The Structure of Human β-Defensin-2 Shows Evidence of Higher Order Oligomerization*

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Defensins are small cationic peptides that are crucial components of innate immunity, serving as both antimicrobial agents and chemoattractant molecules. The specific mechanism of antimicrobial activity involves permeabilization of bacterial membranes. It has been postulated that individual monomers oligomerize to form a pore through anionic membranes, although the evidence is only indirect. Here, we report two high resolution x-ray structures of human β-defensin-2 (hBD2). The phases were experimentally determined by the multiwavelength anomalous diffraction method, utilizing a novel, rapid method of derivatization with halide ions. Although the shape and charge distribution of the monomer are similar to those of other defensins, an additional α-helical region makes this protein topologically distinct from the mammalian α- and β-defensin structures reported previously. hBD2 forms dimers topologically distinct from that of human neutrophil peptide-3. The quaternary octameric arrangement of hBD2 is conserved in two crystal forms. These structures provide the first detailed description of dimerization of β-defensins, and we postulate that the mode of dimerization of hBD2 is representative of other β-defensins. The structural and electrostatic properties of the hBD2 octamer support an electrostatic charge-based mechanism of membrane permeabilization by β-defensins, rather than a mechanism based on formation of bilayer-spanning pores.

Multicellular organisms share an innate defense against microorganisms that is based on small cationic peptides known as defensins (1, 2). Mammals contain two classes of defensins, named α and β, based on the arrangement of cysteines within their sequences. To date, six α-defensins and two β-defensins (hBD1 and hBD2) have been identified in humans. Four of the six α-defensins are sequestered in secretory granules within neutrophils and are termed neutrophil peptides (hNP1–4), whereas the remaining two α-defensins (human α-defensins 5 and 6) are secreted from Paneth cells in the gastrointestinal tract. α-Defensins manifest microbicidal activity only at relatively high concentrations and have a broad spectrum of activity, killing Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses (3). In contrast, β-defensins are inducible, more potent, and selective, killing mainly Gram-negative bacteria and yeast (4). Both α- and β-defensins also mobilize cells engaged in adaptive immune responses (5, 6).

The microbicidal activity of defensins stems from the permeabilization of anionic lipid bilayers and the subsequent release of cellular contents (7–11). Interactions between defensins and bacterial membranes are governed mainly by electrostatic forces (11). One mechanism of permeabilization is thought to involve the formation of ion pores in bacterial membranes (12, 13). The existence of pores and the estimation of their dimensions are based on studies of ion conductance through the bilayer (14, 15) and the passage of molecules of various sizes through model lipid vesicles and bilayers (13, 16). A physical model of such a pore has been constructed based on the x-ray structure of hNP3 (11, 13, 17). Based on its amphiphilic charge distribution and dimeric shape, 12 monomers of hNP3 were arranged to form a membrane-spanning pore of ~20 Å inner diameter (13, 16).

A second model has also been proposed for other small cationic antimicrobial peptides, such as magainins (18) and cecropins (19). According to this model (sometimes called the “carpet” model), the structural aspects of the interaction between peptides and the microbial membrane are of secondary importance. Rather than forming distinct and structurally stable ion pores, these peptides are thought to aggregate into positively charged patches that neutralize anionic lipid headgroups of the membrane over a wide area around the peptides. This neutralization disrupts the integrity of the lipid bilayer, causing transient gaps to arise and allowing ions (and larger

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The atomic coordinates and structure factors (code 1FD3 and 1FD4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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††† The abbreviations used are: hBD, human β-defensin; a.u., asymmetric unit(s); hBD, bovine β-defensin; hNP, human neutrophil peptide; MAD, multiwavelength anomalous diffraction; NMR, nuclear magnetic resonance; PEG, polyethylene glycol; MOPS, 4-morpholinepropanesulfonic acid.
molecules, depending on the extent of local disruption) to permeate the membrane (20).

Except for the x-ray structure of hNP3, no other oligomeric structures of defensins have been determined. Because oligomerization of defensins may occur only at elevated protein concentrations (>3 mM) and possibly in the sterically constraining environment of a bacterial membrane, a crystalline environment is probably suitable for studying the quaternary structure of defensins. To investigate the importance of the structure for the antimicrobial activity of hBD2, we solved the x-ray structure of this defensin by using a novel method of derivatizing the protein crystals, followed by the multiwavelength anomalous diffraction (MAD) phasing protocol. Additionally, we studied the permeabilization of large unilamellar vesicles by native and reductively alkylated hBD2, as well as the oligomerization of native hBD2 in solution.

EXPERIMENTAL PROCEDURES

Crystallization—Protein was obtained from PeproTech (Rocky Hill, NJ). The homogeneity of the preparation was determined by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Crystals were obtained by hanging-drop diffusion with equal volumes of concentrated protein (30 mg/mL) and reservoir solution containing 30% PEG 4000, 0.1 M Tris-HCl (pH 8.5), 0.2 M Li₂SO₄. Two crystal forms grew together in the same hanging drops. The orthorhombic form belongs to space group P2₁2₁2₁ with cell constants a = 50.06 Å, b = 103.91 Å, and c = 28.27 Å. The monoclinic form belongs to space group P2₁ with cell constants a = 54.53 Å, b = 78.95 Å, c = 74.27 Å, and β = 105.30°. Individual crystal forms could be identified by their morphologies, with the orthorhombic form growing as rods and the monoclinic forms growing as square plates. The crystal forms were propagated and grown by macroseeding hanging drops preequilibrated to 20% PEG 4000, 0.066 M Tris-HCl (pH 8.5), 0.133 M Li₂SO₄ with washed seed crystals of the intended form. The monoclinic form was the preferred form.

Data Collection and Helical Derivatization—Data were collected using flash-frozen crystals. Prior to freezing in the 100 K nitrogen stream, crystals were soaked in cryoprotectant solution containing 36% PEG 4000, 0.16 M MOPS (pH 7.1), 0.32 M Li₂SO₄, 10% glycerol for approximately 60 s. Native and derivative data of both crystal forms were collected at beamline X8B (National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY) using an ADSC Quantum 4 CCD detector. CCD images were indexed, processed, merged, and scaled using DENZO and SCALEPACK (21). The data collection statistics are shown in Table I.

Orthorhombic form crystals were soaked briefly (60 s) against KBr and KI (0.25 M) in the cryoprotectant solution immediately prior to freezing in the nitrogen stream and diffraction experiments (22). MAD data sets for nine native crystal forms (see Table I) were collected for the KBr-soaked crystal, and one data set was collected at 1.54 Å for the KI-soaked crystal. The anomalous differences calculated by PHASES (23) were 2.1% (based on F²) and 1.5% (based on F) for (1.4-Å resolution) for the KBr-soaked crystal and data set, and 4.1% (based on F²) and 2.7% (based on F) for (1.9-Å resolution) for the KI-soaked crystal. Bromide and iodide sites were placed automatically with a density cutoff of 2.5σ and hydrogen bonding constraints using the program SHEXL (24), and visually inspected after completion of structural refinement. The final monoclinic model contains 814 water molecules and 5 sulfate ions added. The R value for all reflections (25–1.7 Å) is 18.8% (Rfree 25.5%).

Determination of Aggregation State in Solution—hBD2 was dissolved in 100 mM Tris (pH 8.0) to 30 mg/mL. The protein was cleared by centrifugation and filtered using a 0.05-μm filter immediately prior to light-scattering measurements. A DynaPro-801 instrument was used for the measurements, and multicomponent analysis was performed using the software provided with the instrument.

Membrane Permeabilization—Large unilamellar vesicles were formed by extrusion through 0.2-μm nuclospore polycarbonate membranes under nitrogen pressure as described (15) and loaded with 5,6-carboxyfluorescein (376 Da) fluorescent dextran (3 or 10 kDa). All reagents were from Molecular Probes (Eugene, OR). The reduction and carboxyamidomethylation of hBD2 were performed as described (31). Mass spectrometry analysis of modified hBD2 revealed alkylation of all six cysteine residues.

RESULTS AND DISCUSSION

Antibacterial Properties—To show that the activity of hBD2 is dependent not on charge alone but also on its native structure, we performed experiments on the permeabilization of artificial lipid vesicles, using both native and reduced hBD2. During our experiments, native hBD2 (0.125 μM) induced a 50% release of a small (400 Da) marker from large unilamellar vesicles made from anionic lipid palmitoyloleoylphosphatidylglycerol. Under these conditions, about 20% of 3-kDa dextran and less than 5% of 10-kDa dextran were released. The effect of hBD2 was lipid dependent; the increase in the proportion of neutral palmitoyloleoylphosphatidylcholine led to a dramatically decreased sensitivity to hBD2, indicating the electrostatic nature of hBD2 interaction with lipids. The correct fold of hBD2 is clearly required for this effect, because hBD2 linearized by disulfide bond reduction and carboxyamidomethylation was ineffective even at concentrations that resulted in 100% release of the vesicle contents induced by native hBD2. These results indicate that both the native structure of hBD2 and its charge interaction with the membrane are critical for the membrane permeabilizing activity of this protein.

Crystal Structure of Human β-Defensin-2

The crystal structure of the orthorhombic form was solved by the MAD technique, using an anomalous signal originating from bromide and iodide anions soaked into the crystals of the hBD2 derivatives. The method that we used to derivatize the protein has been successfully applied to several test proteins (22). However, hBD2 is one of the first proteins of unknown structure to be solved using this
technique. The electron density map determined from experimentally derived phases was of excellent quality (Fig. 1), and approximately 90% of the final structure could be automatically traced from the initial map. The remainder of the structure was built using the x-ray data collected for the native hBD2 crystal at 1.35-Å resolution. Four individual protein chains (A–D) are located within the asymmetric unit (a.u.), and all residues, except two C-terminal proline residues of the B and C chains, are traceable in the final electron density maps.

The a.u. of the monoclinic form of hBD2 has a volume nearly 4 times that of the orthorhombic form. Therefore, taking into account the possible solvent contents, we assumed that hBD2 contains 14–20 monomers per a.u. Because the four monomers of the orthorhombic form exist as two topologically identical dimers within the a.u., we expected approximately eight of such dimers to be present in the a.u. of the monoclinic form. We solved phases of the monoclinic form by using the molecular replacement technique, which confirmed the presence of 16 monomers per a.u. All residues for the 16 monomers (A–P) could be easily placed in the electron density, and the structure was refined to 1.7-Å resolution. Data and refinement statistics are shown in Table I.

**Comments on Derivatization Protocol**—After soaking crystals in KBr- and KI-containing solutions (immediately prior to flash freezing and data collection), bromide and iodide anions bind to similar sites on the surface of hBD2. To investigate in more detail the anion-binding sites, after calculating phases from the MAD data and refining the orthorhombic structure, we refined the coordinates of the complete model (including halide anions) against the derivative x-ray data to the maximum resolution (1.4 and 1.9 Å, respectively), using the program CNS. The anion-binding sites are located within cavities and depressions formed by mixed hydrophobic and hydrophilic environments. Coordination is mainly through van der Waals contacts, with few ionic interactions. The occupancies of the ions are relatively low (20–40% as refined by MLPHARE (Ref. 25); during the CNS refinement, these occupancies were fixed to 0.5 (Ref. 29). Despite low occupancies, the high atomic numbers of the halide anions resulted in clear peaks in electron density maps calculated from the derivative x-ray data. Additionally, even though only a moderate concentration (0.25 M) of KBr and KI was used in our experiments (due to problems of crystal stability in the resulting soak solution), the successful derivatization and phasing were the combined result of such factors as the overall positive charge of the protein, attracting anions from the bulk solvent, and the high resolution of anomalous data. The use of surface-bound halide anions to solve the phase problem of protein crystallography provides a simpler, relatively universal, and rapid method of solving crystal structures. Because only moderate concentrations of halides are necessary, this approach can be applied to a wide range of protein crystals. Increasing the concentration of halide anions probably would improve the strength of the anomalous signal sufficiently to allow successful structure solution using significantly lower resolution x-ray data.

**Structural Features**—The hBD2 monomer displays a fold similar to that of hNP3 (17) and bovine β-defensin-12 (bBD12) (32), with a three-stranded β-sheet stabilized by three disulfide bonds (Fig. 2a). We found that hBD2 contains an additional secondary-structure element, an α-helix region spanning residues Pro5–Lys10, held against the sheet by a disulfide bond formed between Cys8 and Cys37. The electron density corresponding to the majority of the structure is very well defined, and several residues could be refined in multiple conformations. Residues Arg22 and Arg23 are highly flexible, as is the entire β-turn between strands β1 and β2. The C termini are also mobile, with Lys39, Lys40, and Pro41 having elevated B factors (>40 Å²).

Formation of the conserved dimer is assisted by interactions between strands β1 of both monomers, resulting in the formation of a six-stranded β-sheet. This interaction, however, only extends through two hydrogen bonds between the backbone atoms of Cys15 (Fig. 2b). We postulate that the presence of Pro17 prevents more extensive hydrogen bonding within the intermolecular sheet, thereby lowering the stability of this motif. The formation of the dimer buries 1,000–1,200 Å², or on average 18.4% of the total surface area of the monomer, and the interface is flat and mainly hydrophobic in nature. Residues Pro4, Ala13, Ile14, Cys15, His16, and Pro17 make the majority of van der Waals contacts across the dimer interface.

The monoclinic form contains two octameric assemblies within the a.u., each composed of four hBD2 dimers arranged with an approximately 422 symmetry (Fig. 3a). The four monomers in the orthorhombic form create one half of the octameric assembly; the full octamer is generated by applying a crystallographic twofold to the contents of the a.u. Thus, the octameric oligomer is conserved across two unique crystal forms. The octamer is formed by a mixture of hydrophobic and hydrogen bonding contacts, with residues Gly1, Asp4, Thr7, Lys10, Gly31, Leu32, Pro33, and Lys39 creating most of the contacts. The octamer is shaped like a distorted square, and is roughly 25 Å × 25 Å × 50 Å. Four inner monomers make up the majority of the octameric interfaces (1, 150–1, 800 Å², or on average 24% of the molecular surface area of the isolated monomer), with the dimer-related outer monomers making fewer contacts (200–500 Å², or on average 7% of the molecular surface area of the monomer).

Dynamic light-scattering experiments performed on concentrated solutions of hBD2 showed a mixture of aggregated molecules, mostly populated by dimers. This result indicates that octamer formation requires additional factors. These may be, for example, the stabilizing interactions with a negatively charged membrane. The distribution of temperature factors
across the octamer, lowest in the center of the octamer and increasing toward the edges, resembles the dynamic properties of many globular proteins in solution and cannot be correlated with interactions resulting from crystal formation. Additionally, a search of the Protein Data Bank for x-ray structures of proteins (except viruses) consisting of 12 or more independent molecules in the a.u. resulted in only six such structures. In all cases, multimeric molecules found in the a.u. were proven to represent biologically relevant assemblies. Therefore, it is quite likely that the hBD2 octamers common to both crystal forms

| Data set | Second | Orthorhombic | Br1 (peak) | Br2 (inflect.) | Br3 (remote) | Total |
|----------|--------|--------------|------------|---------------|--------------|-------|
| Wavelength (Å) | 0.979  | 0.979        | 0.9182     | 0.9203        | 0.91345      | 1.542 |
| Resolution range (Å) | 30–1.7 | 30–1.35      | 25–1.4     | 25–1.4        | 25–2.0       | 30–1.9 |
| Space group | P2₁ | P2₁,2,2      | P2₁,2,2    | P2₁,2,2       | P2₁,2,2      | P2₁,2,2 |
| Unit cell (Å) | a = 54.53 | a = 50.05    | a = 49.18  | a = 49.05     | a = 49.05   | a = 49.05 |
| b = 79.95 | b = 103.91 | b = 102.85   | b = 103.33 | b = 103.33    | b = 103.33 |
| c = 74.27 | c = 28.27  | c = 27.94    | c = 28.03  | c = 28.03     | c = 28.03 |
| β = 105.30 | | | | | |
| Measured reflections | 165,583 | 137,572      | 123,991    | 123,224       | 50,883       | 73,458 |
| Unique reflections | 65,644  | 32,227       | 28,743     | 28,959        | 10,176       | 11,529 |
| Completeness (%) | 97.1 (96.3) | 96.7 (90.1)  | 99.7 (98.2) | 98.4 (86.0)   | 99.9 (100)   | 97.3 (94.8) |
| R_merge (%) | 4.3 (27.3) | 3.1 (20.4)   | 3.4 (32.0) | 3.2 (33.3)    | 3.1 (7.7)    | 7.5 (30.3) |
| R/work (%) | 24.2 (4.7) | 55.0 (6.5)   | 53.6 (4.0) | 47.9 (4.3)    | 42.8 (16.8)  | 23.8 (6.5) |
| Refinement | Rwork,Rfree (%) | 26.4, 18.8   | 23.7, 16.4 | |
| No. of atoms | 5,818 | 1,416        | 1,416      | 1,416         | 1,416       | 1,416 |
| No. of residues | 656   | 162          | 162        | 162           | 162         | 162 |
| No. of waters | 814   | 194          | 194        | 194           | 194         | 194 |
| Root mean square deviation | Bond length (Å) | 0.006       | 0.011      | |
| Bond angles (°) | 1.230  | 1.932        | |
| Overall (Å²) | 29.3  | 29.6         | |
| Protein (Å²) | 28.9  | 28.9         | |
| Solvent (Å²) | 39.0  | 45.0         | |

Numbers in parentheses represent values for only those reflections within the highest-resolution shell, as determined using DENZO (21).

$R_{merge} = \sum |F_o(h) - \langle F_o(h) \rangle|/\sum |F_o(h)|$, $R_{work} = \sum |F_o(h) - kF_c(h)|/\sum |F_o(h)|$, where $T$ represents a test set of reflections (10% of total, chosen at random) not used in the refinement.

**FIG. 2. Stereo drawing of the overall fold and dimerization of hBD2.**

(a) The molecule forms a three-stranded anti-parallel β-sheet with one helix flanking the sheet. Three disulfide bonds are shown to stabilize the global conformation. This figure was made using RIBBONS (38).

(b) The hBD2 dimer is formed by an intermolecular β-sheet via the first β-strands. The interface is stabilized by van der Waals contacts from residues within and flanking β1 (Ala¹³–Pro¹⁷), as well as Pro⁵. This figure was made using RIBBONS (38).
correspond to stable, naturally forming oligomers of this protein.

The core of the octamer is created by the N termini of the four inner monomers (chains A, C, E, G, and I, K, M, O in the monoclinic form, and chains A and C in the octameric form) (Fig. 3a). Gly1, Gly3, Asp4 (the only acidic residue in the protein), and Thr7 create a constellation of hydrogen bonds that close the core of the octamer to solvent movement. The octamer is thus solid, and no solvent-accessible pore or channel is evident without significant movements of the protein backbone in this region. However, there are six water molecules trapped within the core of the octamer. Their mobilities are minimal, as deduced by their low *B* factors, conservation in position among the three independent octamers, and coordination between protein atoms. Four of these water molecules create a cavity within the core of the octamer (Fig. 3b).

The α-helical regions of hBD2 seem to play a key role in formation of the octamer. Aside from positioning Thr7 and Lys10 into contact positions, the orientation of the α-helix is conserved about the octamer core, forming a ridge around it (Fig. 3a). This ridge creates an indentation in the center of the octamer above and below the core. Additionally, the dipoles of the helices would overlap to create a positively charged region near the core, which is further indicated by the clustering of halides in this region. These dipoles may be offset, however, by the presence of Asp4 at the base of the helix.

The two crystal forms of hBD2 provide a detailed independ-
ent structural description of 20 individual monomers, under virtually identical external conditions (pH, temperature, etc.). Such a situation, highly reminiscent of NMR experiments in solution, provides a much more complete image of the dynamic properties of a protein molecule than typically delivered by x-ray crystallography. Fig. 4 shows the superposition of all 20 monomers from the two crystal forms. Because of the high resolution (1.7 Å for the monoclinic form and 1.35 Å for the orthorhombic form) and excellent quality of the x-ray data, the structures of individual monomers are very well defined. Although the structure of each monomer corresponds to its environment in the crystal, due to the high number of independent monomers, the overall ensemble represents the dynamic character of the hBD2 monomer. The overall root mean square deviation, calculated for all atoms, is about 1.1 Å; however, the central core and α-helical region are significantly more rigid and have an average root mean square deviation of 0.3–0.4 Å. The most disordered are the Lys and Arg residues near the C terminus, except Lys36, for which the Cβ atoms are well defined and only the remainder of the side chain is disordered. In contrast, the side chain of Lys36 accommodates two distinctive alternate conformations. Two conformations are also found for the side chains of His36 and Phe19, and are correlated with the location of these residues within the octamer. Thus, although the conformations of some residues, corresponding to their minimum energy, are inevitably affected by the crystal contacts, structural features of the majority are conserved and well defined.

Conformational Changes between the Monoclinic and Orthorhombic Forms—Superpositions of the octameric assemblies from the monoclinic form onto the orthorhombic form show a number of conformational changes resulting from the concerted rearrangement of monomers within the octamer. These changes are unlikely to be due to data quality, refinement errors, or packing, because the superposition of the two independent octamers determined for the monoclinic form does not show the same systematic movements of monomers and displays only minimal flexibility in the backbone atoms of the related monomer chains. The transition from the monoclinic form to the orthorhombic form shows an overall contraction of the monomers into the core of the octamer, and a bending of the octamer along a diagonal of the octamer square. Maximum differences between equivalent residues are on the order of 2 Å, and these occur on the outer edge of the octamer. However, the inner monomers of the octamer show a concerted contraction toward the core. Individual causes and effects related to these conformational changes cannot be discriminated based on the static structure, but their features can be described in better detail. Three such features linked to this conformation change can be identified.

Inspection of the experimentally derived electron density maps shows that the dimer formed between monomers C and D of the orthorhombic form is different from the second dimer in the orthorhombic form as well as all dimers in the monoclinic form by the presence of an isolated peak between Cys15 of each monomer. This peak could be refined as a water molecule (Wat48, B factor 21.39 Å²) coordinated between the carboxyl oxygens and amide nitrogens of these two residues (Fig. 5). Although the hydrogen-bonding distances are close to ideal (2.8 and 2.9 Å), the stereochemistry of the hydrogen bonds is significantly distorted from tetrahedral. As was noted above, the β-sheet formed between strands β1 of individual monomers within the dimer is weakened by the presence of Pro27 (Fig. 2b), and probably can be easily disrupted. Although we cannot identify whether the insertion of Wat48 between monomers C and D of the orthorhombic form is a cause or an effect of the conformational change between the monoclinic and orthorhombic forms, it is linked to the contraction of the inner monomers into the center of the octamer. This water insertion is also related to the twisting of the outer molecules from their relative position in the monoclinic form.

The contraction of the inner monomers into the core of the octamer is related to rearrangements of a hydrogen-bonding network in the core. In the monoclinic form, Gly1:A and Gly1:E form hydrogen bonds with each other, as well as with Asp4:C and Asp4:G within the core cavity. Each of the carboxyl oxygens of both aspartates clearly forms three hydrogen bonds. Based on the extended network of hydrogen bonds, Asp4:C and Asp4:G are protonated (at least partially) and that the electron density peaks that we modeled as water molecules (Wat36 and Wat8) would probably correspond to metal cations (sodium or potassium, perhaps). A sequence alignment of other β-defensins shows a strong degree of structural conservation of the functional groups within the network (Gly1, Asp4, and Thr7); all
other donors and acceptors within the network are backbone atoms. The hydrophobic residues Ile\textsuperscript{2} and Val\textsuperscript{6}, which are involved in contacts with the rest of the protein, are also highly conserved (Fig. 6).

The equivalent N-terminal amino groups in the orthorhombic form are moved closer together as compared with those in the monoclinic form and rotate to alleviate the collision. The two chains (monomer D and its symmetry mate) move toward the center of the octamer, whereas the other two chains (monomer B and its symmetry mate) rotate slightly away from the center. The hydrogen bonds between the N-terminal amino groups and the side chains of aspartate are involved in only five hydrogen bonds in the orthorhombic form, one of which (to Val\textsuperscript{6N:B}) is 3.2 Å, it is unclear whether the aspartate remains protonated.

The rearrangement of the N termini also affects the positions of the waters trapped within the core of hBD2 octamer. There are four waters (two in the a.u. of the orthorhombic form) seen with hBD2. The residues involved in octamer formation, one of which (to Val\textsuperscript{6N:B}) is 3.2 Å, it is unclear whether the aspartate remains protonated.

**Comparison with Other Defensins and Related Peptides**—A sequence alignment of mammalian β-defensins shows several conserved residues in the molecule, namely all of the cysteines and Gly\textsuperscript{26} (Fig. 6). These residues are likely to be involved in maintaining the fold of the molecule. Aspartate oxygens are involved in only five hydrogen bonds in the orthorhombic form, one of which (to Val\textsuperscript{6N:B}) is 3.2 Å, it is unclear whether the aspartate remains protonated.

A structural alignment of known defensin structures (hBD12 (1bnb) (Ref. 32) and hNP3 (1dfn) (Ref. 17)) with hBD2 shows the conservation of the three β-strands and general fold, as well as most of the completely conserved residues seen in the sequence alignment of β-defensins, although the positions of the disulfide bonds differ among the three structures. The octamer structure at the N terminus is loosely conserved among the β-defensins. However, the NMR data for hBD12 do not show a well formed α-helix, although the residues immediately following the first cysteine are near the helical region of the Ramachandran plot. A search of protein structures using DALI (33) found structural homology of hBD2 with the two other defensins, as well as a defensin-like peptide from platypus venom (1b8w) (34) and two sea anemone toxins (35, 36). A comparison of the electrostatic surface potential of hBD2 and these homologous structures (Protein Data Bank accession codes 1bnb, 1dfn, 1b8w, and Gly28 (Fig. 6). These residues are likely to be involved in the hydrogen-bonded neighbors are also shown (in angstroms). This figure was made using RIBBONS (38).
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1ahl, and 1sh1) shows a wide range of charges and charge positions. The proteins whose functions are either toxins (1ahl, 1sh1) or unknown (1b8w) have a uniformly distributed surface of positive and negative charges. Like 1dfn, hBD2 has a generally amphiphilic surface, with patches of hydrophobicity in the flat centers of the monomer and charged residues ringing the edge. This arrangement correlates with the fact that both hBD2 and 1dfn oligomerize in their crystal structures (17). Although hBD2 and 1bnn would have the same net charge at pH 7 (+6), 1bnn displays a uniformly positive charge on its surface, which may explain its lack of oligomerization seen in the NMR experiment (32). hBD2 and 1bnn are 36% identical, and thus very subtle differences would result in the differences in surface charge. hBD2 residues Lys10, Arg22, and Arg23 protrude from the edge of the monomer, whereas these residues in 1bnn (Arg7, Val19, and Pro20) curl closer to the monomer core. Additionally, hBD2 residues Phe19, Pro33, and Leu9 on the flat surfaces are substituted by positively or nonhydrophobic residues (Arg16, Arg20, and Gly6 in 1bnn).

In summary, we present here the first structure of a human β-defensin. Unlike the previously determined structures of either α- or β-defensins, this x-ray structure of hBD2 displays a unique secondary-structure element at its N terminus. The presence of this N-terminal α-helix gives rise to a novel dimer topology. Light-scattering experiments indicate that dimers are the dominant oligomeric form of hBD2 in solution. This mode of dimerization is probably conserved within the β-defensin family and has implications for the differences in activities seen between α- and β-defensins.

The β-defensin mode of dimerization allows the formation of a higher-order oligomerization of the protein as an octamer. The conservation of the octamer across two crystal forms provides evidence that this octamer represents the stable, native quaternary structure of hBD2. The stability of the octamer is further supported by the burial of hydrophobic surface area and the distribution of temperature factors. It is therefore likely that the octameric form of hBD2 represents the form bound to bacterial membranes.

The structural and electrostatic properties of the hBD2 octamer do not show any evidence for burial within bacterial membranes or the formation of a bilayer-spanning pore when bound to the membrane, unlike the mechanism proposed for hNP3. The uniform surface distribution of positively charged residues suggests that hBD2 disrupts the bacterial membrane via electrostatic interactions with the polar headgroups of the membrane. Because of the expected crisis of bacterial antibiotic resistance, understanding the mechanism of defensins might aid the development of novel antibiotics, and the x-ray structure of hBD2 will allow for the design of more streamlined and effective antibiotics.

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