. All solutions used for the experiment were flushed with N_2, and the reaction was carried out in a sealed vial under a N_2 atmosphere.

Construction of Expression Vectors of proGCAP-II. The cDNA encoding human proGCAP-II (residues 27−112) was first subcloned into the expression vector pIN-III-*ompA*-1 (*24*), following introduction by PCR of an *Eco*RI site at its 5′ end and a *Bam*HI site at its 3′ end using pEX2 containing a full cDNA gene of pre-proGCAP-II (*25*) as a template. Thus, three amino acid residues, Ala-Asn-Ser, were introduced at the N terminus of proGCAP-II. The resulting construct for the expression of proGCAP-II is named pIN-III-*ompA*-PU. pIN-III-*ompA*-1 contained the *lpp* promoter, the *lac* promoter, the Shine−Dalgarno sequence, and the sequence encoding an OmpA signal peptide. Next, the cDNA encoding the OmpA signal peptide and proGCAP-II was subcloned into the pET17b expression vector (Novagen), following introduction by PCR of an *Nde*I site at its 5′ end and a *Bam*HI site at its 3′ end. The resulting expression vector, referred to as pET17b-APU, contained the cDNA sequence which encoded the signal peptide of OmpA and Ala-Asn-Ser-proGCAP-II. The DNA sequences of the vectors thus constructed in this study were confirmed by analysis using an Applied Biosystems 373A sequencing system.

Expression of proGCAP-II. *E. coli* BL21(DE3) cells transformed with pET17b-APU were grown at 37 °C in Luria broth medium (1 L) supplemented with ampicillin (50 μg/mL). The expression of proGCAP-II was induced by the addition of 1 mM IPTG at the midlog phase of cell growth. After incubation at 37 °C for 3 h, *E. coli* cells were harvested and washed with 50 mM sodium phosphate (pH 7.8) containing 0.3 M NaCl and 1 mM phenylmethanesulfonyl fluoride. The cells were resuspended in the buffer (20 mL) and treated with lysozyme (1 mg/mL) on ice for 15 min, followed by sonication. The mixture was centrifuged (10000*g* for 20 min), and proGCAP-II was obtained as an insoluble material. The yield of proGCAP-II was approximately 3−5 mg from 1 L of the culture medium.

Renaturation of proGCAP-II from an Inclusion Body. Recombinant proGCAP-II (100 μg), obtained as an insoluble material, was dissolved in 50 mM Tris/HCl (pH 8.0, 1 mL) containing 6 M Gu/HCl, 0.6 M Na_2SO_3, and 0.1 M $Na_2S_4O_6$ and kept at 50 °C for 1 h. After centrifugation, the supernatant was dialyzed against 50 mM Tris/HCl (pH 8.0, 100 mL) at room temperature with two changes of buffer for 24 h. The supernatant, containing sulfonated proGCAP-II, was subsequently dialyzed against 50 mM Tris/HCl (pH 8.0, 100 mL) in the presence of 2 mM GSH and 1 mM GSSG under a N_2 atmosphere at 25 °C for 24 h. Renatured proGCAP-II was purified by HPLC and characterized by mass spectrometry, amino acid analysis, and Edman degradation.

Refolding of proGCAP-II. Renatured proGCAP-II (2 nmol) was dissolved in 0.1 M Tris/HCl (pH 8.0, 500 μL) containing 0.1 M DTT in the presence of 6 M Gu/HCl and kept at 37 °C for 30 min. Reduced proGCAP-II was purified by HPLC and lyophilized. The resulting material was redissolved in 0.1 M Tris/HCl (pH 8.0, 500 μL) containing 10 mM DTT and 6 M Gu/HCl and dialyzed against 50 mM Tris/HCl (pH 8.0, 100 mL) in the presence or absence of 2 mM GSH and 1 mM GSSG with three changes of buffer at 25 °C for 3 days. Refolded proGCAP-II was recovered in 94 and 87% yields, estimated by the HPLC peak area, in the presence or absence of GSH and GSSG, respectively.

Endoproteinase Arg-C Digestion of proGCAP-II. Refolded proGCAP-II (3 nmol) was incubated with Arg-C (30 pmol) in 0.1 M Tris/HCl (pH 8.0, 200 μL) at 37 °C for 12 h. The digest was then subjected to HPLC. The separated peptides were analyzed by mass spectrometry and amino acid analysis. The Arg-C digest of proGCAP-II (1.5 nmol) was dissolved in 0.1 M Tris/HCl (pH 8.0, 200 μL) containing 20 mM $CaCl_2$ and the mixture incubated at room temperature for 30 min with anhydrotrypsin agarose (Takara Shuzo), which had been equilibrated with buffer beforehand. The mixture was centrifuged (5000*g* for 5 min) and the supernatant subjected to HPLC. The recovery of the C-terminal fragment was determined by amino acid analysis.

RESULTS

Chemical Synthesis of GCAP-II. GCAP-II was synthesized by the Fmoc solid-phase method followed by the formation of disulfide linkages in a stepwise manner using two types of selectively removable thiol protecting groups (Trt for Cys^{100} and Cys^{108} and Acm for Cys^{103} and Cys^{111}) for the four Cys residues. Removal of the Trt group and air oxidization gave a peptide with one disulfide bond between Cys^{100} and Cys^{108}. Cleavage of the Acm groups by treatment with iodine then yielded GCAP-II with the second disulfide linkage between Cys^{103} and Cys^{111}. These peptides with one or two disulfide bonds were purified by HPLC, as shown in

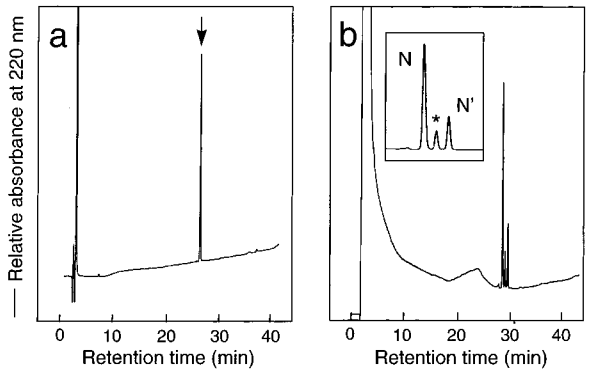

FIGURE 2: HPLC profiles of synthetic GCAP-II analogues: (a) [Cys^{103}(Acm),Cys^{111}(Acm)]GCAP-II with a disulfide bond between Cys^{100} and Cys^{108} and (b) [Cys^{103}(Acm),Cys^{111}(Acm)]GCAP-II oxidized by iodine. The peak with an asterisk is derived from an impurity, produced during the Fmoc solid-phase synthesis. The inset shows an expansion in the range of the 26−32 min retention time. N and N′ represent two isomers of GCAP-II: GCAP-II-N and GCAP-II-N′, respectively.

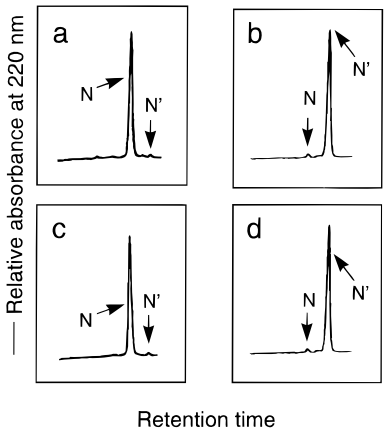

FIGURE 3: HPLC profiles of (a) GCAP-II-N and (b) GCAP-II-N′ separated from Figure 2b and kept in 0.1 M Tris/HCl (pH 8.0) at 25 °C for 2 days and (c) GCAP-II-N and (d) GCAP-II-N′ kept in 0.1 M Tris/HCl (pH 8.0) containing 6 M Gu/HCl at 25 °C for 2 days.

panels a and b of Figure 2, respectively. The peptide (GCAP-II) with two disulfide linkages which should be at the same positions was isolated as two peak fractions (GCAP-II-N and GCAP-II-N′, labeled as N and N′, respectively, in Figure 2b). Chino et al. (*26*) reported that uroguanylin, a form which was secreted into urine and an analogue lacking eight amino acid residues at the N terminus of GCAP-II (Figure 1b), consisted of two topological isomers, when synthesized via a procedure similar to that used for the synthesis of GCAP-II. The ratio (N:N′ = 7:3) of two peak fractions in the synthetic GCAP-II (Figure 2b) was nearly the same as that for the synthetic uroguanylin. This indicates that the N-terminal peptide in GCAP-II has no effect on either the formation of or the ratio of two topological isomers of uroguanylin or GCAP-II.

Interconversion of the Two Topological Isomers of GCAP-II. GCAP-II exists as two topological isomers both in human plasma (*27*) and in the synthetic material (in this experiment), as well as in synthetic uroguanylin (*26*). Recently, Klodt et al. (*28*) reported that the maximum half-life for the conversion to one topological isomer of guanylin was approximately 90 min. We examined the interconversion of each of the two isomers (GCAP-II-N and GCAP-II-N′) of GCAP-II. GCAP-II-N converted to GCAP-II-N′ at a rate of 0.6% per 24 h at 25 °C and vice versa, as shown in panels a and b of Figure 3, respectively, under the conditions described in the legend of Figure 3. The two isomers of GCAP-II also interconverted at a similar rate, regardless of the presence or absence of a strong denaturant (6 M Gu/HCl), as shown in panels c and d of Figure 3. These results suggest that the two isomers of GCAP-II interconvert rather slowly, compared to guanylin, and that they are not constrained by intramolecular hydrogen bonds but, rather, that their interconversion is mainly controlled by steric factors.

To better understand the role of steric effects on the interconversion of the two topological isomers of GCAP-II, we examined the behavior of Thr-Ile-Ala-uroguanylin. This peptide corresponds to the C-terminal fragment of GCAP-II or proGCAP-II prepared by Arg-C digestion, lacks five amino acid residues at the N terminus of GCAP-II, but carries an additional three amino acid residues at the N terminus of the urine form of uroguanylin. Thr-Ile-Ala-uroguanylin consisted of two topological isomers, similar to uroguanylin and GCAP-II, and showed characteristics similar to those observed for GCAP-II-N and GCAP-II-N′ (data not shown). This observation indicates that the N-terminal region of GCAP-II has no effect on the interconversion of the two topological isomers. The Leu residue at the C terminus of GCAP-II, therefore, may contribute to the interconversion of the two isomers as has been reported for uroguanylin (*26*).

The two isomers of GCAP-II or Thr-Ile-Ala-uroguanylin were incubated in the same environment in the absence of Arg-C, like that for the digestion of GCAP-II with Arg-C at 37 °C, to estimate the interconversion of the isomers during the digestion of GCAP-II, as described below. These two isomers interconverted at a rate of approximately 2% per 24 h. On the basis of these observations, we were able to estimate the quantities of the two topological isomers (GCAP-II-N and GCAP-II-N′) in proGCAP-II, if they are latent within the molecule.

Biological Activities of Two Topological Isomers of GCAP-II. Two topological isomers (GCAP-II-N and GCAP-II-N′) of synthetic GCAP-II were assayed with respect to binding to GC-C in 293T cell membranes and the generation of cGMP in 293T cells using procedures described previously (*22*). The biological activities of the two isomers of synthetic GCAP-II were estimated by comparison with those of STp. In this experiment, a shorter analogue [STp(4−17)] which had the same level of biological activity as STp was used (*23*). GCAP-II-N was less active than STp(4−17) and showed approximately 30% of the binding activity of STp-(4−17) for binding to GC-C, while GCAP-II-N′ showed no significant activity, as shown in Figure 4a. The same results were obtained in the assay for cGMP production (Figure 4b). These results suggest that GCAP-II-N represents the biologically active form and the major form of GCAP-II in circulation in human blood.

Circular Dichroism. To obtain further evidence that shows that GCAP-II-N is the active form of GCAP-II, CD spectra were measured in the far-UV region. As shown in Figure 5, the spectrum of GCAP-II-N was nearly the same as that of STp(4−17), which is consistent with a previous report that showed that the active form of guanylin contains a backbone structure similar to STa (*29*, *30*). In addition,

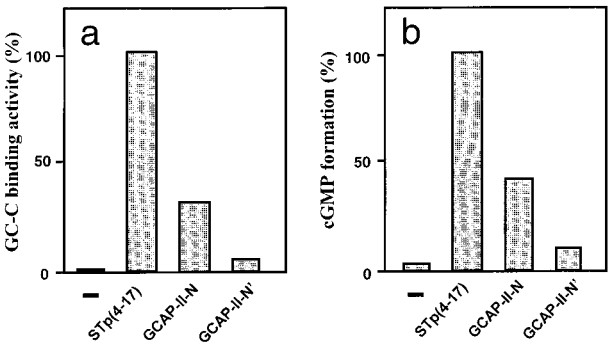

FIGURE 4: Biochemical activities of two isomers of GCAP−II. (a) Activities of binding to GC-C expressed in 293T cells of two topological isomers (GCAP-II-N and GCAP-II-N′) and STp(4−17). The nonspecific binding was less than 10% of the specific binding. (b) The amount of cGMP in 293T cells stimulated by GCAP-II-N, GCAP-II-N′, and STp(4−17). Both sets of data were estimated as the ratios of GCAP-II-N and GCAP-II-N′ to STp(4−17). Experimental data are represented as the average of two data sets. A control experiment was carried out using 293T cells not transfected with pCG-pSTaR (*22*).

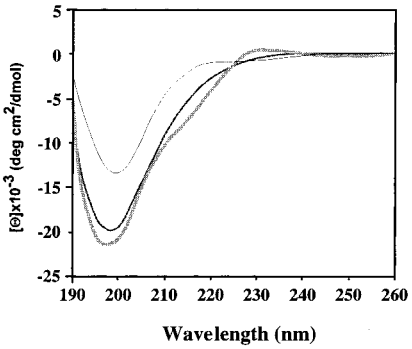

FIGURE 5: CD spectra of the two topological isomers of GCAP-II and STp(4−17). Far-UV CD spectra of GCAP-II-N, GCAP-II-N′, and STp(4−17) were measured at a peptide concentration of 20 nmol/mL in PBS(−) at room temperature. [Θ] is the mean residue ellipiticity: (thin line) GCAP-II-N′, (medium line) GCAP-II-N, and (thick line) STp(4−17).

GCAP-II-N′ showed a much smaller ellipticity at around 200 nm, compared to that of GCAP-II-N or STp(4−17). In a previous paper (*31*), we concluded that STp consists of three β-turn structures: type I β-turns in the N-terminal and central regions and a type II β-turn in the C-terminal portion. The large ellipticity of STp(4−17) in the far-UV region appears to reflect these β-turn structures, since reduced or reduced and carboxymethylated STp had much smaller ellipticities than STp (data not shown). Furthermore, Skelton et al. (*29*) reported that the active form of guanylin is composed of three rigid β-turn structures and that the inactive one assumes a flexible structure. Taken together, these studies provide evidence that GCAP-II-N represents the active form of GCAP-II and has a conformation different from GCAP-II-N′. GCAP-II has a much larger ellipticity at around 200 nm than that reported for guanylin (*30*). This may be due to the fact that the CD spectrum of guanylin was measured for a mixture of two topological isomers. These data also imply that GCAP-II-N contains the β-turn structure which is similar to STp.

Refolding of Reduced GCAP-II. To study the folding of GCAP-II, GCAP-II was reduced by DTT and reoxidized in the presence or absence of 2 mM GSH and 1 mM GSSG in 0.1 M Tris/HCl (pH 8.0) at 25 °C for 2 days. Four peaks

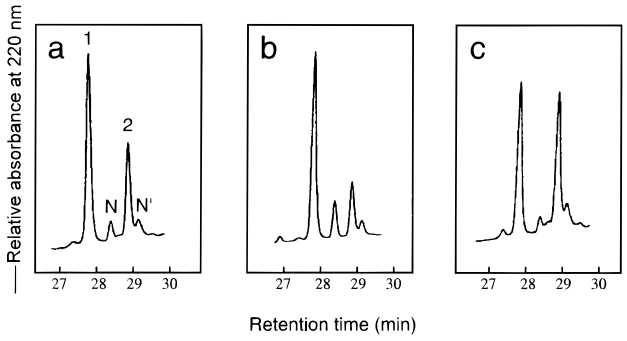

FIGURE 6: Folding of GCAP-II. GCAP-II was oxidatively folded in (a) 0.1 M Tris/HCl (pH 8.0), (b) 0.1 M Tris/HCl (pH 8.0) containing 2 mM GSH and 1 mM GSSG, and (c) 0.1 M Tris/HCl (pH 8.0) containing 6 M Gu/HCl. The 1, 2, N, and N′ represent isomers 1 and 2 and GCAP-II-N and GCAP-II-N′, respectively.

were separated by HPLC, as shown in Figure 6. Of the four peaks, two were assigned to the topological isomers (GCAP-II-N and GCAP-II-N′) of GCAP-II and the remaining two to the disulfide isomers 1 and 2 (shown later), not only by mass spectrometric and amino acid analyses but also by comparison with the HPLC retention times of authentic peptides. These peptides had relatively the same intensity of molar absorbance on HPLC, indicating that the yield of each peptide can be estimated by HPLC peak area measurements. Indeed, the summation of each peak area of the four peaks (Figure 6) was almost equivalent to that of reduced GCAP-II. Two isomers (GCAP-II-N and GCAP-II-N′) of GCAP-II were recovered as minor products regardless of the presence or absence of GSH and GSSG (6% for GCAP-II-N and 8% for GCAP-II-N′ in the absence of GSH and GSSG), while isomers 1 and 2 (disulfide isomers) with disulfide linkages different from those of GCAP-II-N and GCAP-II-N′ were the major components (56 and 30% for isomers 1 and 2, respectively). The same phenomenon was observed in the reoxidation of reduced GCAP-II in PBS(−) (data not shown). Interestingly, the ratio of GCAP-II-N to GCAP-II-N′ increased in the presence of GSH and GSSG in comparison with the ratios in their absence (Figure 6a,b), implying that GCAP-II-N is more thermodynamically stable than GCAP-II-N′. When the refolding of reduced GCAP-II was carried out in the presence of 10 mM GSH and 1 mM GSSG, isomers 1 and 2 were the major products as shown by HPLC, which was similar to that observed above (data not shown). Moreover, the folding efficiency of GCAP-II was not affected by a denaturant (6 M Gu/HCl), as shown in Figure 6c. The experiment regarding the reductive unfolding of GCAP-II in the presence of 2 mM GSH and 1 mM GSSG gave the same HPLC profile as that in Figure 6b. These results suggest that correctly folded GCAP-II is less thermodynamically stable than isomers 1 and 2 and that an additional factor(s) must be involved in the efficient folding of GCAP-II from the reduced structure to the oxidized structure.

To determine the positions of the disulfide linkages of isomers 1 and 2, we synthesized the disulfide isomers of GCAP-II, which possess non-native disulfide paring, on the basis of procedures similar to those used for the synthesis of GCAP-II. Coelution experiments on HPLC of the synthetic isomers and the isomers 1 or 2 (in Figure 6a) indicated that the disulfide linkages of isomer 1 were between Cys^{103} and Cys^{108} and between Cys^{100} and Cys^{111} and those

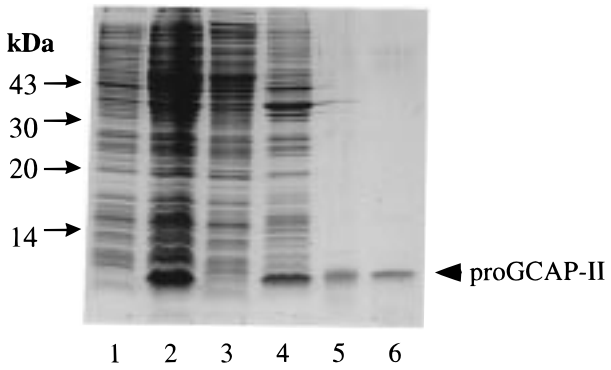

FIGURE 7: SDS−PAGE of proGCAP-II. Proteins were separated on a 15% (w/v) polyacrylamide gel and visualized by staining with Coomassie brilliant blue: lane 1, total proteins expressed in *E. coli* cells without induction by IPTG; lane 2, total proteins in *E. coli* cells after induction by IPTG; lane 3, the supernatant of the cell lysates in lane 2; lane 4, the precipitates of the cell lysates in lane 2; lane 5, the supernatant from the sulfonation and dialysis of the precipitates in lane 4; and lane 6, the supernatant in lane 5 refolded in the presence of GSH and GSSG.

of isomer 2 were between Cys^{100} and Cys^{103} and between Cys^{108} and Cys^{111}.

Expression of proGCAP-II in E. coli. To examine the effect of the pro region of proGCAP-II on the formation of disulfide linkages and the tertiary structure of GCAP-II, proGCAP-II was biosynthetically prepared by a recombinant technique. In a previous report (*30*), proguanylin was expressed as a soluble material in *E. coli* cells using the pAP vector, which carries an alkaline phosphatase promoter and a signal sequence of STII. In this study, we first employed a pIN-III-*ompA*-1 vector with the signal sequence of OmpA (*24*). However, no proGCAP-II was expressed in *E. coli* cells transformed with pIN-III-*ompA*-PU after induction by IPTG. To improve the expression efficiency of proGCAP-II, the cDNA encoding the signal sequence of OmpA and proGCAP-II was subcloned into the pET17b vector, which contains the T7 promoter. proGCAP-II was expressed as an inclusion body in *E. coli* cells and migrated at 10 kDa on SDS−PAGE (lane 4, Figure 7). Although several factors, such as temperature and the concentration of IPTG, were varied, proGCAP-II was obtained as an inclusion body in all protocols used in this experiment. These results imply that the expression rate of proGCAP-II in this system, which consists of pET17b and *E. coli*, was so rapid that proGCAP-II could not be obtained as a soluble material, but, rather, as an insoluble aggregate. Interestingly, the cells transformed with pET17b-APU died after induction of proGCAP-II and lost the ability to express proGCAP-II after a few days of transformation.

Renaturation of proGCAP-II. To solubilize proGCAP-II expressed as an insoluble material in *E. coli* cells, the protein was sulfonated according to previously described methods (*32*, *33*). proGCAP-II was recovered in a soluble fraction after dialysis against 50 mM Tris/HCl buffer (lane 5, Figure 7) and was not observed in precipitates (data not shown). The recovery of the soluble protein was dependent on the concentration of the protein in the sulfonation reaction and the dialysis. The optimal concentration was approximately 100 μg/mL under the conditions used in this experiment. The protein was then renatured in the presence of GSH and GSSG and quantitatively recovered as a soluble material from

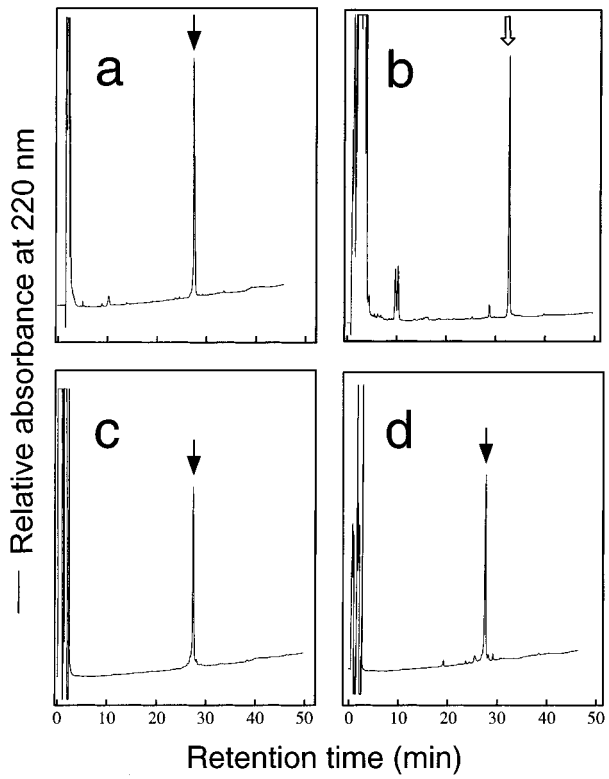

FIGURE 8: HPLC profiles of proGCAP-II. (a) The renatured proGCAP-II in lane 6 of Figure 7. (b) proGCAP-II in panel a reduced by DTT. (c) The refolding of reduced proGCAP-II from panel b in the presence of GSH and GSSG. (d) The refolding reaction of reduced proGCAP-II from panel b in the absence of GSH and GSSG. The thick and open arrows indicate proGCAP-II and reduced proGCAP-II, respectively.

an inclusion body in *E. coli*. Figures 7 and 8a show the SDS−PAGE (lane 6) and the HPLC profile of the renatured protein, respectively. The renatured proGCAP-II was shown to have the same disulfide connectivity as that in native GCAP-II as described below. Mass spectrometric, amino acid, and N-terminal sequence analyses confirmed the primary structure of the renatured proGCAP-II, as summarized in Table 1.

To avoid the influence of the sulfonation of proGCAP-II on its renaturation, the renatured proGCAP-II was again reduced and reoxidized by dialysis against 50 mM Tris/HCl buffer in the presence or absence of GSH and GSSG, as shown in Figure 8b−d. The refolded proGCAP-II's, which were identified by mass spectrometric and amino acid analyses, were recovered at 94 and 87% yields from the reduced form in the presence or absence of GSH and GSSG, respectively. Both were disulfide-bridged at the same positions as those of the sulfonated proGCAP-II (data not shown). Interestingly, proGCAP-II was correctly reconstituted from the reduced form even in the absence of GSH and GSSG. This result implies that the folding of proGCAP-II is more rapid than the formation of the disulfide linkages in proGCAP-II or that the intramolecular disulfide shuffling occurs under thermodynamic control in the folding pathway. Refolding in the presence of 6 M Gu/HCl produced isomers of proGCAP-II with largely non-native disulfide parings, which were eluted at HPLC retention times different from that of proGCAP-II (data not shown).

Determination of the Positions of the Disulfide Linkages of proGCAP-II. To determine the positions of the disulfide

Table 1: Amino Acid Composition and Mass Values of GCAP-II Analogues, proGCAP-II, and Peptide Fragments Derived from proGCAP-II by Arg-C Digestion

	GCAP-II-(2Acm)[a]	GCAP-II-N	GCAP-II-N′	proGDCAP-II	1[b]	2	3	4	5	6
Asp	3.93 (4)[c]	3.99 (4)	4.16 (4)	7.12 (7)	0.97 (1)	1.55 (1)	1.03 (1)	4.26 (4)	1.10 (1)	1.16 (1)
Ser	–	–	–	7.39 (8)	0.73 (1)	1.74 (2)	2.42 (3)	–	3.24 (4)	3.52 (4)
Glu	1.06 (1)	1.07 (1)	1.07 (1)	13.1 (13)	1.76 (2)	2.71 (1)	3.87 (4)	0.94 (1)	5.83 (6)	5.86 (6)
Thr	2.83 (3)	2.80 (3)	2.82 (3)	2.98 (3)	–	–	–	1.77 (2)	–	0.96 (1)
Gly	1.02 (1)	1.04 (1)	1.09 (1)	2.31 (2)	1.03 (1)	–	–	0.92 (1)	–	–
Ala	2.02 (2)	2.02 (2)	2.05 (2)	9.91 (10)	1.00 (1)	2.19 (2)	1.94 (2)	1.91 (2)	4.91 (5)	5.02 (5)
Cys	2.88 (4)	3.30 (4)	3.46 (4)	5.20 (6)	–	–	–	3.21 (4)	1.50 (2)	1.42 (2)
Val	1.93 (2)	2.02 (2)	2.04 (2)	5.83 (6)	0.97 (1)	–	1.02 (1)	1.86 (2)	1.92 (2)	1.89 (2)
Met	–	–	–	0.80 (1)	–	–	0.98 (1)	–	–	–
Ile	0.98 (1)	1.01 (1)	1.03 (1)	2.92 (3)	0.99 (1)	–	–	1.03 (1)	0.97 (1)	1.05 (1)
Leu	3.00 (3)	3.00 (3)	3.00 (3)	11.0 (11)	–	2.00 (2)	3.00 (3)	2.00 (2)	5.00 (5)	6.00)6)
Phe	1.05 (1)	1.03 (1)	1.09 (1)	2.06 (2)	1.13 (1)	–	–	–	1.25 (1)	1.41 (1)
Tyr	–	–	–	1.53 (2)	0.82 (1)	–	–	–	–	–
Lys	3.00 (3)	1.05 (1)	1.07 (1)	2.93 (3)	–	0.88 (1)	1.93 (2)	–	1.00 (1)	0.91 (1)
His	–	–	–	2.10 (2)	–	–	–	–	2.01 (2)	2.00 (2)
Arg	0.94 (1)	0.99 (1)	0.96 (1)	2.86 (3)	0.90 (1)	1.05 (1)	0.94 (1)	–	–	0.90 (1)
Pro	–	–	–	6.71 (60	–	2.48 (2)	2.59 (2)	–	3.71 (4)	4.21 (4)
Trp[d]	–	–	–	–	–	–	–	–	–	–
m/z										
observed[e]	2742.5	2598.5	2598.5	9756.6[g]	1445.7	1597.8	2413.9	1953.0	3627.2	3995.1
calculated[f]	2742.3	2598.2	2598.2	9755.1	1445.6	1597.8	2413.8	1953.2	3626.2	3996.2

[a] GCAP-II-(2Acm) represents [Cys[103](Acm),Cys[111](Acm)]GCAP-II as shown in Figure 2. [b] Peptide number in Figure 9a. [c] Values in parentheses calculated from the sequence. [d] Not determined. [e] The data represent the monoisotopic mass values, obtained by MALDI-MS. [f] Values were calculated from amino acid sequence data. [g] Measured by ESI-MS.

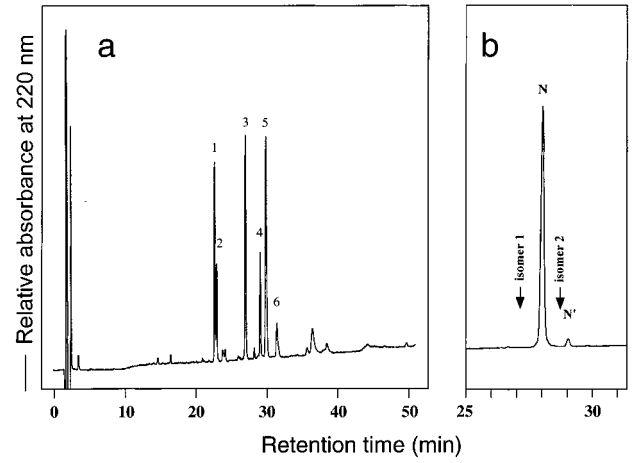

FIGURE 9: HPLC profiles of (a) proGCAP-II digested with endoproteinase Arg-C and (b) the digest treated with a trypsin agarose column. N and N′ represent the N and N′ forms of Thr-Ile-Ala-uroguanylin, respectively. The retention times of isomers 1 and 2 (disulfide isomers) of Thr-Ile-Ala-uroguanylin are indicated.

pairing in proGCAP-II, the renatured protein was digested with endoproteinase Arg-C and the digest subjected to HPLC, as shown in Figure 9a. Six peptide fragments covering the entire sequence of proGCAP-II were identified by mass spectrometric and amino acid analyses, as summarized in Table 1. When the digest was mixed with a trypsin agarose, the C-terminal fragment, corresponding to Thr-Ile-Ala-uroguanylin, remained in solution, while the other peptide fragments, which contain an Arg residue at their C termini, were trapped on the agarose. Similarly, GCAP-II-N was digested with endoproteinase Arg-C to release the N form of Thr-Ile-Ala-uroguanylin. The C-terminal fragment of proGCAP-II was eluted at the same retention time on HPLC as that from GCAP-II-N, along with a small amount of the N′ form of Thr-Ile-Ala-uroguanylin, while no other isomers were detected, as shown in Figure 9b. The recovery of the C-terminal fragment (the N form) was 71% from proGCAP-II. These results clearly show that proGCAP-II is disulfide-bridged at the same positions as GCAP-II.

To determine if proGCAP-II comprises only the N form of GCAP-II or both the N and N′ forms, we estimated the interconversion of the N forms of GCAP-II-N and of Thr-Ile-Ala-uroguanylin to their N′ forms under the same conditions that were used for the endoproteinase Arg-C digestion of proGCAP-II. The digest of proGCAP-II yielded both the N and N′ forms of Thr-Ile-Ala-uroguanylin in a ratio of 98:2, as shown in Figure 9b, as well as those of the N forms of GCAP-II-N and Thr-Ile-Ala-uroguanylin, as described above. The result indicates that the N′ form of Thr-Ile-Ala-uroguanylin in the digest of proGCAP-II was produced during the enzymatic treatment of proGCAP-II and that proGCAP-II contains only the N form (GCAP-II-N, biologically active form) of GCAP-II.

DISCUSSION

The role of a peptide in the pro-leader sequence (amino acid residues 27−88) of proGCAP-II in the formation of both disulfide linkages and tertiary structure of the mature GCAP-II was investigated by examining the in vitro folding of GCAP-II, a circulating form of uroguanylin in human blood, and its precursor proGCAP-II. The choice of GCAP-II for this experiment is explained as follows. (i) GCAP-II is a mature form of proGCAP-II in vivo (*11*, *12*). (ii) The processing of proGCAP-II to yield GCAP-II has been extensively studied (*12*). (iii) GCAP-II exists in human plasma as two topological isomers (one is biologically active and the other biologically inactive) (*27*). (iv) The inactive isomer constitutes approximately 25% of the total GCAP-II in human plasma (*27*). (v) GCAP-II carries only one type of disulfide connectivity, which is important for the expression of the biological activity of the peptides, while GCAP-II having different modes of disulfide linkages has, thus far, not been isolated. Moreover, GCAP-II and proGCAP-II give a peptide having the same sequence (amino acid residues

94−112) located in the C-terminal region of proGCAP-II by treatment with endoproteinase Arg-C, enabling us to easily relate the positions of the disulfide linkages of GCAP-II to those of proGCAPG-II. In addition, an NMR study of guanylin, which is homologous in primary structure to GCAP-II and composed of equal amounts of two topological isomers, showed that the biologically active isomer is stabilized much more strongly by intramolecular hydrogen bonds than the inactive isomer and that the former has a conformation different from the latter but similar to that of STp (*29*). These data allowed us to consider the reason that not only the active form of GCAP-II, but also the inactive form, can be generated during the processing of proGCAP-II in human plasma and how the peptide is folded from the precursor protein, proGCAP-II, to form the correct tertiary structure and disulfide linkages.

Various factors affect the construction of the tertiary structure of proteins during oxidative folding and the formation of disulfide bonds. One of the most important factors in in vivo folding is the environment composed of GSH and GSSG, which maintain an appropriate redox voltage in vivo and, thus, play a beneficial role in the formation of disulfide bonds. Indeed, ω-conotoxin is folded from the reduced form with greatly improved recovery in the presence of GSH and GSSG, although ω-conotoxin with disulfide bridges different from the native disulfide bonds is still produced (*34*, *35*). However, GCAP-II is reconstituted as a minor product and GCAP-II with disulfide pairings different from the native one is predominantly formed from reduced GCAP-II regardless of the presence or absence of GSH and GSSG. The oxidative folding and reductive unfolding experiments of GCAP-II were carried out in the presence of 2 mM GSH and 1 mM GSSG for 2 days at room temperature, both yielding the same ratio of isomers 1 and 2 (disulfide isomers) (oxidative folding is shown in Figure 6). Generally, a disulfide-coupled folding of peptides is completed within a few hours under these conditions, as seen in the case of ω-conotoxin (*34*). Thus, the thermodynamic stability of the disulfide isomers of GCAP-II might be estimated on the basis of the relative abundance of its disulfide isomers in Figure 6. Furthermore, a strong denaturant such as Gu/HCl seems to have a negligible effect on the relative recovery of GCAP-II isomers (Figure 6), suggesting that the folding and the formation of the disulfide bonds of GCAP-II are largely dependent on either steric constraints induced by the peptide chain or the distances between the Cys residues. It is, however, true that the ratio of the two topological isomers of GCAP-II, GCAP-II-N to GCAP-II-N′, increases in the presence of GSH and GSSG rather than in their absence, reflecting the relative differences in the stabilities of GCAP-II-N and GCAP-II-N′. This is an indication of the possibility that noncovalent interactions are partially attributed to the correct folding of GCAP-II. The ratio (1:6) of GCAP-II-N to isomer 1 (disulfide isomer) in the presence of GSH and GSSG (Figure 6b) represents only a marginal stability difference (∼1 kcal/mol) between GCAP-II-N and isomer 1, although it is unlikely that GCAP-II-N is at the thermodynamic ground state. This free energy difference is small but strongly responsible for the folding of GCAP-II. These results show that GCAP-II does not carry sufficient information to permit correct folding and disulfide paring and further suggest that an additional factor(s) such as an intra- or intermolecular chaperone is necessary for the formation of the tertiary structure or disulfide linkages in GCAP-II.

Proteins are folded according to their own amino acid sequences, and their folded native structures are thought to be at the thermodynamic ground states (*36*). Recent studies regarding insulin-like growth factor I suggested that this may not be the case but that they show "non-Anfinsen" folding behavior (*18*, *37*). The production of disulfide-scrambled or aggregated insulin-like growth factor I in the in vitro folding was explained by the existence of thermodynamically unfavorable disulfide bonds in the native structure (*38*). In this study, we demonstrated that the mature peptide (GCAP-II) is unlikely to be at the thermodynamic ground state, suggesting that GCAP-II may behave in obedience to non-Anfinsen folding. Although neither the folding kinetics nor intermediates produced during the folding have yet been determined for the case of GCAP-II, the apparent distribution of the native form and the disulfide isomers in the folding mixture is not affected by the presence of not only GSH and GSSG but also a strong denaturant such as 6 M Gu/HCl. This implies that the rate of exchange of the disulfide bond of the native form with glutathione is not different from that of the disulfide isomers. Thus, we conclude that GCAP-II itself essentially lacks the thermodynamic or kinetic factors required for folding to the native conformation.

Recently, several lines of evidence have been reported which support the hypothesis that a propeptide of the pro-leader sequence of proteins plays a role in the folding of mature products (*14*, *16*−*20*). For example, the propeptide of subtilisin functions as an intramolecular chaperone in the folding of the domain of subtilisin (*19*). Noncovalent interactions between this propeptide and domain are thought to diminish the activation energy in each step along the folding pathway and to stabilize the rate-determining transition states (*17*, *39*, *40*), because the denatured protein appears to be trapped in intermediate states and to rapidly fold to the native protein after the addition of the propeptide. Additionally, BPTI, the folding process of which has been studied in detail, is able to spontaneously achieve the correct disulfide bonds. However, the propeptide of BPTI kinetically accelerates the folding of the protein through a different pathway in the precursor protein (*14*). These provide a classical model for protein folding based on Anfinsen's concept. The thermodynamic role of the pro region (peptide ligand) of neurophysin in its folding has recently been investigated in detail by Deeb and Breslow (*20*). They observed that the efficiency of the disulfide-coupled folding of native neurophysin was low in the absence of the ligand but reached 100% in the presence of the ligand. They also observed the lack of an intermolecular kinetic effect of the peptide ligand on the folding of neurophysin and concluded that the native conformation of neurophysin represents a trapped metastable state and that the propeptide functions in the stabilization of the native conformation of neurophysin during folding. The propeptide of proGCAP-II might have the same effect as the peptide ligand on neurophysin, since the native conformation of GCAP-II is unlikely to be at the lowest-free energy state but appears to be kinetically trapped in proGCAP-II. Furthermore, the experiment regarding the folding of proGCAP-II (see Materials and Methods) showed that the folding efficiency of the native molecules is

unchanged even after 1 week in the presence of GSH and GSSG. Thus, we speculate that the propeptide of proGCAP-II plays a role in the stabilization of the native conformation of GCAP-II during folding. It might follow from this speculation that the folding mechanism of proGCAP-II, in which the N-terminal pro region is fused to the mature peptide, is consistent with the Anfinsen concept.

In this study, in vitro renaturation showed that proGCAP-II is, in fact, folded to a single species of the protein, which carries only one disulfide pairing at the same positions as that of the mature form, GCAP-II. Moreover, proGCAP-II comprises the only active isomer (GCAP-II-N) of GCAP-II and not the inactive isomer (GCAP-II-N′). The active isomer can be converted to the inactive isomer during circulation in human blood. Indeed, two topological isomers (GCAP-II-N and GCAP-II-N′, active and inactive isomers, respectively) of GCAP-II were detected in human blood (*27*). The inactive GCAP-II seems likely to be an artificial product, produced after the processing of proGCAP-II. However, the possibility that the inactive isomer does not take part in a signal transduction system through GC-C but, rather, targets another receptor protein cannot be excluded. We also expressed proGCAP-II in 293T mammalian cells using pEX2, which consists of pcDNA3 (Invitrogen) and the cDNA encoding pre-proGCAP-II. proGCAP-II was secreted into the culture medium as a single protein with the same disulfide linkages as those of GCAP-II (data not shown). These results suggest that proGCAP-II can be spontaneously folded not only in vitro but also in vivo in the same manner.

To obtain large quantities of proGCAP-II for the folding experiment and the structural and biochemical analyses, we attempted to produce proGCAP-II as a soluble material under various conditions using *E. coli*. Although we were not able to obtain proGCAP-II as a soluble fraction, it was expressed as an inclusion body in *E. coli* cells transformed with pET17b-APU, which was constituted from a commercially available pET vector under the control of strong bacteriophage T7 transcription and translation signals. Garcia et al. (*30*) reported that proguanylin was expressed as a soluble material using a pAK19 vector. The pAK19 expression vector might have an advantage in that the induction occurs at a high cell density and the protein is expressed gradually so that the protein is correctly transported to the periplasm and folded. S-Sulfonation has been employed for increasing the solubility of various recombinant proteins, including proinsulin (*32*, *33*, *41*). This method has merit in that the *S*-sulfonate group is easily removed by thiol reagents, such as reduced glutathione. S-Sulfonation of aggregated and insoluble proGCAP-II dramatically increased the solubility of proGCAP-II. proGCAP-II was easily refolded from sulfonated proGCAP-II. Since proGCAP-II possesses only two Cys residues in the pro-leader sequence, the sulfonated propeptide appears to have a tertiary structure similar to the folded structure of the final form, while the portion of GCAP-II is assumed to be completely unfolded by S-sulfonation of the four Cys residues. Therefore, the folding process of proGCAP-II may be explained by studying the conformations of proGCAP-II and sulfonated proGCAP-II. This study is underway in our laboratory.

In conclusion, the results presented here indicate that the mature form of GCAP-II does not possess sufficient information to allow for its correct folding and that the propeptide of proGCAP-II aids in its folding, yielding only the bioactive form. The result implies that a propeptide in the pro-leader sequence of proGCAP-II functions as an intramolecular chaperone in the folding of proGCAP-II. To our knowledge, this is the first report demonstrating that the N-terminal propeptide facilitates the folding of a peptide hormone, suggesting the possibility that the propeptide in the pro-leader sequence plays a role in the folding of small peptides during their biosynthesis.

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