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 due to the shortened side chain. Despite disruption of this structural feature, the peptide variant retains a well-defined native fold due to 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 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 around 40 amino acids and possess a Cys I-CysV, CysII-CysIV, CysIII-CysVI array (3), while the α-defensins comprise around 32-36 amino acids and a characteristic CysI-CysVI, CysII-CysIV, CysIII-CysV arrangement of their disulfide bonds (4). The θ-defensins are considerably smaller at only 18 residues and have a Cys I-CysVI, CysII-CysV, CysIII-CysIV framework, combined 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

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response to cholinergic stimulation or exposure to bacteria or bacterial antigens (12-14). The mouse Paneth cell α-defensins, termed cryptdins (Crps), 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 non-specific 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 positions 7 and 15, respectively, in Crp4 (16-18). The disulfide bonds maintain the α-defensin fold, with mutations in either the Cys I-Cys VI or Cys III-Cys V 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 Gly 19 residue is positioned in a classical β-bulge in the middle of β-strand 2. The ability of Gly to adopt a phi/psi angle combination not normally accessible by L-amino acids, due to 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 NP-2 (20) and NP-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 *E. coli* as N-terminal 6X-histidine tagged fusion proteins from the *EcoRI* and *SalI* 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 GGT TT GTAT GCT AT (ER1-Met-C4-F) paired with reverse primer ATATA TGT GCGG GCCA GCAGT ACA A (SLpMALCrp4R) as reported previously (24). For proCrp4, forward primer pETPCr4-F (5’- GCCGG AATTC ATG TCCTA TCCAA AACAC A) was paired with reverse primer SLpMALCrp4R (5’- ATATA TGT GCGG GCCA GCAGT ACA A), corresponding to nucleotides 104 to 119 and 301 to 327 in preproCrp4 cDNA (24). In all instances, 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 sec, 60°C for 30 sec, and 72°C for 30 sec for 30 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 reaction #3, using T7 promoter and terminator primers as amplimers. Corresponding proCrp4 templates of these variants were prepared as described (16). All 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, Inc., Madison, WI), and transformed into E. coli BL21(DE3)-CodonPlus-RIL cells (Stratagene) for recombinant expression (24,25).

**Mutagenesis at Glu** and Arg 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 reaction #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, Carlsbad, CA), a downstream sequencing primer in the pET-28a vector. In PCR reaction #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 CGAGT TCGTG GG (C4E75D for) and CCCAC GAACT CGATC TCCTC TTTTG CA (C4E75D rev); for (E15K)-Crp4, TGCAA AAGAG GAAAG CGAGT TCGTG GG (C4E75K for) and CCCAC GAACT CGCTT TCCTC TTTTG CA (C4E75K rev); for (E15L)-Crp4, TGCAA AAGAG GACTA CGAGT TCGTG GG (C4E75L for) and CCCAC GAAGT CGTAG TCCTC TTTTG CA (C4E75L rev); for TGCAA AAGAG GAGGA CGAGT TCGTG GG (C4E75G for) and CCCAC GAAGT CGTCC TCCTC TTTTG CA (C4E75G rev). After amplification at 94°C for 5 min, followed by successive cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec for 30 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 reaction #3, using T7 promoter and terminator primers as amplimers.

**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-thiogalactopyranoside for 6 h at 37°C, 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 (Ni-NTA, Qiagen) resin affinity chromatography (24). After CNBr cleavage, Crp4 peptides were purified by C18 reverse-phase high performance liquid chromatography (RP-HPLC), quantitated by bicinchoninic acid (Pierce), and 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, Department of Chemistry, University of California, Irvine.

**Bactericidal peptide assays** Recombinant peptides were tested for microbial activity against E. coli ML35 and Staphylococcus aureus 710a (27). Bacteria (5 x 10⁶ CFU/ml) were resuspended in 10 mM PIPES (pH 7.4) supplemented with 0.01 vol of
trypticase soy broth were incubated with test peptides in 50 µl for 1 h at 37°C, and surviving bacteria were counted as colony forming units per ml (CFU/ml) after overnight growth on semi-solid media (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 (AU-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 N-[2-hydroxyethyl]piperizine-N'-[2-ethanesulfonic acid] (pH 7.4), 150 mM NaCl, 5 mM CaCl2 for 18-24 h at 37 oC, and equimolar samples of all digests were analyzed by AU-PAGE (16,24).

NMR Spectroscopy
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% (v/v) D2O at pH 4.5. All spectral data were recorded at 600 and 500 MHz on Bruker Avance NMR spectrometers. Two-dimensional experiments recorded included DQF-COSY, TOCSY using an MLEV-17 spin lock sequence with a mixing time of 80 ms, and NOESY with mixing times of 100 ms, 150 ms or 200 ms. Selected spectral data, including preliminary data on an additional salt bridge deficient analogue, (R7G)-Crp4, are presented as supplementary information. 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. Spectra were processed on a Silicon Graphics Octane workstation 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 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 carboxyterminal group were estimated by monitoring the effects of pH in the range from 1.1-7.5 on the chemical shifts of resonances within the vicinity of the carboxylate 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 in order to compensate for the significantly lower sample concentration. The spectral data were analyzed and cross peaks were assigned and integrated in CARA (29) and converted to distance restraints using DYANA (30). Backbone dihedral restraints were inferred from 1JHN-Hα coupling constants derived either from the 1D or a high digital resolution DQF-COSY. The dihedral angle ϕ was restrained to -120 ± 30° for 1JHN-Hα greater than 8 Hz (residues 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 Hαi-1-HNi NOE compared to the intra residual Hαi-HNi NOE. Side chain χ1 angles and stereo specific assignments were determined on the basis of observed NOE and 3JHα-Hβ coupling patterns (31). For a t2g3 side chain conformation, the χ1 angles were restrained to -60 ± 30° (residues Cys4, Tyr5, Cys11, Glu15, Tyr25, Cys28, Cys29 and Cys34, Cys31, Asp31, Arg18, Tyr1, Cys34, Cys28 for Crp4 and (E15D)-Crp4 respectively) and for a g2t3 conformation the angles were constrained to 180 ± 30° (residues Val17, Ile23, Tyr27 and...
Cys<sup>6</sup>, Arg<sup>13</sup>, Tyr<sup>27</sup> for Crp4 and (E15D)-Crp4 respectively). No residues could be confirmed to be in the g<sup>2</sup>g<sup>3</sup> conformation based on experimental data. Hydrogen bonds were included into the structure calculations for all amide protons concluded to be slow exchanging, or having a T<sub>c</sub> 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 (32) within the program CNS (33) as described previously (34). The protocol involved a high temperature phase comprising 4000 steps of 0.015 ps of torsion angle dynamics, a cooling phase with 4000 steps of 0.015 ps of torsion angle dynamics during which the temperature is lowered to 0 K, and finally an energy minimization phase comprising 5000 steps of Powell minimization. The refinement in explicit water involves the following steps: first, heating to 500 K via steps of 100K, each comprising 50 steps of 0.005 ps of Cartesian dynamics; second, 2500 steps of 0.005 ps of Cartesian dynamics at 500K, before a cooling phase where the temperature is lowered in steps of 100K, each comprising 2500 steps of 0.005 ps of Cartesian dynamics; finally, the structures were minimized with 2000 steps of Powell minimization.

Protein structures were analyzed using PROMOTIF and PROCHECK and displayed using MOLMOL. Ramachandran analysis showed that ~80% of the residues are in the most favored regions with the remaining in the additionally allowed (~20%). The coordinates representing the solution structure of Crp4 and (E15D)-Crp4 and the experimental restraints have been submitted to the PDB and given the access code 2GW9 and 2GWP respectively.

RESULTS

NMR Spectroscopy and resonance assignments of Crp4 and (E15D)-Crp4

Sets of 2D NMR data were recorded for both Crp4 and (E15D)-Crp4 at 600 MHz. The spectral data were of high quality with excellent signal dispersion indicative of a well-structured peptide and no additional spin systems indicating conformational heterogeneity (Supplementary information). For both peptides full assignments of both backbone and side chain resonances were achieved using 2D sequential assignment strategies. The chemical shift assignments for native Crp4 were in all cases consistent with the assignments reported by Jing et al (23). As expected for a single point mutated peptide, the chemical shifts of most residues of (E15D)-Crp4 were very similar to those of the native peptide, but some significant differences were observed in several regions of the sequence. Most strikingly, the Hε side chain proton of Arg<sup>7</sup>, which in native Crp4 is downfield-shifted by >2 ppm (9.61 ppm), is found at 7.24 ppm in the mutant, close to the expected random coil shift of Arg residues. Other signals from the Arg<sup>7</sup> 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 Arg<sup>7</sup> could not be detected in the mutant. Additional significant differences in the mutant chemical shifts include the two Hβ protons of Cys<sup>28</sup>, which are shifted upfield by 1.5 and 1.0 ppm respectively, Hβ2 of Cys<sup>11</sup> (upfield 0.5 ppm), HN and both Hβ’s of Cys<sup>4</sup> (all downfield ~0.5 ppm), and Hα and Hβ2 of Tyr<sup>5</sup> (both downfield ~0.5 ppm). Crp4 contains one proline residue, Pro<sup>10</sup>, which in both the native and mutant structures was found to be in the trans conformation as evident from strong Hα<sub>i-1</sub>-Hδ<sub>i</sub> 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). Figure 2 shows the secondary Hα shifts for Crp4 and (E15D)-Crp4, from which it is clear that the general trend is stretches of positive numbers, consistent with the triple-stranded β-sheet that is typical of an α-defensin fold. With the exception of Tyr<sup>5</sup> 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, as intramolecularly hydrogen-bonded amides have a low sensitivity to temperature (36,37). For both Crp4 and (E15D)-Crp4 the temperature dependencies of the amide protons 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 ppb/K are involved in intramolecular hydrogen bonds (the probability increases to >93%, if -4.0 < Tc < -1.0 ppb/K) (37). In Crp4 and (E15D)-Crp4 11 amides were found to have temperature coefficients > -4.6 ppb/K. The data are in good agreement with amide D2O exchange experiments, with 7 of 8 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 ppb/K. However, Tc’s 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 likely 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’s 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 bonded from Tc 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 in order to examine the pKₐ of Glu/Asp15. Favorable electrostatic interactions such as salt bridges can have a dramatic effect on the pKₐ 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. Figure 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 (A) and (E15D)-Crp4 (B). It is evident that the ionization states of three groups are affected by the pH changes within this range, namely His10, the Arg8 carboxy terminal group and Glu/Asp15. The data were fitted by non-linear regression analysis, which in both Crp4 and (E15D)-Crp4 gave the pKa values ~5.6 and ~2.3 for His10 and the carboxy terminal respectively. Strikingly, Crp4 Glu15 and (E15D)-Crp4 Asp15 carboxyl groups are largely unaffected prior to reaching a pH below 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 pKₐs of Glu/Asp in Crp4 and (E15D)-Crp4 are 1.1-1.5 and 1.0-1.7 respectively. The expected pKₐs for His, α, β and γ carboxyl groups are ~6.5, 3.5-4.3, 3.9-4.0 and 4.3-4.5 respectively (38,39), hence the non-charged 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 Glu15 carboxyl group is the Hε proton of Arg7 in native Crp4, which at the lower pH is starting to move back from its downfield shifted position towards its random coil value.

Structure Determination and Description of the 3D structure

From the NMR data a set of restraints including upper limit distance restraints based on NOE cross-peak intensities, backbone φ and side chain χ₁ 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 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.

Figure 4 shows a stereoview 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 β-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 residues 18-19 and 21-22. The conformation of these bulges requires Gly residues at positions 19 and 21, due to 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 β-turns comprising residues 12-15 and 22-25, both of which adopt type II conformation, and one γ-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 Figure 5. Also indicated in Figure 5 is the hydrogen bond network that was identified by analysis of amide exchange, amide proton T1c’s 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 Cys11/Val17/Leu26/Cys28, Leu3/Tyr5/Pro30 and Cys5/Cys6/Arg18/Cys21/Tyr27. In addition, in native Crp4 an interaction between the Glu15 and Arg7 side chains can be identified. The positioning of the two side chains, which is confirmed by NOE patterns, indicates that the Glu15 carboxylate group points towards the side of the Arg side chain, coordinating one of its oxygen atoms with the Hε proton and the other with one of the amino protons from the guanidinium group. The salt bridge, together with the Cys18-Cys28 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 side chain, which fills the void left by the larger Glu and the Arg 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 and the positively charged Lys.

The canonical Arg7-Glu15 salt bridge is not a determinant of Crp4 bactericidal activity. To investigate the contribution of the Arg7-Glu15 salt bridge to Crp4 microbicidal function, the bactericidal activities of native Crp4 and Crp4 variants with salt bridge disruptions were compared against E. coli and S. aureus 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 µg/ml (Fig 6, and data not shown). Results of additional assays performed against strains of Vibrio cholerae, Listeria monocytogenes, and wild-type 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 Arg7-Glu15 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 (Arg5-Glu13) of human α-defensin HNP2 have bactericidal activities equivalent to that of native HNP2 (17). Because mutagenesis of the canonical Arg7-Glu15 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 Arg\textsuperscript{7}-Glu\textsuperscript{15} 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 Arg\textsuperscript{7} or Glu\textsuperscript{15} mutants of Crp4 and proCrp4 would be subject to MMP-7-mediated degradation was tested. Native Crp4 is completely resistant to MMP-7 \textit{in vitro}, and MMP-7 activates native proCrp4 without cleaving within the α-defensin moiety of the precursor (Fig 7 and (16,24,25,41). In contrast to the sensitivity of Arg\textsuperscript{5}/Glu\textsuperscript{13} 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 does not ensure sensitivity to proteolysis \textit{per se}, but that variations in peptide primary structure exclusive of canonical positions, in particular the strong electropositive charge of Crp4, also contribute to proteolytic instability.

**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, \textit{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 mis-assignment 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 Glu\textsuperscript{15}-Arg\textsuperscript{7} 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 to other α-defensin structures

The mis-assignment 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. Since the hydrogen bonds were included in all the final structure calculations the other restraints used (eg NOE’s) did not move the structure towards 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 beta 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 nsec the relaxed structure resembled the one reported here and this remained stable during a further 14 nsec of simulation time (Zhou and Vogel, data not shown). 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. Figure 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 only six residues in Crp4 compared to 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 an RMSD of ~1.4Å.

Salt bridges are notoriously difficult to identify by NMR as 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 pKd 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 pKd 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 Hε proton and the slow exchange behaviour 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 Hε proton when Glu15 is protonated support their unusually well-defined position in the structure. The pH titrations also revealed unusually low pKd's for His10 and the C-terminal carboxyl, Arg32. Arg12 in Crp4 is found very close to the N-terminus (Gly1) and the low pKd 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 Arg13 are likely disfavouring 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 co-localizes 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 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 (Figure 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. Figure 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 Asp15 side chain does not differ between the two peptides, Arg7 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 Tyr5 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. The observation that in HNP2 mutations disrupting the salt bridge lead to highly increased susceptibility to proteolytic degradation strongly suggests that the loop structure has been disrupted and is likely flexible. However, no such effect is seen in (E15D)-Crp4. Although structural flexibility is hard to judge from the structure itself there is no evidence from line broadening, lack of NOEs or random coil-like chemical shifts in this area, strongly suggesting that the compensating interactions in (E15D)-Crp4 are enough to retain the well defined stable fold.

CONCLUDING REMARKS
In summary we have reported the solution structures and biochemical properties of Crp4 and a salt-bridge deficient (E15D)-Crp4 mutant. Despite the extensive conservation of this salt-bridge throughout the α-defensin peptide family, our findings show that its disruption does not significantly affect the overall fold of the peptide, but does induce some local structural changes involving side chains around the point of the mutation. These changes compensate for the lack of the salt bridge and appear to be able to stabilize the fold as evident from the apparent well defined structure, as well as the low pKₐ of Asp15 in (E15D)-Crp4. The structural data are consistent with a stable structure and lacks evidence of dynamic processes such as additional resonances from 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 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.

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**Table 1:** NMR and refinement statistics for the families of NMR structures

| NMR distance & dihedral constraints | Crp4  | (E15D)-Crp4 |
|-------------------------------------|-------|-------------|
| Distance constraints                |       |             |
| Total Inter-residue NOE             | 442   | 256         |
| Sequential (|i-j| = 1)                      | 160   | 128         |
| Medium-range (|i-j| ≤ 4)                    | 84    | 38          |
| Long-range (|i-j| ≥ 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 and s.d.)          |       |             |
| Distance constraints (> 0.2 Å)      | 0.1 /structure | 0.6 /structure |
| Max. distance constraint violation (Å) | 0.22 Å | 0.24 Å |
| Dihedral angle constraints (> 3°)   | 0 / structure | 0 / structure |
| Max. dihedral angle violation (°)   | 2.6°  | 3.0°        |
| Deviations from idealized geometry  |       |             |
| Bond lengths (Å)                    | 0.0041 ±0.0001 | 0.0039 ±0.0002 |
| Bond angles (°)                     | 0.56 ±0.037  | 0.56 ±0.027 |
| Improper (°)                        | 0.44 ±0.032  | 0.43 ±0.033 |
| Average pairwise r.m.s.d.* (Å)      |       |             |
| 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%        |

**Pairwise r.m.s.d. was calculated among 20 refined structures over the structured part of the molecules, residues 4-29.**
Figure Legends

**Figure 1:** Sequence comparisons of mouse cryptdins and selected α-defensins from various sources. The characteristic cysteine pattern and the disulfide connectivities are indicated by connecting lines. Other conserved residues, including Arg⁷, Glu¹⁵ and Gly¹⁹ (using the numbering of Crp4) are shown in bold. The sequence of the mutant studied here is given under (E15D)-Crp4.

**Figure 2:** Secondary Hα shifts. The difference between observed chemical shifts and the expected random coil values are shown for all residues in Crp4 and (E15D)-Crp4. Stretches of three or more residues with a secondary shift >0.1 ppm are characteristic of β-strands.

**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 non-linear regression analysis the pKₐ was determined to be ~5.6 for His¹⁰ (affected resonances: HN 9, HN 10, HN 11, HN 12, HB1 10, HB2 10) and ~2.2 for the C-terminus (affected resonances: HN 32, HA 32, HB2 32). Finally at low pH several resonances were affected by the ionization state of Glu/Asp¹⁵ (HE 7, HN 12, HB2 28, HB2 11, HG1 15, HG2 15 in Crp4 and HN 12, HB2 28, HB1 4, HN31, HN16, HB1 15, HB2 15 in (E15D)-Crp4) were identified and indicated a pKₐ of >1.5 in both structures.

**Figure 4:** Stereoview of the structural families of Crp4 (A) and (E15D)-Crp4 (B). The structures are superimposed over the well-defined regions (residues 4-29). Disulfide bonds are shown in grey.

**Figure 5:** Schematic illustration of the secondary structure of Crp4. Observed NOEs are shown as arrows with thick and thin arrows corresponding to strong-medium and weaker NOE interactions respectively. Broken arrows represent NOE connections that are expected to be present but can not be confirmed due to overlap with strong sequential/intra residual cross-peaks. Hydrogen bonds for which donors were identified from amide exchange and Tc’s and acceptors identified by preliminary structure calculations are indicated by thick broken lines. An identical network of NOEs albeit with a few of the weaker long range ones missing due to the lower concentration used is seen in (E15D)-Crp4.

**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 10 mM PIPES (pH 7.4), 1% TSB for 1 h at 37°C (Materials and Methods). Following exposure, bacteria were plated on semi-solid media and incubated for 16 h at 37°C. Surviving bacteria were quantitated as colony forming units per ml (CFU/ml) for each peptide concentration. Bacterial counts below 1 x 10³ CFU/ml indicate that no surviving colonies were detected. Symbols: Crp4 (- ■ -), (E15D)-Crp4 (- ● -), (E15L)-Crp4 (- ▼ -), (E15G)-Crp4 (- ▽ -).

**Figure 7:** Crp4 and proCrp4 Arg⁷-Glu¹⁵ salt bridge variants are insensitive to MMP-7 mediated proteolysis. Samples of Crp4 (5 µg), proCrp4 (11 µg), and Crp4 and
proCrp4 variants with substitutions at Arg\textsuperscript{7} or Glu\textsuperscript{15} were incubated overnight at 37°C in the presence or absence of MMP-7, the mouse pro-α-defensin convertase. In panel A, 5 µg samples of Crp4 (lanes 1, 2), (R7G)-Crp4 (lanes 3, 4), and (E15G)-Crp4 (lanes 5, 6) were incubated with (+) or without (-) MMP-7, subjected to analytical acid-urea PAGE, and stained with Coomassie blue (Materials and Methods). Upper arrow denotes the position of the MMP-7 enzyme. In panel B, 5 µg samples of Crp4 (lanes 1, 2) and (E15D)-Crp4 (lanes 5, 6), or 10 µg samples of proCrp4 (lanes 3, 4) and proCrp4 variants (E15D)-proCrp4 (lanes 7, 8), (R7A)-proCrp4 (lanes 9, 10), or (R7G)-proCrp4 (lanes 11, 12) were incubated without (-) or with (+) MMP-7, analyzed by acid-urea PAGE, and stained with Coomassie blue as in panel A. The difference in position of the bands containing the Crp4 analogues is a result of the electrophoretic mobility in AU-PAGE analysis being affected by the number of charged residues in the peptide.

**Figure 8:** Comparison of the NMR solution structure of Crp4 (A), the crystal structure of HNP3(19) (B) (PDB code 1DFN) and NMR solution structure of RK-1(22) (C) (PDB code 1EWS). All structures are shown in ribbon style with the disulfide bonds in yellow ball-and-stick representation, illustrating the cross-braced central β-sheet. The main structural differences are in the hairpin and are a direct result of the different lengths of the loop between Cys\textsuperscript{IV} and Cys\textsuperscript{V} (6, 8 and 9 residues in Crp4, RK-1 and HNP3 respectively). The side chains of the conserved Arg/Lys-Glu form a tight electrostatic interaction in all three structures. Both termini are flexible in all peptides as evident from disorder in the structural family of Crp4 and RK-1 and from the lack of density for the terminal residues in the x-ray analysis of HNP3.

**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 Tyr\textsuperscript{5} (green) side chain packs into the space left by the Arg\textsuperscript{7} (blue) moving out into solution and it forms electrostatic interactions with Asp\textsuperscript{15} (cyan) as well as stacking of the aromatic side chain with groups from Arg\textsuperscript{7}, Cys\textsuperscript{28} (yellow) and Pro\textsuperscript{30} (grey). The positively charged Lys\textsuperscript{12} (red) may form electrostatic interactions with Glu\textsuperscript{15}/Asp\textsuperscript{15} in both peptides.
Figure 1

**Human**

HNP1       ACYCRIPTACIAGERGYTCIYQGRLLAFCC
HNP2       CYCRIPTACIAGERGYTCIYQGRLLAFCC
HNP3       DCYCRIPTACIAGERGYTCIYQGRLLAFCC
HNP4       VCSCRLVFCRRTTELVGNCLIGGVSFTYCCTRV
5         ATCYCRTGRCATRESLSGVEISGRLYRLCC
6         AFTCHCR-SCYSTSEYSGTCTVMGINHRFCCL

**Rabbit**

RK-1       MFCSSCKY-CDPWEVIDSCGLFNSKI-CCKREK
RK-2       KPYCSCKWR-CGIEEKKICHKFPITYVCCRRP

**Mouse**

Crp1       LRDLVCYCRSRGCKGRERMTGTCKGHLLYTLCCR
Crp2       LRDLVCYCRTRGCKRRERMTGTCRHGLMYTLCCR
Crp3       LRDLVCYCRKRGCKRRERMTGTCRHGLMYTLCCR
Crp5       LSKKLICYCRIRGCKRRERVFGETCRNLFPTFVFCCS

Crp4       GLLCYRCRGCHCKRGERVRGTC-GIRFLY--CCPRR
E15D-Crp4  GLLCYRCRGCHCKRGRDVRGTC-GIRFLY--CCPRR
Figure 2
Figure 3

(A) Crp4

(B) E15D-Crp4
Figure 4

(A)

(B)
Figure 6

![Graph showing bacterial cell survival vs. peptide concentration](https://example.com/graph.png)
Figure 9
Structural and functional characterization of the conserved salt bridge in mammalian paneth cell α-defensins: solution structures of mouse cryptdin-4 and (E15D)-cryptdin-4
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
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