Structural Characterization of the Human Prohormone Proguanylin

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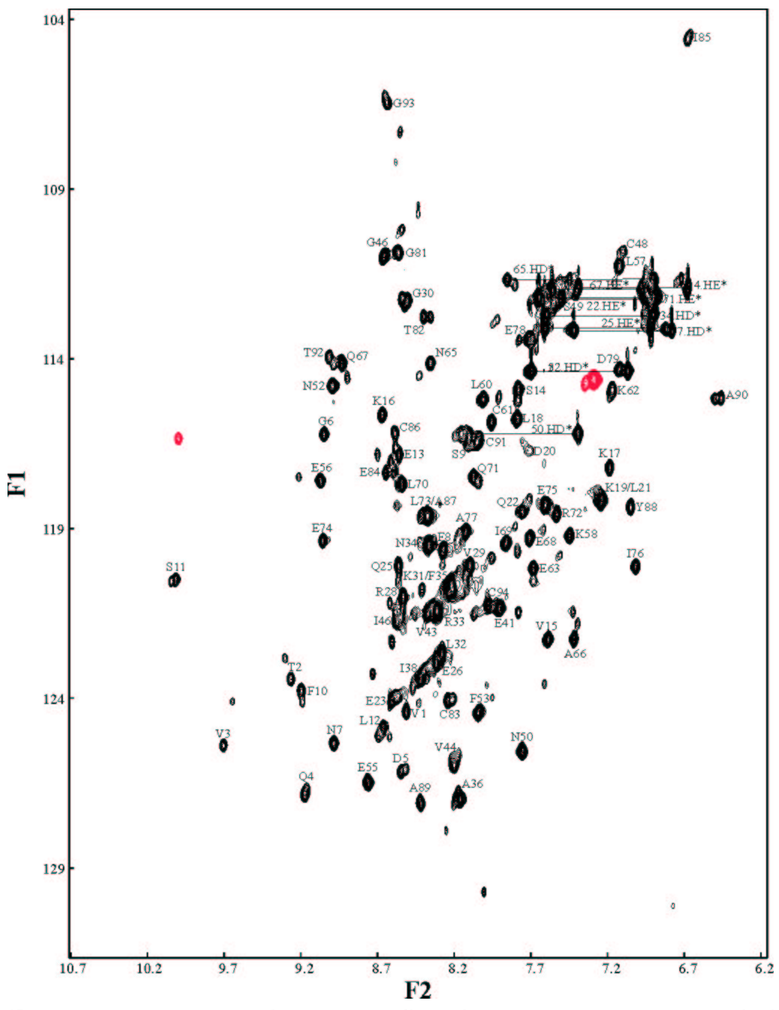
The endogenous ligand of guanylyl cyclase C (GC-C), guanylin, is produced as the corresponding prohormone containing 94 amino acid residues with the mature hormone located at its COOH-terminus. Previously, an essential contribution of the prosequence of guanylin in the disulfide-coupled folding was shown [1], leading to the term of an 'intramolecular chaperone'. Furthermore, in contrast to the mature hormone proguanylin shows only negligible GC-activating potency. Structure determination should reveal the mechanism of inactivation of the hormone part of proguanylin.
An almost complete sequence specific resonance assignment was obtained using H/C/N-triple resonance experiments. The pattern of medium range NOEs, as well as analysis of Hα, Cα, and CO secondary chemical shifts allowed the identification of three helices (13-20; 53-61; 66-78), a putative, small three stranded β-sheet, and a long sequence stretch (22-47) missing regular secondary structural elements. Restrained molecular dynamics calculations revealed a globular folded protein featuring a three helix bundle and a small three stranded β-sheet, made up of two NH_2-terminal strands and one strand of the very COOH-terminus. Thus, as an explanation for the inactivation of the bioactive COOH-terminal domain of proguanylin a shielding by the two NH_2-terminal β-strands can be suggested. This interaction might be comparable to that in the hormone receptor complex, since the hormone binding domain of GC-C exhibits a PBP II fold (periplasmic binding protein fold, type II), mainly consisting of β-sheet structure. Furthermore, the DALI algorithm was not able to identify any structural homologues of proguanylin, indicating a new protein fold.

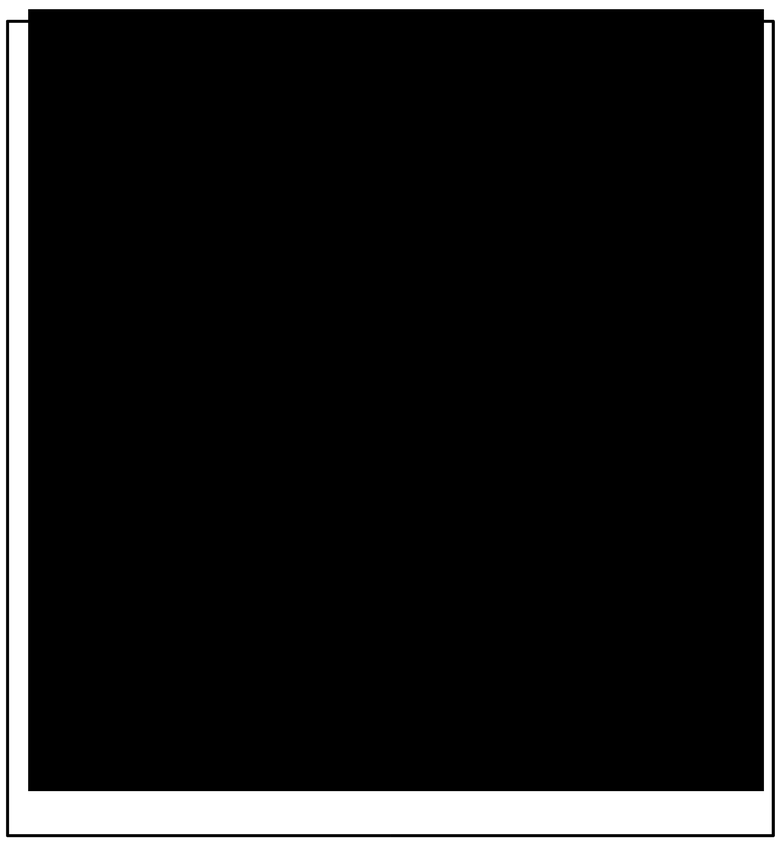
Methods and results

Expression and characterization of rProguanylin
Recombinant proguanylin was expressed and purified from *E. coli* AD494(DE3) cells with its native disulfide connectivity and three-dimensional fold as deduced from comparison with native proguanylin isolated from human blood filtrate [2]. Furthermore, analytical ultracentrifugation studies revealed recombinant proguanylin to be monomeric in solution for concentrations up to the near millimolar range [2]. Isotopic labeled protein was purified from cells grown on M9 minimal media containing $^{15}NH_4Cl$ and ^{13}C-glucose.
NMR samples contained protein at concentrations of 1.2 mM and 1 mM in 50 mM sodium phosphate (10% D_2O), pH 6.0 for ^{15}N labeled and $^{15}N/^{13}C$ labeled proguanylin, respectively.

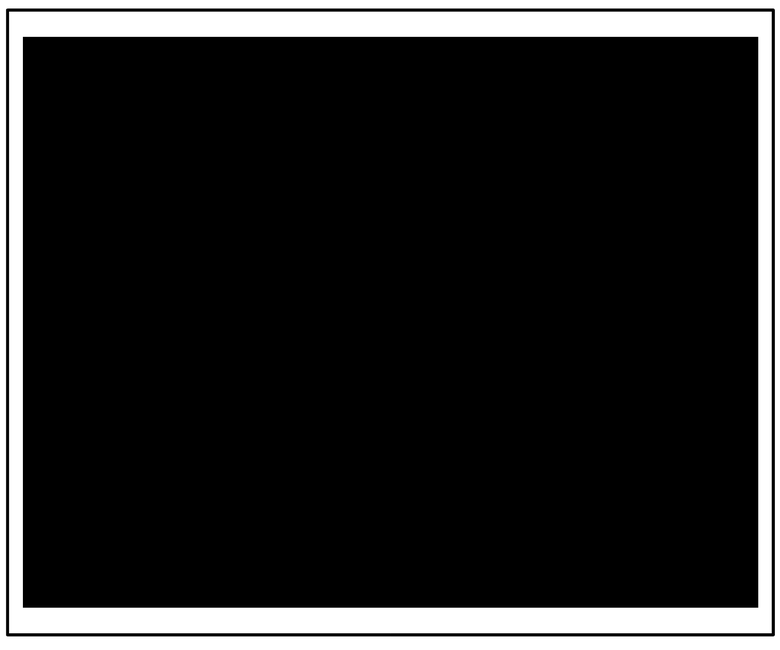
Structure determination
All NMR spectra were recorded on a Bruker DRX600 spectrometer at 20 °C. The following spectra were recorded for the backbone and sidechain assignment of proguanylin: ^{15}N-TOCSY- and NOESY-HSQC, ^{15}N-HMQC-NOESY-HSQC, HNHA, ^{15}N-HSQC, HNCA, HNCO, HBHA(CO)NH, CBCA(CO)NH, HC(CO)NH, C(CO)NH, ^{13}C-NOESY-HSQC, and ^{13}C-ctHSQC spectra. Aromatic amino acid side chains have been assigned using 2D-TOCSY, NOESY, and 2D-D_2O-NOESY spectra of native proguanylin. $^{1}H\alpha$ chemical shifts were taken from HNHA and ^{15}N-TOCSY-HSQC spectra.
The distance restraints for structure calculation were taken from the ^{15}N-NOESY-HSQC spectrum for NOEs involving amide protons and from the ^{13}C-NOESY-HSQC, and 2D-NOESY spectra for NOEs between aliphatic and aromatic protons. $^{3}J(H^N,H^\alpha)$ coupling constants were measured from cross peak to diagonal peak intensity ratios in the HNHA spectrum, corrected by a factor 1.05, as well as from line-shape analysis of the anti-phase cross signal splitting in a high digital resolution 2D-DQF-COSY spectrum using a Lorentzian function for peak fitting.
Slow exchanging amide protons have been identified using ^{15}N-HSQC spectra of a sample containing ^{15}N-labeled protein at 1.2 mM protein concentration in buffered D_2O, pH 6.0.
The solution structures were calculated with XPLOR 3.851, using a modified simulated annealing protocol.



^{15}N-HSQC spectrum of proguanylin. The spectrum was recorded at 293 K and pH 6.0. Assigned backbone and sidechain amide proton resonances are labelled.

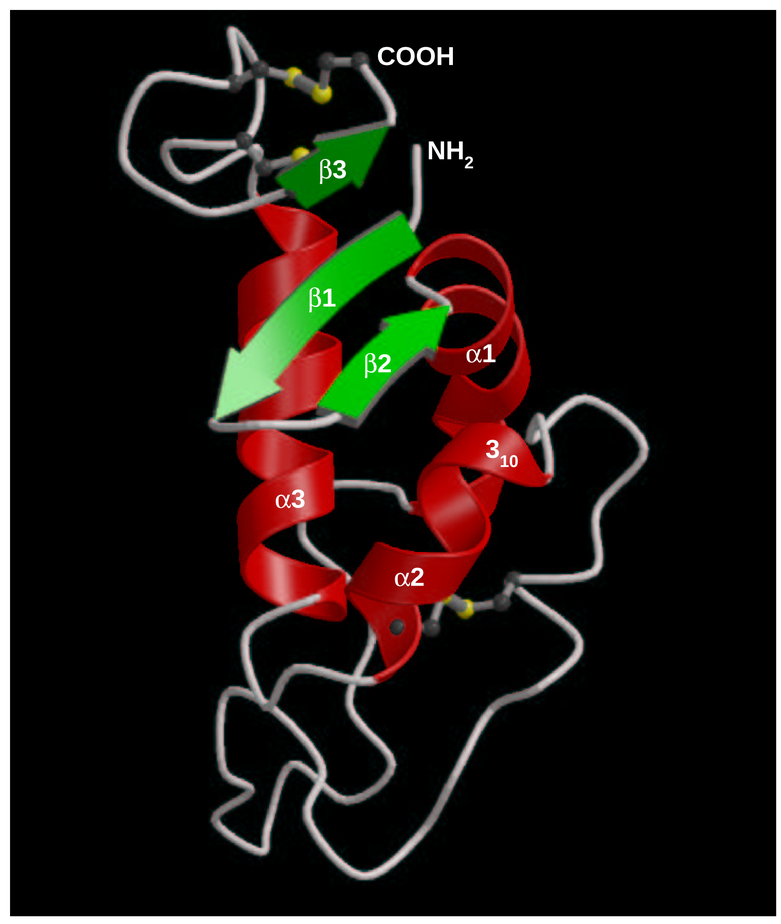


Analysis of secondary chemical shifts compared to random coil values [3]. Positive and negative chemical shift indices (CSI(Hα) and CSI(total)) are typical for β-sheets and α-helices, respectively, as denoted by rectangles above and below the axis. Filled circles represent proline residues, open diamonds leucine residues (only ^{15}N-labeled), and open circles correspond to missing assignment of the indicated resonance (assignment of Leu 47 is missing completely).



Secondary structure of proguanylin: summary of sequential and medium-range NOEs, vicinal NH-$C_\alpha H$ coupling constants (JαN), slow exchanging NH protons (H/D exchange), and chemical shift indices (consensus calculated from Hα, Cα, and CO chemical shift values). The relative strength of the NOEs, classified as weak, medium, and strong from cross-peak intensities of the ^{15}N-NOESY-HSQC spectrum, is indicated by the height of the horizontal bars. Values of $^{3}J(H^NH^\alpha)$ < 6 Hz and > 8 Hz, as typical for α-helices and β-sheets, are illustrated as white and black squares, respectively. Open circles represent slow exchanging NH protons. The secondary structure of proguanylin, determined by molecular dynamics calculations, is depicted in the bottom line.

Heteronuclear NOE of proguanylin at 600 MHz. The high flexibility of the central sequence stretch (residues 22 - 44) is displayed by small values (< 0.6) of the observed heteronuclear NOE. This is supported by chemical shift indices and missing medium and long range NOEs in this region.



A family of 45 structures was calculated by restrained molecular dynamics calculations, using 528 distance constraints obtained from analysis of the NOE connectivities, 18 H-bonds, and 31 coupling constants. One representative structure of proguanylin is shown in the MOLSCRIPT and Raster3D view, indicating regular secondary structural elements. The mature hormone guanylin (comprising residues Pro80 - Cys94) is fixed in its bioactive topology (A-form). Sidechains of cysteine residues and disulfide bridges are shown in stick representation (Cys48 - Cys61; Cys83 - Cys91; Cys86 - Cys94).

Conclusion

The structure of proguanylin features a three helix bundle and a small three stranded antiparallel β-sheet and indicates a new protein fold.
In its precursor protein the peptide hormone guanylin (comprising residues 80-94) is fixed in its bioactive topology and is involved in β-sheet formation. These inter-β-strand interactions, especially between the COOH- and NH_2-terminal residues suggest a shielding of the bioactive domain as an explanation for the negligible GC-activating potency of proguanylin. Furthermore, the same interactions can explain the important role of the first NH_2-terminal residues in the disulfide-coupled folding of proguanylin.

References

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[2] Lauber, T., Nourse, A., Schulz, A., and Marx, U.C.: Native and recombinant proguanylin feature identical biophysical properties and are monomeric in solution. s*ubmitted*
[3] Wishart, D. S., Bigam, C. G., Holm, A., Hodges, R. S., Sykes, B. D.: 1H, 13C and 15N random coil NMR chemical shifts of the common amino acids. I. Investigations of nearest-neighbor effects. (1995), *J. Biomol. NMR* **5**, 67-81.