Structural and Functional Characterization of the Conserved Salt Bridge in Mammalian Paneth Cell α-Defensins

The Journal of Biological Chemistry, Volume 281, Number 38, pp. 28068–28078, September 22, 2006

K. Johan Rosengren, Norelle L. Daly, Liselotte M. Fornander, Linda M. H. Jönsson, Yoshinori Shirafuji, Xiaqing Qu, Hans J. Vogel, Andre J. Ouellette, and David J. Craik

From the Department of Chemistry and Biomedical Sciences, University of Kalmar, SE-391 82 Kalmar, Sweden, the Institute for Molecular Bioscience and Australian Research Council Special Research Centre for Functional and Applied Genomics, University of Queensland, Brisbane, Queensland 4072, Australia, the Departments of Pathology & Laboratory Medicine and Microbiology & Molecular Genetics, School of Medicine, University of California, Irvine, California 92697-4800, and the Structural Biology Research Group, Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada

α-Defensins are mediators of mammalian innate immunity, and knowledge of their structure-function relationships is essential for understanding their mechanisms of action. We report here the NMR solution structures of the mouse Paneth cell α-defensin cryptdin-4 (Crp4) and a mutant (E15D)-Crp4 peptide, in which a conserved Glu residue was replaced by Asp. Structural analysis of the two peptides confirms the involvement of this Glu in a conserved salt bridge that is removed in the mutant because of the shortened side chain. Despite disruption of this structural feature, the peptide variant retains a well defined native fold because of a rearrangement of side chains, which result in compensating favorable interactions. Furthermore, salt bridge-deficient Crp4 mutants were tested for bactericidal effects and resistance to proteolytic degradation, and all of the variants had similar bactericidal activities and stability to proteolysis. These findings support the conclusion that the function of the conserved salt bridge in Crp4 is not linked to bactericidal activity or proteolytic stability of the mature peptide.

Broad spectrum endogenous antimicrobial peptides, including defensins, contribute to the innate immune response (1). The mammalian defensins are all characterized by a central β-sheet that is cross-braced by an array of three disulfide bonds but can be further divided into three classes: α-, β-, and θ-defensins, based on their disulfide bond connectivities and topology (1, 2). β-Defensins are the largest at ~40 amino acids and possess a CysI-CysVI, CysII-CysIV, CysIII-CysVII array (3), whereas the α-defensins comprise ~32–36 amino acids and a characteristic CysI-CysV, CysII-CysII, CysIII-CysV framework, combined with the remarkable arrangement of their disulfide bonds (4). The θ-defensins are considerably smaller at only 18 residues and have a CysI-CysVI, CysII-CysV, CysIII-CysIV framework, with the remarkable feature of a head-to-tail cyclic peptide backbone, resulting from two 9-residue gene products being joined into a circle by the post-translational formation of two peptide bonds (5).

Mammalian α-defensins were first identified in myeloid cells (6) but have since been found in Paneth cells of the small intestine (7, 8) and in rabbit kidney (9, 10). Paneth cell α-defensins, which play an important role in enteric mucosal immunity (11), are secreted as components of granules into the lumen of small intestinal crypts in response to cholinergic stimulation or exposure to bacteria or bacterial antigens (12–14). The mouse Paneth cell α-defensins, termed cryptdins (Crps) (15) are secreted into the crypt lumen at concentrations of 25–100 mg/ml, orders of magnitude above their minimal inhibitory concentrations (14). Their antimicrobial activity is equivalent against Gram-positive and Gram-negative bacteria, with cryptdin-4 (Crp4) displaying the greatest mouse α-defensin bactericidal activity in in vitro assays (15). The mode of action of these peptides, which involves nonspecific interactions and disruption of bacterial membranes, is dependent on peptide surface positive charge and amphipathicity, a feature common to most mammalian α-defensins.

Despite the apparent positive selection of gene duplication and diversification evident in alignments of known α-defensin primary structures (Fig. 1), recent studies have reported on the structural and functional roles of canonical residues conserved in all α-defensins. These include the spacing and disulfide connectivities of the six Cys residues, a Gly at the position corresponding to residue 19 in Crp4, and a positively charged residue (Arg/Lys) and a negatively charged residue (Glu) found at posi-
the middle of conserved Gly19 residue is positioned in a classical by matrix metalloproteinase-7, the proCrp convertase (16). The disulfide-deficient Crp4 mutants retain or exceed native Crp4 complete disruption of the fold (16). Despite being unfolded, all residues, including Arg7, Glu15, and Gly19 (using the numbering of Crp4) are disulfide connectivities are indicated by. Other conserved residues, including Arg7, Glu15, and Gly19 (using the numbering of Crp4) are shown in bold type. The sequence of the mutant studied here is given under (E15D)-Crp4. tions 7 and 15, respectively, in Crp4 (16–18). The disulfide bonds maintain the α-defensin fold, with mutations in either the Cys1-Cys16 or Cys3-Cys3 disulfide pairings, resulting in a complete disruption of the fold (16). Despite being unfolded, all disulfide-deficient Crp4 mutants retain or exceed native Crp4 bactericidal activity but are sensitive to proteolytic degradation by matrix metalloproteinase-7, the proCrp convertase (16). The conserved Gly19 residue is positioned in a classical β-bulge in the middle of β-strand 2. The ability of Gly to adopt a d/∅ angle combination not normally accessible by l-amino acids, because of its small size and less stringent conformational restrictions, is crucial for the structure of the sheet. Mutational studies on human neutrophil α-defensin 2 (HNP2) have revealed that although it cannot be replaced by any other l-amino acid, a correctly folded product can be achieved by the inclusion of a D-amino acid, for which the required backbone conformation is energetically favorable (18). The final α-defensin canonical feature is the occurrence of Arg and Glu, respectively, at positions 7 and 15 in mouse Crp4, which are predicted to form a conserved salt bridge (17). The role of this salt bridge in HNP2 was investigated by site-directed mutagenesis, which showed that salt bridge disruption or removal did not diminish HNP2 antibacterial activity or HNP2 precursor folding in vitro (17). However, the mutated analogues were susceptible to proteolysis by human neutrophil elastase, again emphasizing the need for a well defined stable fold to prevent attack from proteases (17).

Here, we have investigated the role of this canonical salt bridge on the native structure of α-defensins. To date, reported α-defensin structures include the crystal structures of HNP3 (19) and HNP2 (18) and the NMR solution structures of the rabbit neutrophil defensins 2 (20) and 5 (21), the rabbit kidney defensin-1 (RK-1) (22), and mouse Crp4 (23). Although HNP2, HNP3, and RK-1 all show the presence of a salt bridge, this structural feature was not identified in Crp4 (23). After analysis of NMR data for both native Crp4 and analogues, we suggest that in the published structure a small part of the sheet has been incorrectly aligned, leading to a lack of recognition of the key salt bridge. Here we present the corrected high resolution solution structure of Crp4 and the structure of the mutant (E15D)-Crp4 in which the conserved salt bridge has been removed by effectively shortening the side chain of the Glu residue but otherwise making no change to the charge state of the native peptide. This study improves the understanding of the structural and functional roles of this conserved structural feature of the α-defensin family.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Crp4 Peptide Variants—Recombinant Crp4 peptides were expressed in *Escherichia coli* as N-terminal hexahistidine-tagged fusion proteins from the EcoRI and Sall sites of the pET28a expression vector (Novagen, Inc., Madison, WI) as described previously (24, 25). The Crp4 coding cDNA sequences were amplified using forward primer ATATA TGAAT TCATG GGTTC GTTAT GCTAT (ER1-MET-C4-F) paired with reverse primer ATATA TGTCC GACTG GCAGT AA stressed to GCAGTG ACTTG GCGG GGCGG GAGAT ACA (SLPMALCRP4R) as reported previously (24). For PRECP, forward primer pETPCr4-F (5’-GCCGCC AATTC ATGGA TCCTA TCCAA AACAC A) was paired with reverse primer SLPMALCRP4R (5’-ATAAT TGTCC GACTG GCaget GCAGTG ACTTG GCGG GGCGG GAGAT ACA), corresponding to nucleotides 104–119 and 301–327 in preproCrp4 cDNA (24). In all instances, the reactions were performed using the GeneAmp PCR core reagents (Applied Biosystems, Foster City, CA) by incubating the reaction mixture at 94 °C for 5 min, followed by successive cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 30 cycles, followed by a final extension reaction at 72 °C for 7 min.

**Mutagenesis at Glu15 and Arg7 Residue Positions—Mutations were introduced into Crp4 by PCR as described previously (16) in the order described below. In the first round of mutagenesis the Crp4 construct in pET-28a (25) was used as template. In PCR 1, a mutant forward primer, e.g. C4E75D for, containing the Asp for Glu mutation at Crp4 residue position 15 flanked by three natural codons was paired with reverse primer T7 terminator (Invitrogen), a downstream sequencing primer in the pET-28a vector. In PCR 2, the mutagenizing reverse primer C4E75D rev, the reverse complement of the mutant forward primer, was paired with the T7 promoter forward primer, again from the pET-28a. Mutagenizing forward and reverse primers were: for (E15D)-Crp4, TGCAA AAGAG GAGAT TGCGT GG (C4E75D for) and CCCAC GAATC GACTG TCCTCT TTTTG CA (C4E75D rev); for (E15K)-Crp4, TGCAA AAGAG GAGAT TGCGT GG (C4E75K for) and CCCAC GAATC GACTG TCCTCT TTTTG CA (C4E75K rev); for (E15G)-Crp4, TGCAA AAGAG GAGAT TGCGT GG (C4E75G for) and CCCAC GAATC GACTG TCCTCT TTTTG CA (C4E75G rev); and for (E15L)-Crp4, TGCAA AAGAG GAGAT TGCGT GG (C4E75L for) and CCCAC GAATC GACTG TCCTCT TTTTG CA (C4E75L rev). After amplification at 94 °C for 5 min, followed by successive cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 30
Structures of Crp4 and (E15D)-Crp4

cycles, followed by a final extension reaction at 72 °C for 7 min, samples of purified products from reactions 1 and 2 were combined as templates in PCR 3, using T7 promoter and terminator primers as amplifiers. Corresponding proCrp4 templates of these variants were prepared as described (16). All of the mutated Crp4 and proCrp4 templates were cloned in pCR-2.1 TOPO, verified by DNA sequencing, excised with SalI and EcoRI, subcloned into pET28a plasmid DNA (Novagen), and transformed into *E. coli* BL21(DE3)-CodonPlus-RIL cells (Stratagene) for recombinant expression (24, 25).

**Purification of Recombinant Crp4 Proteins**—Recombinant proteins were expressed and purified as His-tagged Crp4 fusion peptides (16, 24). Briefly, recombinant proteins were expressed at 37 °C in Terrific Broth medium by induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside for 6 h at 37 °C, the cells were lysed by sonication in 6 M guanidine-HCl in 100 mM Tris-Cl (pH 8.1), and the soluble protein fraction was clarified by centrifugation (24–26). His-tagged Crp4 fusion peptides were purified using nickel-nitrilotriacetic acid (Qiagen) resin affinity chromatography (24). After CNBr cleavage, Crp4 peptides were purified by C18 reverse phase high performance liquid chromatography and quantitated by bicinchoninic acid (Pierce), and the molecular masses of purified peptides were determined using matrix-assisted laser desorption ionization mode mass spectrometry (Voyager-DE MALDI-TOF; PE Biosystems, Foster City, CA) in the Mass Spectroscopy Facility of the Department of Chemistry at the University of California, Irvine.

**Bactericidal Peptide Assays**—Recombinant peptides were tested for microbicidal activity against *E. coli* ML35 and *Staphylococcus aureus* 710a (27). Bacteria (5 × 10⁶ colony-forming units/ml) resuspended in 10 mM PIPES (pH 7.4) supplemented with 0.01 volume of trypticase soy broth were incubated with test peptides in 50 μl for 1 h at 37 °C, and the surviving bacteria were counted as colony-forming units/ml after overnight growth on semi-solid medium (24, 25).

**Exposure of Glu¹⁵ Crp4 and proCrp4 Variants to MMP-7, the Mouse Paneth Cell Pro-α-defensin Convertase**—Recombinant Crp4, proCrp4, and variants with site-directed mutations at position 15 were digested with MMP-7 and analyzed for evidence of proteolysis by acid-urea PAGE (24, 28). Samples of Crp4 variants (5 μg) and proCrp4 variants (11 μg) were incubated with activated recombinant human MMP-7 (0.3 ~ 1.0 μg) catalytic domain (Calbiochem, La Jolla, CA) in buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM CaCl₂, for 18–24 h at 37 °C, and equimolar samples of all digests were analyzed by acid-urea PAGE (16, 24).

**NMR Spectroscopy**—The samples for structure determination contained ~2 mg of native Crp4 or ~0.6 mg of (E15D)-Crp4 dissolved in either 90% H₂O and 10% D₂O (v/v) or 100% D₂O at pH 4.5. All of the spectral data were recorded at 600 and 500 MHz on Bruker Avance NMR spectrometers. Two-dimensional experiments recorded included double quantum filtered correlation spectroscopy (DQF-COSY), TOCSY using an MLEV-17 spin lock sequence with a mixing time of 80 ms, and NOESY with mixing times of 100, 150, or 200 ms. Selected spectral data, including preliminary data on an additional salt bridge-deficient analogue, (R7G)-Crp4, are presented as supplementary material. The spectra were generally acquired with 4096 complex data points in F2 and 512 increments in the F1 dimension over a spectral width of 12 ppm. The spectra were processed on a Silicon Graphics Octane work station using XWINNMR (Bruker). The F1 dimension was generally zero-filled to 1024 real data points, and 90° phase-shifted sine bell window functions were applied before Fourier transformation. Chemical shifts were internally referenced to 2,2-dimethyl-2-silapentane sulfonic acid (DSS) at 0.00 ppm. Slowly exchanging NH protons were detected by acquiring a series of one-dimensional and TOCSY spectra of the fully protonated peptide immediately after dissolution in D₂O. Further evidence for hydrogen bonds was deduced from amide temperature coefficients, which were determined by recording TOCSY spectra at 288, 293, 298, 303, and 308 K and plotting the amide shifts as a function of temperature. The pKa of Glu¹⁵ in Crp4 and Asp¹⁵ in (E15D)-Crp4 and the C-terminal group were estimated by monitoring the effects of pH in the range of 1.1–7.5 on the chemical shifts of resonances within the vicinity of the carbonyl group.

**Structure Determination**—Distance restraints for Crp4 were derived primarily from 150-ms NOESY spectra recorded at 298 K and 600 MHz with additional restraints derived from a NOESY recorded at 500 MHz with a cryogenic probe added during refinement stage. Distance restraints for (E15D)-Crp4 were derived from a 600-MHz NOESY recorded at 298 K with a mixing time of 200 ms to compensate for the significantly lower sample concentration. The spectral data were analyzed, and the cross-peaks were assigned and integrated in CARA (29) and converted to distance restraints using DYANA (30). Backbone dihedral restraints were inferred from 3JHN-Hα coupling constants derived from either the one-dimensional or a high digital resolution double quantum filtered correlation spectroscopy. The dihedral angle φ was restrained to −120 ± 30° for 3JHN-Hα greater than 8 Hz (resides Cys4, Tyr5, Cys6, Arg16, Arg18, Cys21, Leu26, Tyr27, Cys28, and Cys29 for both Crp4 and (E15D)-Crp4). Additional φ angle restraints of −100 ± 80° were included where the positive angle could be excluded based on strong sequential Hα to Hα NOE compared with the intra-residual Hα-Hα NOE. Side chain χ and stereo-specific assignments were determined on the basis of observed NOE and 3Jα-Hα coupling patterns (31). For a χ²-g¹ side chain conformation, the χ angles were restrained to −60 ± 30° (resides Cys4, Tyr5, Cys11, Glu15, Phe25, Cys28, and Cys29 for Crp4 and residues Cys4, Cys11, Asp15, Arg18, Phe25, Cys28, and Cys29 for (E15D)-Crp4), and for a χ² conformation the angles were constrained to 180 ± 30° (resides Val17, Ile23, and Tyr27 for Crp4 and residues Cys6, Arg13, and Tyr27 for (E15D)-Crp4). No residues could be confirmed to be in the g²-g³ conformation based on experimental data. Hydrogen bonds were included into the structure calculations for all of the amide protons concluded to be slow exchanging or having a T1 consistent with a hydrogen bond, only once a suitable acceptor could be identified in the preliminary structures. In all cases these hydrogen bonds were found between the backbone atoms within the elements of secondary structure.

Three-dimensional structures were calculated using simulated annealing and energy minimization protocols from ARIA...
the Arg7 side chain are shifted downfield in the mutant by between 0.7 and 0.2 ppm, and a sharp resonance at 6.55 ppm, which originated from the guanidinium group of Arg7, could not be detected in the mutant. Additional significant differences in the mutant chemical shifts include the two Hβ protons of Cys28, which are shifted upfield by 1.5 and 1.0 ppm, Hβ2 of Cys11 (upfield 0.5 ppm), HN and both Hβs of Cys4 (all downfield ~0.5 ppm), and Hα and Hβ2 of Tyr5 (both downfield ~0.5 ppm). Crp4 contains one proline residue, Pro30, which in both the native and mutant structures was found to be in the trans-conformation as evident from strong Hαi−1-Hαi NOEs to the preceding residue.

The presence of secondary structure in peptides can generally be readily identified by an analysis of the deviation of the Hα shifts from random coil shifts (35). Fig. 2 shows the secondary Hα shifts for Crp4 and (E15D)-Crp4, from which it is clear that the general trend is stretches of positive values, consistent with the triple-stranded β-sheet that is typical of an α-defensin fold. With the exception of Tyr5, only small differences in the Hα secondary shifts are seen between the two peptides, suggesting that despite the large effects on the chemical shifts of some residues, the mutation does not significantly affect the backbone fold.

Temperature Variation and pH Titration Studies of Crp4 and (E15D)-Crp4—Monitoring the amide chemical shifts of a protein as a function of temperature is a rapid and powerful method for identifying hydrogen bond donors in a three-dimensional structure, because intramolecular hydrogen-bonded amides have a low sensitivity to temperature (36, 37). For both Crp4 and (E15D)-Crp4 the temperature dependences of the amide chemical shifts were determined from TOCSY spectra over the temperature range 288–308 K. Generally 85% of amides that have a temperature coefficient (Tc) more positive than ~4.6 ppm/K are involved in intramolecular hydrogen bonds (the probability increases to >93%, if −4.0 < Tc < −1.0 ppm/K) (37). In Crp4 and (E15D)-Crp4 11 amides were found to have temperature coefficients >−4.6 ppm/K. The data are in good agreement with amide D2O exchange experiments, with seven of eight amides identified as slow exchanging, having a Tc consistent with a hydrogen bond. The exception, Arg7, has a slow exchange with the solvent but a Tc of −5.8 ppm/K. However, Tc values are known to be affected by strong shielding/deshielding (36) and may give false positives/negatives if the amide resonance has an unusual shift. This is the case for Arg7, which at 9.88 ppm is the most downfield resonance in Crp4. For all amides identified as hydrogen bond donors by Tc values or D2O exchange, suitable acceptors were identified within elements of secondary structure in the preliminary structures, with the slow exchanging amides being part of the core of the β-sheet and the additional amides identified as hydrogen
Structures of Crp4 and (E15D)-Crp4

FIGURE 3. NMR monitored pH titrations of Crp4 and (E15D)-Crp4. The chemical shift versus pH is plotted for all resonances significantly affected by pH. By nonlinear regression analysis the $pK_a$ was determined to be $-5.6$ for His$^{10}$ (affected resonances: HN 9, HN 10, HN 11, HN 12, HB1 10, and HB2 10) and $-2.2$ for the C terminus (affected resonances: HN 32, HA 32, and HB2 32). Finally, at low pH several resonances were affected by the ionization state of Glu/Asp$^{15}$ (HE 7, HN 12, HB2 28, HB1 15, and HG2 15 in Crp4 and HN 31, HN 16, HB1 15, and HB2 15 in (E15D)-Crp4) were identified and indicated a $pK_a$ of $>1.5$ in both structures.

| TABLE 1 |
| --- |
| **NMR and refinement statistics for the families of NMR structures** |
| | Crp4 | (E15D)-Crp4 |
| **NMR distance and dihedral constraints** |
| Total inter-residue nuclear distance constraints | 442 | 256 |
| Overhauser effect | | |
| Sequential ($|\rho| = 1$) | 160 | 128 |
| Medium range ($|\rho| = 4$) | 84 | 38 |
| Long range ($|\rho| = 5$) | 198 | 90 |
| Hydrogen bonds | 26 (13 H-bonds) | 22 (11 H-bonds) |
| **Total dihedral angle restraints** |
| $\phi$ | 25 | 24 |
| $\chi_1$ | 10 | 11 |
| **Structure statistics** |
| Violations (mean $\pm$ S.D.) | | |
| Distance constraints ($>0.2$ Å) | 0.1/structure | 0.6/structure |
| Maximum distance constraint violation (Å) | 0.22 Å | 0.24 Å |
| Dihedral angle constraints ($>3^\circ$) | 0/structure | 0/structure |
| Maximum dihedral angle violation ($^\circ$) | 2.6 | 3.0 |
| Deviations from idealized geometry | | |
| Bond lengths (Å) | 0.0041 $\pm$ 0.0001 | 0.0039 $\pm$ 0.0002 |
| Bond angles ($^\circ$) | 0.56 $\pm$ 0.037 | 0.56 $\pm$ 0.027 |
| Improper ($^\circ$) | 0.44 $\pm$ 0.032 | 0.43 $\pm$ 0.033 |
| Average pairwise root mean square deviation (Å)$^a$ | | |
| Heavy | 1.54 | 1.77 |
| Backbone | 0.36 | 0.48 |
| Ramachandran statistics (%) | | |
| Most favored regions | 79.6 | 71.0 |
| Additionally allowed | 20.0 | 28.4 |
| Generously allowed | 0.4 | 0.6 |

$^a$ Pairwise root mean square deviation was calculated among 20 refined structures over the structured part of the molecules, residues 4–29.

bonded from $T_c$ data being found in turns, around the edges of the sheet, and in bulge regions.

In addition to the temperature variation experiments, both Crp4 and (E15D)-Crp4 were subjected to pH titrations to deter-

mine the $pK_a$ of Glu/Asp$^{15}$. Favorable electrostatic interactions such as salt bridges can have a dramatic effect on the $pK_a$ values of the groups involved and by monitoring the chemical shift dependence of resonances adjacent to the titrating groups the degree of stabilization of the charged state from such interactions may be determined. Fig. 3 shows the chemical shift as a function of pH for all resonances having a significant pH dependence ($>0.1$ ppm) in the pH range 1.1–7.5 for Crp4 (Fig. 3A) and (E15D)-Crp4 (Fig. 3B). It is evident that the ionization states of three groups are affected by the pH changes within this range, namely His$^{10}$, the Arg$^{32}$ C-terminal group, and Glu/Asp$^{15}$. The data were fitted by nonlinear regression analysis, which in both Crp4 and (E15D)-Crp4 gave $pK_a$ values of $-5.6$ and $-2.3$ for His$^{10}$ and the C terminus, respectively. Strikingly, the Crp4 Glu$^{15}$ and (E15D)-Crp4 Asp$^{15}$ carboxyl groups are largely unaffected as the pH is lowered to 3, and as a result the full titration curves cannot be obtained without subjecting the proteins to extreme conditions. However, based on curve fitting of the available data for several resonances affected by protonation/deprotonation, the 95% confidence intervals for the $pK_a$ values of Glu/Asp in Crp4 and (E15D)-Crp4 are 1.1–1.5 and 1.0–1.7, respectively. The expected $pK_a$ values for His, $\alpha$, $\beta$, and $\gamma$ carbonyl groups are $-6.5$, $3.5$–$4.3$, $3.9$–$4.0$, and $4.3$–$4.5$, respectively (38, 39); hence the noncharged state of the His and the charged state of both the termini and the Glu/Asp are significantly stabilized by interactions in the folded structure. The resonance that is most affected by the titration of the Glu$^{15}$ carboxyl group is the H proton of Arg$^2$ in native Crp4, which at low pH start to move back from its downfield shifted position toward its random coil value.

**Structure Determination and Description of the Three-dimensional Structure**—From the NMR data a set of restraints including upper limit distance restraints based on NOE cross-peak intensities, backbone $\phi$ and side chain $\chi_1$ dihedral angles, and hydrogen bond restraints was derived and used for structure determination of the two peptides. Both structures were calculated by simulated annealing and refined in explicit solvent and the structural and energetic statistics for the final families of 20 structures are summarized in Table 1. All of the structures are in good agreement with the experimental data and have good covalent geometries, as evident from low deviations from optimal bond lengths and angles and from the Ramachandran statistics.

Fig. 4 shows a stereo view of the families of 20 structures representing the solution structures of Crp4 and (E15D)-Crp4. Both structures, with the exception of the termini, are well defined with the main element of secondary structure being the central $\beta$-sheet. The sheet is made up by strands comprising residues 5–8, 15–21, and 25–29, with the second strand having two bulges around res-
The conformation of these bulges requires Gly residues at positions 19 and 21, because of the ability of Gly to adopt backbone conformations normally not accessible by other L-amino acids. The three strands are linked by two well-defined \( \beta \)-turns comprising residues 12–15 and 22–25, both of which adopt a type II conformation, and one \( \gamma \)-turn comprising residues 8–10. The arrangement of the sheet and turns is supported by a large number of cross-strand NOEs, as illustrated in Fig. 5. Also indicated in Fig. 5 is the hydrogen bond network that was identified by analysis of amide exchange, amide proton \( T_1 \) values, and structure calculations.

Given the small size of Crp4 the structure lacks a distinct hydrophobic core, and the main stabilizing features of the fold are the disulfide bonds and hydrogen bonds between the polar groups of the backbone. Some hydrophobic interactions between side chains are present, including those involving residues Cys\(^{13} \)/Val\(^{17} \)/Leu\(^{27} \)/Cys\(^{28} \), Leu\(^{7} \)/Tyr\(^{5} \)/Pro\(^{30} \), and Cys\(^5 \)/Cys\(^6 \)/Arg\(^{18} \)/Cys\(^{21} \)/Tyr\(^{27} \). In addition, in native Crp4 an interaction between the Glu\(^{15} \) and Arg\(^7 \) side chains can be identified. The positioning of the two side chains, which is confirmed by NOE patterns, indicates that the Glu\(^{15} \) carboxylate group points toward the side of the Arg side chain, coordinating one of its oxygen atoms with the H\(^{\alpha} \) proton and the other with one of the amino protons from the guanidinium group. The salt bridge, together with the Cys\(^{11} \)/Cys\(^{28} \) disulfide bond, appears to stabilize the residue 9–15 loop, which is the only part of the molecule, apart from the two termini, not involved in elements of regular secondary structure. In (E15D)-Crp4 the shortened carboxyl-bearing side chain makes this interaction impossible, and the mutation results in a reorientation of the Tyr\(^{5} \) side chain, which fills the void left by the larger Glu and the Arg\(^7 \) side chains, with the latter moving away from the molecular core, out into solution. Electrostatic interactions that apparently compensate for the lack of the salt bridge are formed between the Asp and the phenolic hydrogen of Tyr\(^{5} \) and the positively charged Lys\(^{12} \).

The Canonical Arg\(^7 \)–Glu\(^{15} \) Salt Bridge Is Not a Determinant of Crp4 Bactericidal Activity—To investigate the contribution of the Arg\(^7 \)–Glu\(^{15} \) salt bridge to Crp4 microbicidal function, the bactericidal activities of native Crp4 and Crp4 variants with salt bridge disruptions were compared with \( E. \) coli and \( S. \) aureus in \textit{in vitro} assays (Fig. 6). Under the conditions of these assays, the overall bactericidal activities of Crp4, (E15D)-Crp4, (E15L)-Crp4, and (E15G)-Crp4 were similar, reducing bacterial cell survival by at least three log values at or below 10 \( \mu \)g/ml (Fig. 6 and data not shown). The results of additional assays performed against strains of \textit{Vibrio cholerae}, \textit{Listeria monocytogenes}, and wild-type \textit{Salmonella enterica} serovar Typhimurium were reproducibly similar to those in Fig. 6, as were assays performed with a (R7G)-Crp4 variant, which also contains a salt bridge disruption (data not shown). These findings show that Crp4 bactericidal activity is independent of the Arg\(^7 \)–Glu\(^{15} \) salt bridge, although the dose-response curves of certain peptides differed modestly (Fig. 6). This finding is consistent with the fact that corresponding salt bridge mutants (Arg\(^{5} \)–Glu\(^{13} \)) of human \( \alpha \)-defensin HNP2 have bactericidal activities equivalent to that of native HNP2 (17). Because mutagenesis of the canonical Arg\(^7 \)–Glu\(^{15} \) salt bridge had little or no effect on Crp4 bactericidal action, alternative roles for the...
salt bridge were considered, including protection from proteolysis by matrix metalloproteinase-7 (MMP-7), the activating convertase for mouse Paneth cell pro-α-defensins.

**Disruption of the Arg⁷–Glu¹⁵ Salt Bridge Does Not Induce Crp4 Susceptibility to MMP-7 Proteolysis**—Mouse Paneth cell α-defensin biosynthesis requires MMP-7-mediated proteolytic conversion of inactive proCrps to their functionally active forms (40, 41). Because mutations to the mouse Crp4 disulfide array result in Crp4 proteolysis by MMP-7 (16) and because mutations in the HNP2 salt bridge induce susceptibility to neutrophil elastase, the hypothesis that Arg⁷ or Glu¹⁵ mutants of Crp4 and proCrp4 would be subject to MMP-7-mediated degradation was tested. Native Crp4 is completely resistant to MMP-7 in vitro, and MMP-7 activates native proCrp4 without cleaving within the α-defensin moiety of the precursor (Fig. 7 and Refs. 16, 24, 25, and 41). In contrast to the sensitivity of Arg⁷/Glu¹³ HNP2 variants (17), none of the Crp4 or proCrp4 salt bridge variants tested, including (E15D)-Crp4, (R7G)-Crp4, (E15G)-Crp4, (E15D)-proCrp4, (R7A)-proCrp4, and (R7G)-proCrp4, displayed evidence of proteolysis by MMP-7 in this highly sensitive assay (Fig. 7). These findings suggest that structural alterations induced in Crp4 by disrupting the canonical α-defensin salt bridge do not ensure sensitivity to proteolysis per se, but that variations in peptide primary structure exclusive of canonical positions, in particular the strong electropositive charge of Crp4, also contribute to the proteolytic stability.

**DISCUSSION**

The Glu residue at position 15 is the only negatively charged residue in Crp4 and strikingly is almost completely conserved throughout the α-defensin family. Based on structural data, this Glu has been proposed to be involved in a conserved salt bridge with an Arg/Lys, and in a recent study a number of mutants of HNP2 exploring the role of this salt bridge were generated and analyzed with respect to bactericidal activity, in vitro folding ability, and proteolytic stability (17). Here, we have investigated the biological and structural consequences of removing this conserved salt bridge in a mouse Paneth cell α-defensin, Crp4. The NMR structure of native Crp4 was recently reported, but apparent misassignment of a few crucial hydrogen bonds between the β2 and β3 strands resulted in a small part of one strand being incorrectly aligned with the sheet, and as a consequence, the Glu¹⁵-Arg⁷ salt bridge was not identified (23). However, as we show here, the conserved salt bridge is indeed present in the corrected structure of Crp4, and its structural role has been evaluated by structural analysis of the analogue (E15D)-Crp4, in which it has been removed.

The Structure of Crp4 and Comparison with Other α-Defensin Structures—The misassignment of a few hydrogen-bonding partners in the earlier reported Crp4 solution structure by Jing et al. (23) led to a different arrangement of a small part of the β2 strand with respect to the β3 strand. Nevertheless the overall shape of the molecule was very similar to that obtained in the present study. Unexpectedly the incorrect alignment of these two β strands did not give rise to obvious problems in the energy of the original structure. Because the hydrogen bonds were included in all the final structure calculations the other restraints used (e.g. NOEs) did not move the structure toward a more correct positioning of the sheet. However, in subsequent unrestrained molecular dynamics calculations, movement of the β2 strand and increased hydrogen bonding in Crp4 was observed. Movement of β strands is not normally observed in such unrestrained molecular dynamics simulations, indicating that part of the original structure had considerable strain associated with it. During this movement process the other two strands of the sheet remained in their correct orientation, which is identical to that observed in this study. After 6 ns the relaxed structure resembled the one reported here and this remained stable during a further 14 ns of simulation time.

The results obtained in the unrestrained simulations are clearly consistent with the outcome of the current structural study of Crp4.

The present solution structure of Crp4 adopts a typical α-defensin fold that is characterized by a triple-stranded antiparallel β-sheet. Fig. 8 shows a comparison of Crp4, HNP3, and RK-1, and it is clear that the most significant difference between Crp4 and most other α-defensin is in the hairpin region. This is a direct result of the loop between Cys²¹ and Cys²⁸ comprising

---

*6. N. Zhou and H. J. Vogel, data not shown.*
Salt bridges are notoriously difficult to identify by NMR because side chain orientations are not always well defined, and proton-proton distances across salt bridges are in most cases too long to lead to detectable NOEs. However, the existence of a salt bridge may be deduced by determining the pKₐ of the interacting carboxylate group, which is typically lowered several pH units relative to a free carboxylate (42). By monitoring the chemical shifts as a function of pH, we determined that the pKₐ of Glu¹⁵ in Crp4 is ~1.5, consistent with the presence of a salt bridge. The structure shows that the salt bridge interacts “side-on” with Arg⁷, which provides a definitive explanation for both the unusual shift of the Arg⁷ H proton and the slow exchange behavior observed for the amino protons from the guanidinium group. Both protons have several NOEs to surrounding groups, and the large chemical shift changes observed for the Arg⁷ H proton when Glu¹⁵ is protonated support their unusually well defined position in the structure. The pH titrations also revealed unusually low pKₐ values for His¹⁰ and the C-terminal carboxyl, Arg³². Arg³² in Crp4 is found very close to the N terminus (Gly¹), and the low pKₐ is likely a reflection of the proximity of the positive charges of Gly¹ as well as the positive charge of its own side chain. Similarly, His¹⁰ does not have a direct interaction with another charged group, but the overall positive nature of Crp4 and the presence of several close by positive charges including Arg⁷, Lys¹², and Arg¹⁴ are likely disfavoring the protonated form.

Structural Effects of the Glu¹⁵ to Asp¹⁵ Mutation in Crp4—With the only exception of two sequences from guinea pig and one from rhesus enteric defensin-6, the Arg/Lys-Glu pair of oppositely charged residues is conserved throughout more than 40 known α-defensins. Interestingly, no members of the family have an Asp rather than a Glu at the corresponding position, despite the two residues only differing in a single base pair on a genetic level and both potentially having the ability to form salt bridges with positively charged residues. This observation leads to the question: what is the role of the salt bridge in the mammalian α-defensins? In a recent study on HNP2, several mutants disrupting the salt bridge were generated, and it was found that the salt bridge is not needed for biological activity nor in vitro folding of HNP2 precursors, although the in vitro folding of mature domains was affected (17). In contrast, the mutated analogues were more susceptible to degradation by neutrophil elastase, a major protease that colocalizes with HNP2 in neutrophil azurophilic granules, suggesting that the salt bridge may contribute to protease resistance in vivo. Here, despite clear structural implications of a conservative substitution at the Arg⁷-Glu¹⁵ salt bridge disruption to the Crp4 9–15 loop, biological and biochemical consequences of those structural modifications were not evident from assays of bactericidal activity and in vitro proteolytic precursor activation of Crp4 salt bridge variants, strongly suggesting a different primary role for the salt bridge at least in the case of the more highly cationic mouse Paneth cell α-defensins.

FIGURE 6. Bactericidal activity of Crp4 Arg⁷–Glu¹⁵ salt bridge variants. Exponentially growing E. coli ML 35 (A) or S. aureus (B) were exposed to the peptide concentrations shown 50 μl of 10 mM PIPES, pH 7.4, 1% TSB (trypticase soy broth) (v/v) for 1 h at 37°C (“Experimental Procedures”). Following exposure, bacteria were plated on semi-solid medium and incubated for 16 h at 37°C. Surviving bacteria were quantitated as colony-forming units/ml for each peptide concentration. Bacterial counts below 1 × 10⁵ colony-forming units/ml indicate that no surviving colonies were detected. •, Crp4; ○, (E15D)-Crp4; ▽, (E18L)-Crp4; ▽, (E15G)-Crp4.

only six residues in Crp4 compared with nine in HNP3 and eight in RK-1. The longer loop changes not only the structure of the hairpin turn but also quite dramatically the direction in which the turn projects away from the core of the molecule. Although the number of residues in this loop in HNP3 allows the two anti-parallel strands to be linked by a regular β-turn, the lack of three residues, an odd number, means that in Crp4 a bulge has to be formed for the turn to be able to adopt a regular conformation. This is facilitated by Gly²², which allows a classic β-bulge to be formed. A similar conformation is seen for Gly¹⁹ in all known α-defensin structures, and it has been shown to be crucial for the structure of the sheet and thus is likely responsible for the evolutionary conservation of a Gly at this position (18). The structure of the molecular core with the central β-sheet and the disulfide bonds is highly conserved, and the three molecules superimpose over this region with a root mean square deviation of ~1.4 Å.

Salt bridges are notoriously difficult to identify by NMR because side chain orientations are not always well defined, and proton-proton distances across salt bridges are in most cases too long to lead to detectable NOEs. However, the existence of a salt bridge may be deduced by determining the pKₐ of the interacting carboxylate group, which is typically lowered several pH units relative to a free carboxylate (42). By monitoring the chemical shifts as a function of pH, we determined that the pKₐ of Glu¹⁵ in Crp4 is ~1.5, consistent with the presence of a salt bridge. The structure shows that the salt bridge interacts “side-on” with Arg⁷, which provides a definitive explanation for both the unusual shift of the Arg⁷ H proton and the slow exchange behavior observed for the amino protons from the guanidinium group. Both protons have several NOEs to surrounding groups, and the large chemical shift changes observed for the Arg⁷ H proton when Glu¹⁵ is protonated support their unusually well defined position in the structure. The pH titrations also revealed unusually low pKₐ values for His¹⁰ and the C-terminal carboxyl, Arg³². Arg³² in Crp4 is found very close to the N terminus (Gly¹), and the low pKₐ is likely a reflection of the proximity of the positive charges of Gly¹ as well as the positive charge of its own side chain. Similarly, His¹⁰ does not have a direct interaction with another charged group, but the overall positive nature of Crp4 and the presence of several close by positive charges including Arg⁷, Lys¹², and Arg¹⁴ are likely disfavoring the protonated form.

Structural Effects of the Glu¹⁵ to Asp¹⁵ Mutation in Crp4—With the only exception of two sequences from guinea pig and one from rhesus enteric defensin-6, the Arg/Lys-Glu pair of oppositely charged residues is conserved throughout more than 40 known α-defensins. Interestingly, no members of the family have an Asp rather than a Glu at the corresponding position, despite the two residues only differing in a single base pair on a genetic level and both potentially having the ability to form salt bridges with positively charged residues. This observation leads to the question: what is the role of the salt bridge in the mammalian α-defensins? In a recent study on HNP2, several mutants disrupting the salt bridge were generated, and it was found that the salt bridge is not needed for biological activity nor in vitro folding of HNP2 precursors, although the in vitro folding of mature domains was affected (17). In contrast, the mutated analogues were more susceptible to degradation by neutrophil elastase, a major protease that colocalizes with HNP2 in neutrophil azurophilic granules, suggesting that the salt bridge may contribute to protease resistance in vivo. Here, despite clear structural implications of a conservative substitution at the Arg⁷-Glu¹⁵ salt bridge disruption to the Crp4 9–15 loop, biological and biochemical consequences of those structural modifications were not evident from assays of bactericidal activity and in vitro proteolytic precursor activation of Crp4 salt bridge variants, strongly suggesting a different primary role for the salt bridge at least in the case of the more highly cationic mouse Paneth cell α-defensins.
As is evident from an initial analysis of the NMR data, replacement of the conserved Glu does not affect the well defined overall fold, which is characterized by excellent chemical shift dispersion typical of β-sheet structures. However, although the secondary shifts (Fig. 2) are generally very similar in the two peptides, there are some significant differences, both locally around the mutations and in other regions, confirming structural changes at a minimum associated with side chain orientations. Fig. 9 shows a comparison of the lowest energy structures of native Crp4 and (E15D)-Crp4 and illustrates the main differences around the mutation. Although the projection of the Asp\(^{15}\) side chain does not differ between the two peptides, Arg\(^{7}\) has rotated, and its charged side chain projects into the solution in the mutant structure. However, the most striking difference is the position of the Tyr\(^{5}\) side chain, which folds in and fills the space left by the mutation. As a result a large number of new NOEs are seen for the Tyr side chain aromatic protons, and its position explains the large chemical shift differences observed in (E15D)-Crp4. Tyr\(^{5}\) forms new interactions with the Cys\(^{11}\)–Cys\(^{28}\) disulfide, as well as Arg\(^{7}\) and Pro\(^{30}\) side chains. The observation that the Asp\(^{15}\) \(pK_a\) in (E15D)-Crp4 is similar to what is seen for Glu\(^{15}\) in native Crp4 was initially a surprise. However, from the structure it is clear that new interactions that stabilize the charged state of Asp\(^{15}\) are formed. The reorientation of Tyr\(^{5}\) allows a strong electrostatic interaction between the Asp\(^{15}\) and the phenolic hydrogen on the Tyr\(^{5}\) side chain. Interactions between Asp and hydrogen bond donors can be highly stabilizing in protein structures (42). Furthermore, the positively charged Lys\(^{12}\) side chain can interact closely with the Asp/Glu\(^{15}\) carboxyl. Although Lys\(^{12}\) is in the proximity of the Glu/Asp\(^{15}\) in both Crp4 and (E15D)-Crp4, it appears from the structure that they interact more closely in (E15D)-Crp4. This observation is supported by the fact that in (E15D)-Crp4 the Lys\(^{12}\) side chain displays a chemical shift separation of both the δ and ε methylene protons, strongly suggesting an ordered conformation. This is in contrast to what is seen in Crp4 where the Lys\(^{12}\)
multiple conformations and exchange broadening. Furthermore, mutations of the salt bridge do not affect in vitro biological activity or the proteolytic stability of Crp4, which has been shown to be the case in HNP2 (17). Although HNP2 precursors with a disrupted salt bridge can be folded in vitro (17), perhaps the conserved salt bridge is under positive selection in α-defensins to facilitate folding or trafficking in the endoplasmic reticulum in vivo rather than determining the stability or activity of the final folded product.

**REFERENCES**

1. Ganz, T. (2003) *Nat. Rev. Immunol.* 3, 710–720
2. Selsted, M. E., and Ouellette, A. J. (2005) *Nat. Immunol.* 6, 551–557
3. Tang, Y. Q., and Selsted, M. E. (1993) *J. Biol. Chem.* 268, 6649–6653
4. Tang, Y. Q., and Selsted, M. E. (1989) *J. Biol. Chem.* 264, 4003–4007
5. Tang, Y. Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C. J., Ouellette, A. J., and Selsted, M. E. (1999) *Science* 286, 498–502
6. Ganz, T., Selsted, M. E., Sklarek, D., Harwig, S. S., Daher, K., Bainton, D. F., and Lehrer, R. I. (1985) *J. Clin. Investig.* 76, 1427–1435
7. Ouellette, A. J., Miller, S. I., Henschen, A. H., and Selsted, M. E. (1992) *FEBS Lett.* 304, 146–148
8. Jones, D. E., and Bevins, C. L. (1992) *J. Biol. Chem.* 267, 23216–23225
9. Bateman, A., MacLeod, R. J., Lembessis, P., Hu, J., Esch, F., and Solomon, S. (1996) *J. Biol. Chem.* 271, 10654–10659
10. Wu, E. R., Daniel, R., and Bateman, A. (1998) *Peptides* 19, 793–799
11. Salzman, N. H., Chou, M. M., de Jong, H., Liu, L., Porter, E. M., and Paterson, Y. (2003) *Infect. Immun.* 71, 1109–1115
12. Satoh, Y., Ishikawa, K., Ono, K., and Vollrath, L. (1986) *Digestion* 34, 115–121
13. Satoh, Y. (1988) *Cell Tissue Res.* 251, 87–93
14. Ayabe, T., Satchell, D. P., Wilson, C. L., Parks, W. C., Selsted, M. E., and Ouellette, A. J. (2000) *Nat. Immunol.* 1, 113–118
15. Ouellette, A. J., Hsieh, M. M., Nosek, M. T., Cano-Gauci, D. F., Huttner, K. M., Buick, R. N., and Selsted, M. E. (1994) *Infect. Immun.* 62, 5040–5047
16. Maemoto, A., Qu, X., Rosengren, K. J., Tanabe, H., Henschen-Edman, A., Craik, D. I., and Ouellette, A. J. (2000) *J. Biol. Chem.* 275, 44188–44196
17. Wu, Z., Li, X., de Leeuw, E., Ericksen, B., and Lu, W. (2005) *J. Biol. Chem.* 280, 43039–43047
18. Xie, C., Prahl, A., Ericsson, B., Wu, Z., Zeng, P., Li, X., Lu, W. Y., Lubkowski, J., and Lu, W. (2005) *J. Biol. Chem.* 280, 32921–32929
19. Hill, C. P., Yee, J., Selsted, M. E., and Eisenberg, D. (1991) *Science* 251, 1481–1485
20. Pardi, A., Zhang, X. L., Selsted, M. E., Skalicky, J. J., and Yip, P. F. (1992) *Biochemistry* 31, 11357–11364
21. Pardi, A., Hare, D. R., Selsted, M. E., Morrison, R. D., Bassolino, D. A., and Bach, A. C, II (1988) *J. Biol. Chem.* 263, 625–636
22. McManus, A. M., Dawson, N. F., Wade, J. D., Carrington, L. E., Winzor, D. J., and Craik, D. I. (2000) *Biochemistry* 39, 15757–15764
23. Jing, W., Hunter, H. N., Tanabe, H., Ouellette, A. J., and Vogel, H. J. (2004) *Biochemistry* 43, 15759–15766
24. Shirafuji, Y., Tanabe, H., Satchell, D. P., Henschen-Edman, A., Wilson, C. L., and Ouellette, A. J. (2003) *J. Biol. Chem.* 278, 7910–7919
25. Satchell, D. P., Sheynis, T., Shirafuji, Y., Kukushina, S., Ouellette, A. J., and Jelink, R. (2003) *J. Biol. Chem.* 278, 13838–13846
26. Satchell, D. P., Sheynis, T., Kukushina, S., Cummings, J., Vanderlick, T. K., Jelink, R., Selsted, M. E., and Ouellette, A. J. (2003) *Peptides* 24, 1795–1805
27. Lehrer, R. I., Barton, A., and Ganz, T. (1988) *J. Immunol. Methods* 108, 153–158
28. Selsted, M. E. (1993) *Genet. Eng.* 15, 131–147
29. Keller, R. (2004) *The Computer Aided Resonance Assignment Tutorial*, 1st Ed., Cantina Verlag, Goldau, Switzerland
30. Guntert, P., Mumenthaler, C., and Wuthrich, K. (1997) *J. Mol. Biol.* 273, 283–298
31. Wagner, G. (1990) *Prog. NMR Spectrosc.* 22, 101–139
32. Linge, J. P., and Nilges, M. (1999) *J. Biomol. NMR* 13, 51–59

---

**Figure 9**. Structural comparison of the NMR structures of native Crp4 and (E15D)-Crp4. The structures are shown in ribbon representation with side chains of key residues around the site of the mutation in ball-and-stick. The E15D mutation has little effect on the backbone fold but results in side chain reorientations. Most strikingly the Tyr5 (green) side chain packs into the space left by the Arg7 (blue) moving out into solution, and it forms electrostatic interactions with Asp15 (cyan) as well as stacking of the aromatic side chain with groups from Arg7, Cys28 (yellow) and Pro30 (gray). The positively charged Lys12 (red) may form electrostatic interactions with Glu15/Asp15 in both peptides.
33. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **54**, 905–921
34. Rosengren, K. I., Daly, N. L., Plan, M. R., Waine, C., and Craik, D. J. (2003) *J. Biol. Chem.* **278**, 8606–8616
35. Wishart, D. S., Bigam, C. G., Holm, A., Hodges, R. S., and Sykes, B. D. (1995) *J. Biomol. NMR* **5**, 67–81
36. Cierpicki, T., Zhukov, I., Byrd, R. A., and Otlewski, J. (2002) *J. Magn. Reson.* **157**, 178–180
37. Cierpicki, T., and Otlewski, J. (2001) *J. Biomol. NMR* **21**, 249–261
38. Bundi, A., and Wuthrich, K. (1979) *Biopolymers* **18**, 285–297
39. Matthew, J. B., Gurd, F. R., Garcia-Moreno, B., Flanagan, M. A., March, K. L., and Shire, S. J. (1985) *CRC Crit. Rev. Biochem.* **18**, 91–197
40. Wilson, C. L., Ouellette, A. J., Satchell, D. P., Ayabe, T., Lopez-Boado, Y. S., Stratman, J. L., Hultgren, S. J., Matrisian, L. M., and Parks, W. C. (1999) *Science* **286**, 113–117
41. Ayabe, T., Satchell, D. P., Pesendorfer, P., Tanabe, H., Wilson, C. L., Hagen, S. J., and Ouellette, A. J. (2002) *J. Biol. Chem.* **277**, 5219–5228
42. Joshi, M. D., Hedberg, A., and McIntosh, L. P. (1997) *Protein Sci.* **6**, 2667–2670