Prosequence-mediated disulfide coupled folding of the peptide hormones guanylin and uroguanylin

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Abstract
In contrast to their prohormones the mature peptide hormones guanylin and uroguanylin are not able to fold to their native disulfide connectivities upon oxidative folding. Structural properties of both peptide hormones and their precursor proteins as well as the role of their prosequences in proper disulfide coupled folding are reviewed. In addition, the structural behavior of a proguanylin mutant that closely resembles prouroguanylin has been investigated to gain further insight into structural properties of this homologous precursor protein.

Keywords: Guanylin, uroguanylin, peptide hormone, proguanylin, prouroguanylin, prosequence, folding, disulfide bonds.

Introduction
There is a strong correlation between the three-dimensional structure - the fold - of a protein and its biological activity. Therefore, understanding of protein folding to reach the protein's native state is an important branch of structural biology and still represents a challenge in this discipline. In his pioneering work in the early 1970s Anfinsen has shown that the three-dimensional structure of a protein is determined by its amino acid sequence [1] and therefore sequence based structure prediction should be possible. However, diseases associated with protein misfolding demonstrate the existence of more than one folded (but not native) state of a protein [2]. Another complicacy concerning the relation between the primary sequence and the three-dimensional structure of a protein is the existence of so-called intramolecular chaperons. Usually, these intramolecular chaperons are
covalently attached NH$_2$-terminal propeptides that are assumed to help an intermediate folded state to overcome a kinetic or thermodynamic barrier on the folding pathway [3]. Many proteins are produced with an NH$_2$-terminal propeptide sequence that is located between the signal peptide and the mature protein. These prosequences usually play an important role in intracellular trafficking and secretion of the corresponding mature protein. In some cases, however, they are also essential for mediating proper folding of their corresponding proteins [4]. Propeptide mediated protein folding was initially demonstrated for proteinases such as subtilisin [5] and α-lytic protease [6]. The prosequence mediated folding behavior of these two proteins has been studied extensively [3]. Other proteins that contain prosequences which are crucial for protein folding are for example growth factors [7,8] and peptide hormones. Examples for the latter are the prohormones proguanylin [9] and prouroguanylin [10,11]. These two proteins represent special cases compared to other proteins containing prosequences with intramolecular chaperon-like features as their prosequences are about four to five times longer than the mature hormones guanylin and uroguanylin. Structural properties of these two peptide hormones and their precursor proteins as well as analysis of mutational data that lead to a better understanding of the role of these prosequences in proper disulfide coupled folding are reviewed in the present article. Furthermore, we have investigated the structural behavior of a proguanylin mutant that closely resembles prouroguanylin in order to gain further insight into the structural properties of this homologous protein.

**Guanylin and Uroguanylin exhibit a unique topological isomerism**

Guanylin and uroguanylin are guanylyl cyclase C (GC-C) activating peptide hormones of 15 (guanylin) respectively 16 or 24 (uroguanylin) amino acid residues. The different uroguanylin peptides found in urine (16 residues) [12,13] and in blood ultra filtrate (24 residues) [14,15] correspond to different products of prouroguanylin processing in the kidney and in circulation. Both peptide hormones contain four cysteine residues that have to be connected in a 1-3/2-4 pattern (Fig. 1 A) to produce biological activity [16,17]. In addition to their high sequence similarity (Fig. 1 A) and similar GC-C activating potencies [17], guanylin and uroguanylin fold into similar three-dimensional structures [18]: The 1-3/2-4 disulfide bond pattern of both peptide hormones located within a short sequence stretch of only 12 amino acid residues leads to the unique existence of two topological isoforms of each peptide as demonstrated by HPLC chromatography and NMR spectroscopy [17-21]. Although both isomers exhibit the same cysteine connectivity, they differ in their three-dimensional conformations, referred to as A- and B-isomers, with only one of them - the A-isomer - showing biological activity [18,20]. Even though an equilibrium of interconversion prevents separation of the guanylin isomers, NMR analysis revealed two conformationally different species present in a 1:1 ratio [17,19]. In contrast, the topological isomers of uroguanylin can be separated by HPLC allowing discrete structural and biochemical characterization [18]. The structures of the respective guanylin and uroguanylin A-isomers can be described as a right-handed spiral stabilized by the two 1-3/2-4 disulfide bonds, respectively, whereas the structures of the B-form can be depicted as a distorted left-handed spiral (Fig. 2). Apart from reverse turns, the
structures of both isomers do not show any elements of regular secondary structure [18,19]. Comparison of the structures of the respective uroguanylin [18] and guanylin isoforms [19] confirms the high structural similarity between these peptides with backbone RMSD values for the cysteine rich region of the respective average structures of 1.4 Å for the A-isomers and 1.5 Å for the B-isomers. As mentioned above, only the A-isomers are able to activate GC-C [18,20], and their structures closely resemble that of the agonistic E. coli heat-stable enterotoxin STa [22].

**Oxidative folding of guanylin and uroguanylin requires the respective prosequences**

Both peptide hormones are secreted as the corresponding biologically inactive prohormones of 94 (proguanylin) and 86 (prouroguanylin) amino acid residues with the mature hormones located at the very COOH-termini (Fig. 1 B) [15,23-26]. Oxidative folding of reduced 15-residue guanylin under various refolding conditions predominantly results in formation of the two inactive disulfide isomers with only 5 to 10% showing the native disulfide connectivities [9,16]. Although the isomer with the native 1-3/2-4 disulfide pattern was slightly preferred using a Cys/Cys2-containing refolding buffer, the ratio of the guanylin isomers could not be shifted towards the native isomer by varying temperature, buffer, or redox conditions [9]. Similar observations have been made for uroguanylin-24 for which the native disulfide pairing also was the minor product, independent of the redox conditions used [10]. These results suggested that correctly folded guanylin and uroguanylin possessing the native disulfide connectivities are thermodynamically less stable than the two non-native disulfide isomers, implying additional factors to facilitate correct disulfide coupled folding of both peptide hormones. As the reduced guanylin and uroguanylin precursor proteins containing the entire prosequences are able to predominantly fold to products with native cysteine connectivity under optimized refolding conditions [9,10], these factors have been identified to be the respective prosequences.

Proguanylin lacking the first 31 NH2-terminal residues of the prosequence, however, almost exclusively folds to products with non-native disulfide connectivities even though the same redox conditions were used as for correct refolding of proguanylin [9]. From this experiment it was evident that the NH2-terminal region of proguanylin significantly contributes to correct disulfide-coupled folding. This crucial role of the NH2-terminal residues of the prosequence for proper folding was also demonstrated for the closely related uroguanylin-prohormone [10,11] of which various NH2-terminally truncated mutants have been produced to further identify amino acid residues of the prosequence that are involved in disulfide coupled folding of this precursor protein [11]. Both in vivo and in vitro refolding experiments performed with these mutants revealed one or two residues at the very NH2-terminus of prouroguanylin to be critical for correct disulfide bond formation. For in vitro studies, five mutant proteins of prouroguanylin have been analyzed in which amino acid residues were sequentially deleted from the NH2-terminus but contained an additional NH2-terminally methionine residue. Met1-prouroguanylin(2-86) predominantly exhibited the native disulfide connectivity in the peptide hormone region. For the mutant proteins Met2-prouroguanylin(3-86) and Met3-prouroguanylin(4-86), however, the native isomer only made up 1/3 respectively 1/9 of the product, suggesting that deletion of the very first residues of prouroguanylin greatly affects the construction of the native tertiary
structure of mature uroguanylin. This suggestion was confirmed by the mutant Met\textsuperscript{12}-prouroguanylin(3-86) in which the first two residues are substituted by methionine, therefore containing the same number of residues as the wildtype protein. For this mutant protein, predominantly uroguanylin with the native fold was obtained after oxidative folding [11], implying rather important backbone/backbone interactions than a role of the side chain of Tyr2 during the folding process. Indeed, Lauber \textit{et al.} [27] have shown that the very NH\textsubscript{2}-terminal part of the prosequence and the hormone region of the closely related guanylin prohormone interact \textit{via} backbone/backbone interactions with each other (see below). The same kind of interactions can also be assumed for prouroguanylin as discussed below. Furthermore, for \textit{in vivo} studies Hidaka \textit{et al.} [11] have expressed wildtype prouroguanylin as well as three prouroguanylin mutants lacking the first 6, 11, or 17 residues in human embryonic kidney 293T cells. Wildtype prouroguanylin was expressed as a monomeric protein showing the correct disulfide pattern within the hormone region. In contrast, prouroguanylin(7-86), prouroguanylin(12-86), and prouroguanylin(18-86) were expressed as dimers covalently linked \textit{via} intermolecular disulfide bonds [11]. These findings together with the unusual elution behavior of both proguanylin and prouroguanylin in size exclusion chromatography led Hidaka \textit{et al.} [11] to the conclusion that both proguanylin as well as prouroguanylin form stable non-covalently bound homodimers in solution and that dimerization would also play a role in the disulfide dependent folding pathway of both precursor proteins. At least for proguanylin, this assumption, however, could be contested by extensive analytical ultracentrifugation studies and NMR diffusion measurements at various concentrations [9,28].

Taken together, the prosequences of both guanylin and uroguanylin, particularly their NH\textsubscript{2}-terminal residues play a crucial role in the oxidative folding of both proteins and can be considered as intramolecular chaperones.

**Oligomerization state of proguanylin and prouroguanylin**

From their unusual elution behavior in size exclusion chromatography prouroguanylin and proguanylin have been assumed to be dimers in solution [11]. These results, however, contradicted former suggestions of proguanylin being monomeric in solution [9]. As structure determination of proguanylin required unequivocal knowledge of the association state of this protein, accurate determination of the oligomerization state was carried out by extensive biophysical studies on both natural and recombinant proguanylin [28]. Even though recombinant proguanylin as produced by Lauber \textit{et al.} [28] possesses two additional amino acid residues at its NH\textsubscript{2}-terminus, recombinant and natural proguanylin feature identical biophysical properties as deduced from capillary zone electrophoresis, HPLC, and mass spectrometry. Identical structural properties were also confirmed by CD and NMR spectroscopy. From analytical ultracentrifugation studies applying both sedimentation equilibrium and sedimentation velocity measurements, natural as well as recombinant proguanylin have been shown to be monomeric in solution for protein concentrations up to the millimolar range [28]. These results together with the data from NMR diffusion measurements [9] verified that proguanylin is monomeric even at the concentration of 2.4 mM [28].

Comparison of the monomeric proguanylin with prouroguanylin reveals that both proteins exhibit a sequence identity of 35 % and show almost identical predicted secondary structures (Fig. 1 B). Main differences are
found in the central part of the proteins with the most striking difference being a deletion of 7 amino acid residues within this region whereas the terminal amino acid residues (1-22 and the last 30 residues) are quite similar. This deletion mainly “aligns” with residues that are unstructured and flexible in the proguanylin structure (Fig. 1 B, Fig. 3 A). Since for proguanylin the predicted secondary structure closely resembles the experimentally determined one, the same can be assumed for prouroguanylin. Additionally, it was shown for prouroguanylin that the hormone part exclusively adopts the A-form topology after digestion of the prohormone and release of the mature hormone region [10], which is the same as observed in the proguanylin structure (Fig. 3 B) [27]. Based on these findings it has been suggested that prouroguanylin adopts a fold similar to that of proguanylin [27], allowing comparative modelling of the prouroguanylin structure (Fig. 3 C). Besides this striking similarity of both precursor proteins on a structural level, prouroguanylin also likely exists as a monomer in solution. This assumption is supported by biophysical analysis of a proguanylin mutant that lacks residues Arg28 to Pro37 (Fig. 1 B) resulting in a sequence identity to prouroguanylin of 38.6 %. Like wildtype proguanylin, this protein - proguanylin-Δ(28-37) - is exclusively monomeric in solution, as shown by analytical ultracentrifugation at protein concentrations of 450 and 900 µM [29]. Also, as confirmed by NMR spectroscopy and comparison with wildtype proguanylin, the core structure of proguanylin containing all elements of regular secondary structure is not influenced upon this deletion of residues 28 to 37. The same applies for the formation of the native disulfide bonds [29].

**The three-dimensional structure of proguanylin and its implications for folding**

The recent determination of the solution structure of proguanylin [27] revealed a globularly folded protein composed of a three helix bundle and a small triple-stranded antiparallel β-sheet of two NH$_2$-terminal strands and one strand of the very COOH-terminus (Fig. 3 A). The loop region connecting the first two helices (residues 22 to 45; Fig. 3 A) exhibits a significantly increased inherent flexibility on a pico- to nanosecond time scale [27] and neither has an impact on the folding of the proguanylin core structure comprised of all elements of regular secondary structure nor on disulfide coupled folding of this precursor protein as proven by the analysis of proguanylin-Δ(28-37) described above.

As mentioned earlier, the prohormone itself is almost inactive with respect to GC-C activation [9,28,30]. This is despite the fact that the sequence corresponding to mature guanylin (residues Pro80 to Cys94) is fixed in its bioactive A-isomer topology (Fig. 3 B) [27]. With a more detailed analysis of the proguanylin structure, however, this issue can be explained as follows: in addition to β-sheet formation with the NH$_2$ terminus of the protein, the hormone region is involved in numerous contacts to the NH$_2$-terminal β-strand in that it partly wraps around the first four residues of the prosequence (Fig. 4) [27]. On the one hand, these interactions stabilize the A-isomer topology of the hormone region and sterically prevent a B-form like conformation, on the other hand, they lead to shielding of part of the hormone surface (Fig. 4) that is therefore not accessible for interactions with GC-C, explaining the significantly decreased receptor binding affinity [27]. Furthermore, the same interactions provide an explanation for the important role of the first NH$_2$-terminal residues in the disulfide coupled folding of proguanylin. As outlined above, disulfide coupled folding of the
homologous guanylin and uroguanylin precursor proteins requires the NH$_2$-terminal residues of the respective prosequence of which the first few residues are of particular importance [9,11]. This is underlined by the fact that all NH$_2$-terminally truncated variants of both proguanylin and prouroguanylin exhibit non-native disulfide connectivities. As known from the three-dimensional structure all truncations affect the central strand of the triple-stranded β-sheet and thus the interactions between the termini [27]. These results show that the NH$_2$-terminal residues of the prosequence are essential for and involved in the correct assembly of the three-dimensional structure of proguanylin and as a result in the assembly of the three-dimensional structure of mature guanylin.

Further experiments performed on a disulfide deletion mutant support this assumption of a straight influence of the prosequence on the structure of the hormone region [31]. Substitution of cysteine residues 86 and 94 by serine - resulting in proguanylin-C86S/C94S - leads to strong weakening of the tertiary contacts between guanylin and its prosequence whereas the helical core structure remains rigid as deduced by comparison of the NMR spectra of this mutant protein with those of wildtype proguanylin. Secondary chemical shift analysis, however, reveals that the conformation of the hormone region of proguanylin-C86S/C94S remains more similar to that of wildtype proguanylin than to mature guanylin [31] indicating residual tertiary contacts to be present that lead to structural stabilization of the hormone region. In contrast, isolated guanylin lacking the respective disulfide bond is biologically inactive [17] which can be interpreted as a complete loss of tertiary structure. Therefore, the conformation of the hormone region of proguanylin-C86S/C94S has to be induced by the prosequence. In agreement with the observation that non-random conformations on the folding pathway of a protein will favor formation of a specific disulfide bond and *vice versa*, a specific disulfide bond will favor and stabilize a specific conformation [32], these studies suggest two different processes that are likely to contribute to formation of the native disulfide connectivities of the hormone region in a cooperative manner. First, the structure of the prosequence - in particular the NH$_2$-terminal β-hairpin - has a straight effect on hormone topology (see above) and thereby favors formation of the native disulfide bonds, and second, the native disulfide bonds exhibit a stabilizing effect on the tertiary structure of the prohormone and thus trap the native structure and disulfides.

All experiments described above support the opinion that the structural requirements of the prosequence of guanylin are crucial and sufficient for correct structure and disulfide formation in the hormone region of proguanylin. This precursor protein, however, contains a third disulfide bond (Cys48 - Cys61) whose function was only investigated recently [31]. Deletion of this disulfide bond, respectively substitution of both cysteine residues by serine, resulted in loss of the pronounced secondary and tertiary structure of this protein. Additionally, no disulfide intermediates connecting cysteine residues of the prosequence (i.e. Cys48 or Cys61) with those of the hormone region could be observed during *in vitro* oxidative folding of wildtype proguanylin [31]. The crucial role of this disulfide bond (Cys48 - Cys61) during disulfide coupled folding is therefore not the formation of intramolecular disulfide intermediates but rather the stabilization of the hydrophobic core and thus of the overall proguanylin structure.
Taken together, all data available concerning the structure and folding of proguanylin imply that the interactions between the termini of proguanylin function to force formation of the native disulfide bonds of guanylin and stabilize the bioactive conformation of this peptide hormone during the folding of the prohormone.

References


Legends to Figures

Figure 1:
A: Amino acid sequences and disulfide pattern of GC-C activating peptides: human guanylin, human uroguanylin-16 (isolated from urine), human uroguanylin-24 (isolated from human blood ultrafiltrate), and *E. coli* heat-stable enterotoxin STa. Identical residues are highlighted in black.
B: Amino acid sequence alignment [33] of the respective prohormones human proguanylin and human prouroguanylin. Identical residues are highlighted in black, similar residues are highlighted in gray. The sequence deleted in prouguanylin-Δ(28-37) is highlighted in light gray. Experimentally determined (a) and predicted [34] (b) secondary structures are indicated.

Figure 2:
A: Ribbon drawing of guanylin A- (left) and B-isomer (right) (generated with MolMol [35]). Disulfide bonds are indicated in yellow. The homologous peptide hormone uroguanylin shows an almost identical fold with RMSD values of the backbone heavy atoms of 1.4 Å and 1.5 Å for the A- and B-isomer, respectively [18,19].
B: Schematic drawing of the backbone folds and the disulfide connectivities of guanylin isomers A (left) and B (right). The termini are indicated, cysteine residues are represented by circles and the disulfide bonds are given as zig zag lines. Amino acid numbering is according to the prohormone sequence.

Figure 3:
A: Representative structure of proguanylin. Elements of regular secondary structure are emphasized by ribbons, with helical structure indicated in red and β-sheet structure in green. Residues of the hydrophobic core (Phe10, Val15, Leu18, Phe53, Leu57, Leu60, and Leu73) are displayed in cyan, residues with enhanced inherent flexibility, i.e. \(1^H\)\(^15\)N NOE values < 0.6, are highlighted in blue, side chains of cysteine residues and disulfide bonds are indicated in yellow (Cys48 - Cys61; Cys83 - Cys91; Cys86 - Cys94) (generated with Molmol (Koradi 1996)).
B: Topology of the hormone region of proguanylin (residues 80 - 94).
C: Homology model [36,37] of prouroguanylin. Disulfide bonds between residues 41 – 54, 74 – 82, and 77 – 85 are displayed in yellow (same color code as in A; Figure generated with MOLMOL [35]).

Figure 4:
Interaction of the proguanylin hormone region with the NH\(_2\) terminus. A representative structure of proguanylin is shown. Compared to the view in Fig. 3A the molecule is rotated by 45° around a vertical axis. Helices and the NH\(_2\)-terminal β-sheet are colored red and green, respectively. Residues Val1 to Gln4 are displayed as spacefilled atoms, residues 80 to 94 are shown in stick representation (blue; spacefill is indicated), the disulfide bond between Cys48 and Cys61 is displayed in yellow (Figure generated with MolMol [35]).
Figure 2:
Figure 3:
Figure 4: