

Role of Pro-leader Sequence of *de novo* Designed-Disulfide Hybrid Peptides

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Introduction

Peptide hormones are produced *in vivo* in the form of precursor proteins (prohormones) with pro-leader sequences and subsequently processed into a mature form. However, little is known concerning the role of the pro-sequence in precursor proteins. There are a few examples, such as subtilisin, of the role of the propeptide region. The pro-region of those proteins aids the mature proteins in the proper assembly as intra-molecular chaperones. In this decade, it has been established that the pro-region of peptide hormones also plays a role in the correct folding of the mature hormone as an intra-molecular chaperone.

Guanylin and uroguanylin, endogenous ligands of particulate guanylyl cyclase C (GC-C), are thought to function in regulating the level of cGMP as a second messenger in intestinal and kidney cells, resulting in the regulation of chloride and water secretion from the inside of cells to the outside. We previously reported that the pro-peptide region of uroguanylin functions as an intramolecular chaperone in the proper folding of the mature peptide, uroguanylin, and that the tertiary structure of the mature form is kinetically trapped by the pro-peptide region [1,2]. In this report, we explored the kinetically trapped structure of the pro-peptide peptide for possible drug design using the function of the intra-molecular chaperone.

The core region of heat-stable enterotoxin (STh), an exogenous ligand of GC-C, consists of 13 amino acid residues and possesses a 50% homology to uroguanylin. In addition, STh possesses three intra-molecular disulfide bonds, in which two disulfide bonds are localized at relatively same positions as that in uroguanylin (Fig. 1A), and simultaneously folds into the native form without the pro-region. The disulfide pairings of uroguanylin and STh play a critical role in the expression of biological activity. However, little is known concerning between the disulfide pairings and pro-region. Therefore, two disulfide hybrid peptides were *de novo* designed as hybrid peptide 1 (NDDCCELACNVACTGAL) and hybrid peptide 2 (NDCAELCCNVAATGCL) to investigate whether the pro-peptide region of uroguanylin is able to kinetically trap the designed hybrid peptides (Fig. 1A).

Results and Discussion

The hybrid peptides were chemically synthesized by Boc solid phase method. Pro-hybrid proteins 1 and 2, in which the pro-peptide region of uroguanylin was fused to the N-termini of the hybrid peptides 1 and 2 (Fig. 1B), were prepared by *E. coli* expression system using the pET17b

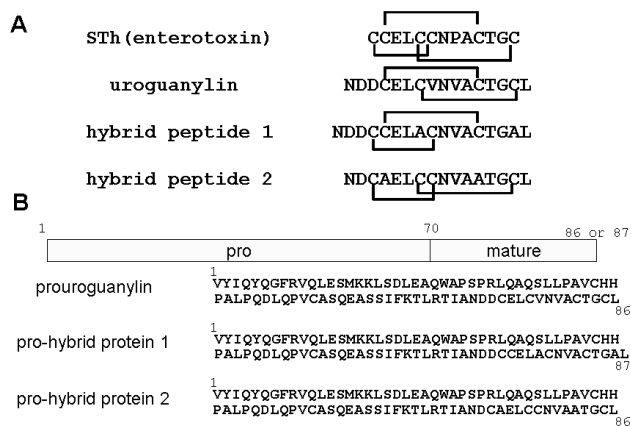


Fig. 1. (A) Amino acid sequences of the core region of STh, uroguanylin, hybrid peptide 1, and hybrid peptide 2. (B) Primary structures of prouroguanylin, pro-hybrid protein 1, and pro-hybrid protein 2. The disulfide linkages in STh, uroguanylin, hybrid peptide 1, and hybrid peptide 2 are shown by solid lines. Pro at the box in B represents the pro-leader sequence of uroguanylin.

vector. The proteins were purified by RP-HPLC, then renatured by the dilution method [1,2].

In Vitro Folding of the hybrids peptide 1 and 2—Interestingly, hybrid peptide 1 has the binding activity of GC-C, while hybrid peptide 2 does not (data not shown).

In order to estimate folding reaction of the hybrid peptides 1 and 2, the peptides 1 and 2 were folded by the dilution method in the presence of 2 mM GSH and 1 mM GSSG at room temperature. The folding products were analyzed by RP-HPLC and MALDI-TOF/MS. The folding reaction of the hybrid peptide 1 yielded three products consisting of the designed peptide, a topological isomer and disulfide isomer (peak 1, 2, and 3 in Fig. 2A, respectively), confirmed by MALDI-TOF/MS. Interestingly, only the *de novo* designed-peptide possessed the GC-C activating ability. These results indicate that the mature sequence of the hybrid peptide 1 does not carry enough information to achieve the correct disulfide bonds.

On the other hand, folding products of hybrid peptide 2 included the designed form and disulfide isomer (products ratio, wild type : disulfide isomer = 3 : 1), as shown in Fig. 2B. These results indicate that these *de novo* designed peptides are unable to completely achieve correct folding. In addition, the hybrid peptide 2 did not show the GC-C activating ability.

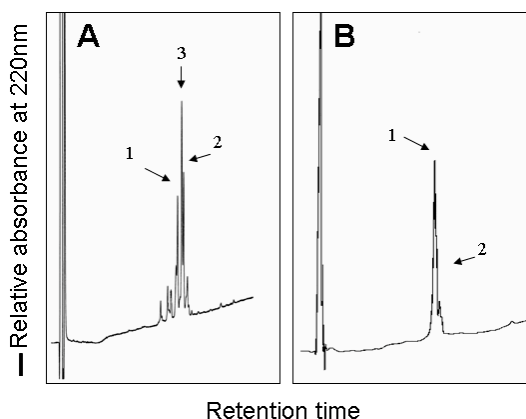


Fig. 2. (A) RP-HPLC analysis of the folding of hybrid peptide 1 (1: wild type, 2: topological isomer, 3: disulfide isomer). (B) RP-HPLC analysis of the folding of hybrid peptide 2 (1: wild type, 2: disulfide isomer).

In Vitro Folding of the pro-hybrid proteins 1 and 2- In order to estimate the intra-molecular chaperone function of the propeptide region of uroguanylin for the folding of the hybrid peptides 1 and 2, pro-hybrid proteins 1 and 2 were prepared by *E. coli* expression system. After purification of the pro-hybrid peptides, folding reactions were performed by the dilution method in the presence of 2 mM GSH and 1 mM GSSG.

In the folding reaction of pro-hybrid protein 1 (three disulfide linkages), an intermediate with two disulfide bonds (2SH in Fig. 3A) were specifically produced at the first stage of the folding. These disulfide bonds may be localized at the mature region, hybrid peptide 1. Then, the folding reaction was completed after the formation of the third disulfide bond, which may be localized in the propeptide region. This result indicates that the production of the misfolding protein is not significant for the folding and the formation of the disulfide bond in the propeptide region is the rate determining step, including the disulfide shuffling reaction. On the HPLC profile, only one folding intermediate (2SH in Fig. 3A) was observed, but it was not clear that the peak-labeled with 2SH in Fig. 2 consisted of single protein. It is still possible that several intermediates were eluted at the same retention time on the HPLC profile. In addition, the folding pathway of the pro-hybrid protein 1 seems to be different from that of prouroguanylin (data not shown) since many intermediates were observed in the folding of prouroguanylin.

In the folding of pro-hybrid protein 2 (contained three disulfide linkages), an intermediate with two disulfide bonds (2SH in Fig. 3B) were specifically produced at first stage, and these disulfide bonds may be localized at the mature region. Then, the folding completes after the formation of the disulfide bond in the propeptide region. This result indicates that the production of the misfolding protein is not significant and the disulfide shuffling occurs very quickly, resulting in the production of one intermediate. In this aspect, the folding pathway of the

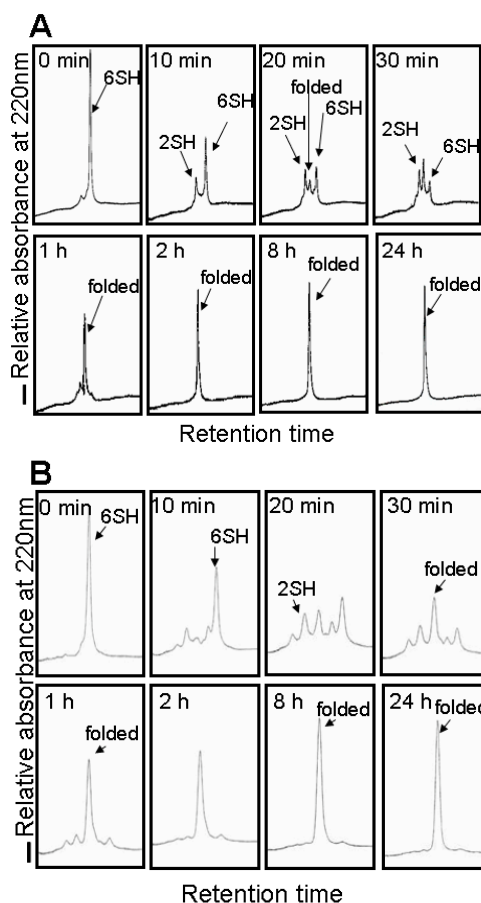


Fig. 3. (A) RP-HPLC profiles of the folding reaction of pro-hybrid protein 1 (6SH : 0 disulfide linkage, 2SH : 2 disulfide linkages, folded : 3 disulfide linkages). (B) RP-HPLC profiles of the folding reaction of pro-hybrid protein 2 (6SH : 0 disulfide linkage, 2SH : 2 disulfide linkages, folded : 3 disulfide linkages).

pro-hybrid protein 2 is similar to that of pro-hybrid 2. Thus, compared with the refolding of hybrid peptide 1 and 2, the pro-peptides of the pro-hybrid 1 and 2 function as an intra-molecular chaperone to the objective conformation of de novo designed peptides.

Conclusion

The pro-region of the hybrid proteins 1 and 2, as well as prouroguanylin, recognized the mature region, however the folding pathway of the hybrid proteins 1 and 2 are a little different from that of prouroguanylin.

References

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2. Yuji, H., Chisei, S., Megumu, O., Nobuaki, O., Kunt, A., Wolf-Georg, F., and Yasutsugu, S., (2000) *J. Biol. Chem.*, **275**, 25155-25162.