Unique Dimeric Structure of BNip3 Transmembrane Domain Suggests Membrane Permeabilization as a Cell Death Trigger

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BNip3 is a prominent representative of apoptotic Bcl-2 proteins with rather unique properties initiating an atypical programmed cell death pathway resembling both necrosis and apoptosis. Many Bcl-2 family proteins modulate the permeability state of the outer mitochondrial membrane by forming homodimeric and hetero-oligomers. The structure and dynamics of the homodimeric transmembrane domain of BNip3 were investigated with the aid of solution NMR in lipid bicelles and molecular dynamics energy relaxation in an explicit lipid bilayer. The right-handed parallel helix-helix structure of the domain with a hydrogen bond-rich His-Ser node in the middle of the membrane, accessibility of the node for water, and continuous hydrophilic track across the membrane suggest that the domain can provide an ion-conducting pathway through the membrane. Incorporation of the BNip3 transmembrane domain into an artificial lipid bilayer resulted in pH-dependent conductivity increase. A possible biological implication of the findings in relation to triggering necrosis-like cell death by BNip3 is discussed.

Mitochondria hold a crucial role in programmed cell death required to control cell development and to maintain homeostasis in multicellular organisms (1). Mitochondria-mediated cell death is both promoted and suppressed by apoptotic proteins of the Bcl-2 family, most of which contain a C-terminal hydrophobic domain essential for membrane targeting (2). A major function of Bcl-2 family proteins is to regulate the permeability state of the outer mitochondrial membrane by forming homo- and hetero-oligomers inside the membrane that determine cell fate (3–5). The pro-apoptotic protein BNip3 (Bcl-2 Nineteen-kDa interacting protein 3) with a single Bcl-2 homology 3 (BH3) domain is one of the most intensively studied members of the family (6). BNip3 and its homologues belong to an independent monophyletic branch with individual evolutionary history (2) and are essentially different from other BH3-only proteins such as Bid/Bik not only in that they do not require BH3 domain for their function but also because they directly cause changes of mitochondrial potential (7). BNip3-induced cell death is independent of caspases and cytochrome c release; it is believed to represent a novel form of programmed cell death, resembling necrosis rather than classical apoptosis (8).

For all cells, loss of nutrient supply represents a potent signal for programmed death. BNip3 plays an important role in hypoxic cell death of normal and malignant cells (9). Hypoxia induces expression and accumulation of cytoplasmic or loosely membrane-bound BNip3; however, in order to activate cell death pathway acidosis is required (10). Transition from respiratory to glycolytic metabolism with increased glucose consumption, lactic acid production, and decrease of cytosolic pH causes redistribution of BNip3 to the outer mitochondrial membrane and integration of homodimeric BNip3 into it, triggering a cell death cascade, which ultimately leads to opening of the mitochondrial permeability transition pore (8, 10). BNip3 inserts into the outer mitochondrial membrane with its N terminus in the cytoplasm and C terminus inside the mitochondria. It was demonstrated that the BNip3 transmembrane domain (TM)1 is crucial for pro-apoptotic activity, mitochondrial localization, and homodimerization of the protein.

The atomic coordinates and structure factors (code 2j5D) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The chemical shift assignments of the BNip3tm dimer have been deposited at BioMagResBank (www.bmrb.wisc.edu) under accession code BMRB 7288.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental data and supplemental Figs. S1–S5.

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§ The abbreviations used are: TM, transmembrane; BNip3tm, TM fragment 146–190 of human pro-apoptotic protein BNip3; GapAtm, TM fragment 61–98 of human protein glycophorin A; BLM, bilayer lipid membrane; MHP, molecular hydrophobic potential; DMPC, dimyristoyl-phosphatidylcholine; DPhPC, diphytanoyl-phosphatidylcholine; NOE, nuclear Overhauser effect; MD, molecular dynamics; MES, 4-morpholineethanesulfonic acid; VDAC, voltage-dependent anion channel.

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whereas its N-terminal region can be involved in interaction with Bcl-2 proteins (11, 12).

As was demonstrated recently, the BNip3 TM segment exists in the form of a tightly associated dimer, in both the detergent and lipid environments (13). So far, the structure of the transmembrane domain of a representative of the Bcl-2 family in the lipid environment has not been reported. We describe the spatial structure and internal dynamics of the homodimeric TM domain of human pro-apoptotic protein BNip3 in a membrane-mimicking lipid environment obtained with the aid of a combination of NMR spectroscopic technique and molecular dynamics (MD) energy relaxation. The structure suggests a possibility that the BNip3 TM domain alone can form an ion-conducting pathway in the membrane, as supported by direct measurements on the bilayer lipid membranes (BLMs). This property of the TM domain can help to explain the mechanism of BNip3 action, in particular in hypoxia acidosis-induced cell death.

EXPERIMENTAL PROCEDURES

Construct Design, Cloning, and Expression—The DNA sequence encoding human BNip3 fragment 146–190 (BNip3tm) was synthesized from six oligonucleotides via PCR. The TrxA-BNip3tm fusion protein was constructed by fusing the gene for thioredoxin of Escherichia coli with an N terminus His tag extension to the gene for BNip3tm in the pGEMEX1 (Promega) vector. To facilitate purification, a His tag and enterokinase cleavage site were placed between the genes for the thioredoxin and BNip3tm fragment. The fusion protein was expressed in E. coli BL21(DE3)pLysS and grown in 2-liter flasks at 37 °C. For the study of hydrogen bonding and water accessibility to protein groups, slowly exchanging amide protons were identified by reconstituting lyophilized 15N-labeled sample in D2O and recording a series of 1H-15N HSQC spectra over 1 week at 30 °C. In addition, two-dimensional 1H ROESY spectra and homonuclear 1H NOE spectra (60-ms mixing time) were acquired for the unlabeled sample as described (15). The effective rotation correlation times, τgz, for the individual 15N nuclei were calculated from a 1/T1/T2 ratio using DASHA (16).

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The values of heteronuclear 15N{1H} steady state NOE, 15N longitudinal (T1), and transverse (T2) relaxation times were obtained for the 15N-labeled sample as described (16). The effect of Mn2+ on the 1H NMR spectra was determined by recording spectra in H2O containing 1 mM MnCl2.

Protein Purification—Cell pellets (1-liter equivalents) were resuspended in 50 ml of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM 2-mercaptoethanol, 10 mM imidazole, 15 mM Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride) and lysed by ultrasonication. Centrifugally clarified lysate was applied to chelating Sepharose FF beads (Amersham Biosciences) pre-treated with NiSO4 and eluted with 200 mM imidazole. After overnight incubation with recombinant light chain of human enterokinase, the cleaved BNip3tm fragment was passed through chelating Sepharose FF, loaded onto an SP-Sepharose FF column (Amersham Biosciences), and eluted by gradient of NaCl. Yields of ~10 mg of protein/liter of cell culture could be obtained by this procedure. Protein concentration was determined by A280 values. Protein identity and purity were confirmed by gel electrophoreses, mass spectrometry, and NMR spectroscopy in a methanol/chloroform (1/1) mixture containing 5–10% of water. The unlabeled human glycophorin A fragment 61–98 (GpAtm), including a GpA TM segment with adjacent N- and C-terminal regions, was obtained in a similar way as the BNip3tm fragment.

NMR Spectroscopy and Structure Calculations—Four BNip3tm samples were prepared: uniformly 15N/13C-labeled, 15N-labeled; unlabeled; and a 1:1 mixture of uniformly 15N/13C-labeled and unlabeled protein (“heterodimer” sample). The lyophilized protein was solubilized in the form of an aqueous suspension of dimyristoyl-phosphatidylcholine (DMPC)/dihexanoyl-phosphatidylcholine (DHPC) lipid (Avanti Polar Lipids) bicelles prepared with a lipid molar ratio of 0.25, and then the samples were subjected to several freeze/thaw cycles resulting in uniform protein distribution among the lipid bicelles. To verify the validity of NMR experimental conditions, circular dichroism spectra of BNip3tm in the DMPC/DHPC bicelles and in DMPC monolamellar liposomes (phospholipid bilayer) were recorded. After base-line subtraction, the spectra proved virtually identical (for results and experimental procedures, see the supplemental data).

NMR experiments were performed on a 600-MHz (1H) Varian Unity spectrometer equipped with a pulsed field gradient unit and triple resonance probe. NMR spectra were acquired at 40 °C using 1-mm samples of BNip3tm incorporated into lipid bicelles (with a lipid/protein molar ratio of 40) dissolved in buffer solution (pH 5.0) containing 20 mM deuterated sodium acetate, 0.15 μM sodium azide, 1 mM EDTA, and either 5 or 99.9% D2O unless otherwise specified. The backbone and side chain 1H, 13C, and 15N resonances of BNip3tm were assigned using standard triple resonance techniques (14, 15). Two- and three-dimensional heteronuclear 1H-15N HSQC, 1H-13C HSQC, 15N-edited TOCSY (40-ms mixing time), HNCA, HN(CO)CA, HNCA, and CBCA(CO)NH spectra in H2O provided backbone and partial side chain assignments, while HCCH-TOCSY (15.6- and 23.4-ms mixing times) and homonuclear 1H NOESY (60-ms mixing time) experiments in D2O facilitated side chain assignments. Resonance assignments were performed with CARA (www.nmr.ch).

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For the study of hydrogen bonding and water accessibility to protein groups, slowly exchanging amide protons were identified by reconstituting lyophilized 15N-labeled sample in D2O and recording a series of 1H-15N HSQC spectra over 1 week at 30 °C. In addition, two-dimensional 1H ROESY (50-ms mixing time) and 1H NOESY (60-ms mixing time) spectra were acquired for the unlabeled sample in H2O using the watergate scheme. The water-protein cross-peaks in these spectra were narrowed by suppressing radiation dumping in evolution and mixing periods with the aid of weak (50 mG) magnetic field gradients (18).

NMR structural analysis of the BNip3 transmembrane domain was performed using CYANA (19). Intramonomeric nuclear Overhauser effect (NOE) distance restraints were identified with CABA through the analysis of three-dimensional 15N- and 13C-edited NOESY experiments (60-ms mixing time) (15) performed for 15N- and 15N/13C-labeled samples in H2O and D2O, respectively. Two-dimensional 1H NOESY (60-ms mixing time) spectra acquired for the unlabeled sample was used as an additional source of the structural information concerning aromatic ring protons. Intermonomeric distance restraints

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were derived from two-dimensional $^{15}$N,$^{13}$C F1-filtered/F3-edited-NOESY and three-dimensional $^{13}$C F1-filtered/F3-edited-NOESY spectra (20) acquired with an 80-ms mixing time for the "heterodimer" sample in H$_2$O and D$_2$O, respectively. Protein-lipid NOE contacts were identified from two-dimensional $^{15}$N,$^{13}$C F1-filtered/F3-edited-NOESY and three-dimensional $^{13}$C F1-filtered/F3-edited-NOESY spectra acquired with an 80-ms mixing time for the $^{15}$N/$^{13}$C-labeled sample. Stereospecific assignments and torsion angle restraints for $^{1}$H$_{9272}$, $^{1}$H$_{9274}$, and $^{1}$H$_{9273}$ were obtained by the analysis of local conformation in CYANA using sequential NOE data and the available $^{3}$J$_{HN}$ and $^{3}$J$_{N}$ coupling constants evaluated quantitatively from three-dimensional $^{1}$H-$^{15}$N HNHA and qualitatively from three-dimensional $^{1}$H-$^{15}$N HNHB experiments (15). Backbone dihedral angle restraints were also estimated based on the assigned chemical shifts using TALOS (21). The slowly exchanging amide protons were assigned as hydrogen bond donors with related hydrogen-acceptor partners on the basis of preliminary structure calculations. Corresponding hydrogen bond restraints were employed in subsequent calculations for $d$(O,N), $d$(O,H$^\alpha$), $d$(C,H$^\alpha$) distances according to Ref. (22). The standard CYANA simulated annealing protocol was applied to 200 random structures, and the resulting 16 structures with the lowest target function were selected. Constrained energy relaxation of the 16 best CYANA structures of the BNip3tm dimer was performed using available distance restraints by MD in the explicit DMPC bilayer.

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A twin range (12/20 Å) spherical cutoff and PME algorithm were used to treat van der Waals and electrostatic interactions, respectively. In total, 16 NMR structures of the BNip3tm dimer with different tautomeric forms of His$^{173}$ were refined: 12 $^{4}$His$^{173}$-BNip3tm and 4 $^{6}$His$^{173}$-BNip3tm dimers with a protonated Ne or N$\delta$ group of the imidazole ring, respectively. In each case, a 5-ns collection MD run with applied intra- and intermonomeric NOE distance restraints was carried out. Furthermore, two additional MD simulations were performed starting from coordinates of the resulting systems. In the former one, a 5-ns continuation MD run without restraints was carried out in order to check the stability of the dimer. The latter simulation (1-ns MD with NMR distance restraints followed by 5-ns free MD) was done with protonated His$^{173}$ in one subunit of the $^{4}$His$^{173}$/^{6}His$^{173}$-BNip3tm dimer in order to assess the effect of the ionization state of His$^{173}$ on the dimeric structure. Equilibrium parts of MD trajectories (last 2 ns) were analyzed using original software developed by the authors and utilities supplied with the GROMACS package. Hydrophobic properties of $^{\alpha}$-helices were calculated using the molecular hydropathicity potential approach as described elsewhere (24).

**Electrochemical Measurements**—Bilayers were formed either on 1–1.2-mm aperture in a vertical or on 200-$\mu$m aperture in a horizontal teflon partition with the aid of the Mueller-Rudin method. The membranes were formed from a 20-mg/ml solution of diphytanoyl-phosphatidylcholine (DPhPC; Avanti Polar Lipids) in decane. The measurements were performed at 23 °C in 100- or 10-mM KCl solutions containing 1 mM EDTA and
buffered with 10 mM Hepes at pH 7.0 or 30 mM MES at pH 4.0. BNip3tm and GpAtm were added either into water solution in the form of solution in Me2SO (1 μg/μl) or directly into the solution of the lipid in decane used for formation of the membranes (to the final concentration of 0.5 μg/μl).

For electrical measurements, silver chloride electrodes with standard agar-salt bridges with the total resistance not exceeding 50 kΩ were used. The capacitance of BLM was determined from the electric current under applied alternating voltage with the aid of homemade software. The voltage was applied from analog output of a ADC-DAC board (Lcard L780). Conductivity to direct current was determined by applying constant voltage to the membrane for a period significantly exceeding the RC constant of the membrane and recording the current.

RESULTS

Structure Determination of the BNip3tm Dimer—The 45-residue BNip3 fragment 146–190 (BNip3tm), including the BNip3 TM segment with adjacent N- and C-terminal regions, was solubilized in an aqueous suspension of DMPC/DHPC lipid bicelles, and the standard heteronuclear NMR techniques were applied to determine the spatial structure and to describe the internal dynamics. Directly identified intra- and intermonomeric NOE contacts (a representative strip is shown on Fig. 1B) confirmed the dimeric helical structure of the BNip3 TM domain in the bicelles and demonstrated that the dimer sub-units are in a parallel mutual arrangement. The presence of the single cross-peak set in the 1H-15N HSQC spectrum (Fig. 1A) implies that the BNip3tm dimer is symmetrical on the NMR time scale. Therefore, the dihedral angle restraints and both intra- and intermonomeric distance restraints were symmetrical.

| NMR distance and dihedral restraints | Total unambiguous NOE restraints | 608 |
|-------------------------------------|---------------------------------|-----|
| Total intra-residue                 | 202                             |     |
| Total inter-residue                 | 406                             |     |
| Sequential (i–i + 1)                | 162                             |     |
| Medium range (i–i + 6)              | 240                             |     |
| Long range (i–i + 4)                | 4                              |     |
| Inter-monomeric NOE                 | 28                              |     |
| Hydrogen bond restraints (upper/lower) | 114/114                        |     |
| Total torsion angle restraints      | 162                             |     |
| Backbone ϕ                         | 82                              |     |
| Backbone ψ                         | 50                              |     |
| Side chain χ3                        | 30                              |     |

Structure calculation statistics

| CYANA target function (Å²) | 0.37 ± 0.06 |
|---------------------------|-------------|
| Restraint violations      | 0           |
| Dihedral (>5°)            | 0           |
| Average pairwise root mean square deviation (Å) | |
| All folded regions-(159–187)₂ | 2.03 ± 0.53 |
| Backbone atoms             | 2.83 ± 0.52 |
| Stable α-helical region-(166–184)₂ | 0.95 ± 0.30 |
| Backbone atoms             | 1.54 ± 0.29 |
| Ramachandran analysis⁶     | 85.8        |
| % Residues in most favored regions | 85.8 |
| % Residues in additional allowed regions | 12.7 |
| % Residues in generously allowed regions | 0.8¹⁰¹ |
| % Residues in disallowed regions | 0.7¹⁰¹ |

Helix-helix packing

| Lennard-Jones contact energy (kJ/mol) | 510 ± 100 |
| Contact surface area (Å²) | 255 ± 30 |
| Stable α-helical region-(166–184)₂ | 650 ± 90 |
| Stable α-helical region-(166–184)₁ | 375 ± 25 |
| Angle θ (°) between the TM helix axes | 45 ± 5 |
| Distance d (Å) between the TM helix axes | 5.7 ± 0.5 |

⁶ Ramachandran statistics were determined using PROCHECK_NMR (48).
¹ Residues from unfolded and flexible regions.
cally doubled for each dimer subunit that resulted in a dimer with 2-fold symmetry averaged over the ensemble of calculated NMR structures. The full set of input data for NMR structure calculation included 608/28 intra/intermonomeric unambiguous NOE distance restraints, distance restraints for 38 hydrogen bonds, and 162 backbone $\varphi$, $\psi$ and side chain $\chi$ dihedral angle restraints. From the observed protein-lipid NOE contacts (Fig. 1C, supplemental Fig. S1) with lipid polar heads and hydrophobic tails we can conclude, similarly to Ref. (26) where analogous contacts have been analyzed, that the DMPC/DHPC bicelles mimic the embedding of the BNip3 TM domain into a double layer lipid membrane reasonably well. As for the protein-lipid interaction, around the central part of the molecular surface of the BNip3tm dimer we identified many protein-lipid NOE contacts with lipid hydrophobic tails, whereas the protein-lipid NOE contacts with lipid polar heads were found only in the N- and C-terminal parts of the dimer helices. Thus, the dimer spans the hydrophobic phase of the bicelle.

The resulting NMR structures of the BNip3tm dimers were subjected to energy relaxation using MD in an explicit hydrated lipid bilayer with the imposed experimentally derived distance restraints. The MD energy relaxation allowed adapting the structure to the lipid environment, with improvement of the quality (distribution of the torsions of the side chains, backbone conformation), while the geometry of interaction of the TM fragments remained practically unchanged. Moreover, a 5-ns continuation of MD without NMR restraints did not cause any change of the dimer structure or violations of the restraints. Hence, the obtained structure of the BNip3tm dimer is stable in the membrane. A survey of the structural statistics for the final ensemble of the 16 structures of the BNip3tm dimer (Fig. 2A) is provided in Table 1.

**Tertiary Fold of the BNip3tm Dimer in Lipid Bicelles**—Analysis of the calculated spatial structure revealed that the BNip3tm integrated in the lipid bicelles mostly adopted an $\alpha$-helical structure. The membrane-spanning $\alpha$-helices cross at the angle $\theta$ of $-45 \pm 5$° with the distance $d = 5.7 \pm 0.5$ Å between helix axes and form a right-handed parallel symmetric dimer (Fig. 2) typical for membrane-soluble helical pairs (27). For the BNip3tm dimer, the overall rotation correlation time $\tau_\text{R}$, estimated from a $T_1/T_2$ ratio and averaged over $^{15}$N nuclei with $^{1}$H/$^{15}$N NOE higher than 0.6, is $\sim 18$ ns (Fig. 3). Based on the empirical dependence (28), this $\tau_\text{R}$ value corresponds to the effective molecular mass of $\sim 50$ kDa that almost exactly coincides with the size of the DMPC/DHPC bicelle (29) composed of the BNip3tm dimer surrounded by 80 lipid molecules.

The $^{15}$N/$^{1}$H NOE, $^{15}$N $T_1$, and $T_2$ values (Fig. 3) measured for the backbone $^{15}$N nuclei exhibit significant variations along the protein sequence and are indicative of a stable TM $\alpha$-helical region (residues 166–184) adjacent to more flexible N- and C-terminal $\alpha$-helices (residues 159–165 and 185–187, respectively) located in the membrane/water interface. The TM helix has the highest positive $^{15}$N/$^{1}$H NOEs, which suggests restricted internal mobility for the NH vectors in a pico-nano-second time scale. In contrast, the residues 146–158 and 188–190 from the N- and C termini, respectively, have nearly unrestricted mobility, resulting in low and negative $^{15}$N/$^{1}$H NOEs and decreased local rotation correlation times $\tau_\text{R}$, estimated from the $T_1/T_2$ ratio (Fig. 3). For more detailed discussion of the stable and transitory structures of the BNip3tm dimer see the supplemental data.

**BNip3tm Dimerization Interface**—The TM domain, though generally hydrophobic, contains certain relatively hydrophilic regions. The dimerization interface includes both polar and non-polar residues. A convenient parameter for visualization of spatial distribution of hydrophilic/hydrophobic properties is molecular hydrophobicity potential (MHP) (24). On Fig. 4A, contour isolines on a two-dimensional hydrophobicity map for
the BNip3tm helices encircle hydrophobic regions with high values of MHP, and the red-hatched area (≈650 Å²) indicates the helix-packing interface of the symmetrical BNip3 dimer. It can be seen that the N-terminal helices interact exclusively through hydrophobic segments on the outer surface. All the stable intermonomeric contacts (preserved over the MD energy relaxation) in the dimer are along the TM helices, whereas the less stable ones are along the mobile N-terminal helices, where the intermonomeric stacking interactions occur in a group of six phenylalanine residues (Phe157/Phe161/Phe165) forming a labile Phe ring hydrophobic cluster (Fig. 2B). The contact area between the TM helices includes Phe165, Leu169, Ser172, His173, Ala176, Ile177, gly178, Ile181, Ile183, gly184, and Arg185 (Fig. 4). It is important to note that among the residues found on the helix packing interface are Ser172, His173, Ala176, gly180, Ile181, and Gly184 whose significance for BNip3 dimerization in the native membrane was established recently via site-directed mutagenesis (30). This supports the assumption that the structure of the BNip3tm in the bicelles is essentially the same as in the membrane. The side chains of Ser172 and His173 connect the monomers through the formation of intermonomeric hydrogen bonds (Fig. 5C), consistent with the predictions from the mutagenesis data (30). In addition to that, they are involved in intramonomeric hydrogen bonding with carbonyl groups of Leu169 and Ser168. Thus, the (Ser172/His173)₂ hydrophilic motif in the center of the dimerization interface of the BNip3 TM domain forms a compact His-Ser node (Fig. 2B) of inter- and intramonomeric hydrogen bonds. The C-terminal TM part of the BNip3tm dimer is stabilized by hydrophobic side chain contacts of Ala176, Ile177, Ile181, and Ile183 and by weakly hydrophilic backbone contacts of the helices tightly self-associated through a tandem variant of the glycophorin motif (31) A₁⁷⁶XXXG₁⁸⁰XXXG₁⁸⁴, formed by residues with small side chains allowing close approach of the helices. The tandem motif appears to be essential for proper alignment of the side chains in the His-Ser node required for formation of hydrogen bonds.

The experimental and modeling results reveal that a certain degree of structural heterogeneity might exist in the interfacial region of the dimer. The ¹H-¹⁵N HSQC spectra of BNip3tm (Fig. 1A) revealed the presence of a minor component for HN cross-peaks of Ser172 and Ala176. NMR experiments (¹H-¹⁵N HSQC, ¹H NOESY, ¹H ROESY) at different temperatures (30–55 °C) confirmed this to be due to slow conformational
exchange. Under the base experimental conditions (pH 5.0, 40 °C), the exchange rate does not exceed 20 Hz and the contribution of the minor component is ~10%. Because the HN groups of Ser172 and Ala176 are in the dimerization interface, it appears quite probable that the slow exchange is associated with variations of local structure of the dimerization interface and/or of the mutual orientation of the monomers. Indeed, the energy relaxation of NMR structures identified several options for the local structure of the His-Ser node in the dimerization interface, with the overall dimeric structure preserved (see the supplemental data for details). Possible continuous rearrangements, in the course of which the imidazole and hydroxyl groups of His173 and Ser172 serve both as donors and acceptors of the intermonomeric hydrogen bonds, can explain the slow exchange observed in the experiment.

Penetration of Water Molecules into the BNip3tm Dimerization Interface—The N-terminal part of the BNip3tm TM helices exhibits a relatively fast exchange of amide protons with the solvent deuterium (Fig. 2B), which suggests that the His-Ser node is accessible to water. In support of this, we identified intensive ROE cross-peaks between water and His173 imidazole resonances in the 1H-ROESY spectrum. Moreover, lack of NH signal from the imidazole ring implies that there is a fast exchange between imidazole and water molecules, resulting in

FIGURE 5. Water penetration into the BNip3tm dimer. A and B, water penetration into the *His173,His173-BNip3tm dimer and the *His173/*His173-BNip3tm dimer with one protonated imidazole ring, respectively. The α-helical regions are shown with ribbons. The carbon, nitrogen, and oxygen atoms of the dimer are green, blue, and red, respectively. The water oxygen and lipid head phosphorus atoms are presented as magenta and yellow balls. C and D, enlarged pictures of His-Ser node conformations from panels A and B, respectively. Only HN, CO, and heavy side chain bonds of the Ser168, Ser172, Leu169, His173, and Ala176 residues are shown. Residues of the second monomer are marked with asterisks. The hydrogen bonds are shown by dotted lines.
the appearance of rotating frame NOE cross-peaks between the \( \text{COH} \) and \( \text{CeH} \) groups of the ring and water. Analysis of the BNip3tm dimer structure reveals that a region rich in hydrogen bond donors and acceptors is formed between the N-terminal fragments of the TM helices, mostly including the side chains of (Ser\textsuperscript{172}/His\textsuperscript{173})
. Effectively, a hydrophilic cavity shielded from the lipid tails is formed, facilitating penetration of water molecules between the monomers up to the middle of the membrane despite the Phe ring hydrophobic cluster (Fig. 2B).

Interactions with water molecules are also identified (2–3\% of MD trace) for the side chains of the Ser\textsuperscript{172} and His\textsuperscript{173} residues (Fig. 5, A and C). Contacts of these residues with water are only observed if their side chains do not participate in forming inter- or intramonomeric hydrogen bonds. The deficit of hydrogen bonds in the latter case is compensated by formation of hydrogen bonds with water molecules. The structural heterogeneity revealed by NMR data confirms the possibility of the existence of different hydrogen bonding with water molecule in the His-Ser node, which is almost in the middle of the membrane at a distance of \( \sim 12 \) Å from the polar head plane (as determined by phosphorus atoms). Consequently, the BNip3 binding into the lipid bilayer would facilitate penetration of water inside the membrane. On the C-terminal side of the TM domain no such penetration of water is observed. Over the MD energy relaxation the depth of penetration of water in this case is the same as for pure DMPC bilayer (\(< \) 5 Å from the polar head plane).

Possibility and Consequences of His\textsuperscript{173} Imidazole Ring Protonation—A very intriguing feature of the BNip3 dimerization interface is the presence of two imidazole rings of His\textsuperscript{173} deeply buried inside the membrane. Accessibility of the histidine to water raises the question about possibility of its protonation. This is especially interesting because intracellular acidosis was shown to be an important step of BNip3 cell death pathway actuation (10). In the pH titration experiments, no protonation of the imidazole group was observed in the NMR spectra down to pH 4 (the lowest pH achievable with DMPC/DHPC bicelles). In other words, the apparent pK of the imidazole group is below 4, and under the possible experimental conditions the situation with protonation of the detectable portion of the His\textsuperscript{173} imidazole group is unattainable. The lack of apparent (integrated over NMR time scale) protonation of the His\textsuperscript{173} imidazole group can be explained, in addition to intramembrane localization, by hydrogen bonding in the His-Ser node. However, protonation of the undetectably small fraction of these groups can have interesting implications. To assess this possibility, additional MD energy relaxations of NMR structure with protonation of one or two His\textsuperscript{173} residues were performed. Simultaneous protonation of two His\textsuperscript{173} residues destabilized the lipid bilayer. In the case of protonation of one of two His\textsuperscript{173} in the dimer, the general packing of the lipid bilayer and the overall structure of the dimer are preserved (Fig. 5B), but accessibility of His\textsuperscript{173} and Ser\textsuperscript{172} residues to water increases substantially, up to 90\% of states covered by the 5-ns MD trace. Consequently, the water permeability of the membrane with the incorporated BNip3 dimer is quite sensitive to protonation/deprotonation of the His\textsuperscript{173} imidazole ring. The structure of the His-Ser node is also significantly affected (Fig. 5D). The side chains of Ser\textsuperscript{172} residues in this case leave the helix packing area and form intramonomeric hydrogen bonds with the carbonyl groups of Ser\textsuperscript{166} and Leu\textsuperscript{169} as well as with water molecules. The stacking interaction of the imidazole rings is abolished. The protonated imidazole ring of His\textsuperscript{173} turns toward the lipid polar head plane to become oriented parallel to the helix axis and forms hydrogen bonds with the uncharged imidazole ring and with water molecules simultaneously. Thus, a chain of hydrogen bonds is created involving several molecules of water and imidazole rings. This chain reaches the middle of the lipid bilayer. Besides, rearrangements of the backbone hydrogen bond network in the C-terminal TM part of the BNip3tm dimer occur in response to the histidine protonation, e.g. during MD simulation the \( \alpha \)-helical hydrogen bond CO(Ile\textsuperscript{181})-HN(Arg\textsuperscript{185}) is switching to CO(Ile\textsuperscript{181})-HN(Gly\textsuperscript{184}), whereas the hydrogen bond CO(Tyr\textsuperscript{182})-HN(Arg\textsuperscript{186}) disappears.

BNip3tm Conductive Properties—Taking into account the structure and dynamic properties of the BNip3 TM domain, as well as the fact that some of the Bcl-2 apoptotic proteins form ion channels or pores (3, 5), we performed a series of experiments with incorporation of the TM domain into artificial BLM in order to test possible membrane permeabilization by the protein. In the experiments where the protein was directly added into the lipid solution used to form the BLM (as opposed to addition of the BNip3tm Me\textsubscript{2}SO solution into water; see the supplemental data), stable and reproducible conductivity, notably exceeding that of the BLM from pure DPhPC, was detected. The conductivity weakly depended upon pH, being 360 \pm 30 pS at pH 4.0 and 210 \pm 20 pS at pH 7.0 (the values correspond to the excess of conductivity over that of pure DPhPC membrane, \( \sim 40 \) pS) but did not detectably change within KCl concentration range from 10 to 100 mM (Fig. 6). In the controls with
addition of similar amount of TM fragment 61–98 of glycophorin A (GpAtm), forming homodimers of well known structure in the membranes (31), the conductivity under constant voltage was, within the accuracy of measurement, the same as in case of pure DPhPC membranes (Fig. 6).

**DISCUSSION**

**Conformation of BNip3tm Dimer Suggests Conductive Transmembrane Pathway**—The structural properties of the BNip3tm dimer suggesting its ability to conduct ions include the hydrogen bond–rich His-Ser node in the middle of the membrane, accessibility of the node for water, and a continuous hydrophilic track along the entire length of the membrane-spanning region. BLM conductivity measurements support this possibility. For the conductivity induced by the homodimeric BNip3 alone, apparently, the most likely candidate for the conducted ion is proton, taking into account the structure and dynamic properties of the TM domain. The observed pH dependence of the conductivity induced by BNip3tm appears to favor the assumption of proton conductivity, i.e. the increase of conductivity at decreasing pH (in the presence of much higher concentrations of other ions) along with the lack of conductivity dependence on potassium concentration can be considered circumstantial evidence of proton-selective conduction through the dimer.

Proton permeability requires the presence of a water-accessible pathway or a system of hydrogen bonds (32). Besides that, many proton channels are characterized by the presence of functionally essential histidine residues inside the membrane, involved in formation and regulation of the proton–conducting pathway (33). The well known tetrameric viroporin M2 responsible for influenza A virus acidification forms a pH-gated proton channel with a TM histidine tetrad essential for conductive properties. Moreover, another viroporin P7 from hepatitis C virus annotated as an oligomeric ion channel (34) has a TM region SLAGTH similar to the BNip3 region $S^{168}$LLSH$^{173}$ forming the His-Ser node. As pointed out above, in the BNip3tm dimer there is a water-accessible cavity spanning half of the membrane thickness and covered by residues Ser$^{168}$, Ser$^{172}$, and His$^{173}$, whereas the rest of the TM dimer is tightly packed through small residues Ala$^{176}$, Gly$^{180}$, and Gly$^{184}$, backbone atoms of which form weakly hydrophilic contacts. Apparently, there is a certain energy barrier for proton translocation through the second part of the domain, but certain conditions, e.g. sufficient electrical potential difference, can make the translocation possible through proton transfer along the chain of hydrogen bonds. Importantly, the TM voltage will be non-uniformly distributed along the dimer axis, with a larger portion of it dropping across the second, non-water-accessible part of the BNip3 TM domain. Besides that, as mentioned above, according to the MD simulations protonation of one His$^{173}$ residue initiates changes of the local structure of the His-Ser node and its water accessibility, as well as distortion of the backbone hydrogen bond network in the vicinity of the second glycophorin motif G$^{180}$XXXG$^{184}$. Thus, despite low pK of the His$^{173}$ residue in the His-Ser node, it can participate in a relay-like transfer of protons via a chain of proton acceptors. Having reached the His-Ser node from the N-terminal side through the hydrophilic water-accessible part of the dimer, the proton might then be transferred in a relay-like manner through a chain of backbone hydrogen bonds to the C-terminal side of the domain, where there are two Tyr$^{182}$/Arg$^{186}$ pairs with tight $\pi$-interaction of aromatic rings and guanidine groups. They can also play a role in conductivity pathway. The slow conformational exchange observed around the His-Ser node indicating that the system of hydrogen bonds undergoes certain rearrangements supports the possibility of a relay-like mechanism. Additionally, it can be hypothesized that the Phe ring hydrophobic cluster on the N-terminal juxtamembrane side of the domain serves as a plug, regulating the BNip3-induced membrane permeability through shielding the inner polar TM part of the dimer from water molecules (Fig. 2B).

**The Unique Structure of the TM Domain of Pro-apoptotic Protein BNip3 Can Be Closely Related to Its Biological Function**—Cell death pathway induced by BNip3 is atypical, combining some features of both necrosis and apoptosis (9). BNip3 uniqueness is the most pronounced in cell death caused by hypoxia acidosis (35). Cell death occurs through the opening of a highly conductive nonselective mitochondrial permeability transition pore (8), a multiprotein complex at the contact site between the inner and outer mitochondrial membranes. This pathway does not require caspase activation and cytochrome c release (1). Presumably, opening of the permeability transition pore is preceded by mitochondrial hyperpolarization, i.e. an increase in inner membrane potential (3, 36, 37). The mechanism of BNip3 pro-apoptotic activity is not yet understood; however, the available phenomenology of the cell death process and recent findings related to the permeability of the mitochondrial outer membrane for small ions suggest a mechanism relating the possible ion conductance induced by BNip3 TM domain and its structural properties with BNip3 biological function. Similarly, it was recently suggested, a plant saponin avicin forming in the outer mitochondrial membrane channels conducting only small ions may favor apoptosis, presumably by altering the potential across this membrane and the intermembrane space pH (38).

The outer mitochondrial membrane is normally permeable for charged metabolites and inorganic ions, mainly due to the existence of voltage-dependent anionic channels (39). Closure of VDAC is believed to occur under a wide range of abnormal cellular conditions and in particular to be an early event in response to many cell death stimuli (40). Historically, free permeability of VDAC in all states for small metal cations and protons was assumed, and consequently their concentrations on different sides of the outer membrane were believed to be in equilibrium with TM electric potential difference. Although still debatable, there is growing evidence that permeability of the outer mitochondrial membrane varies in a wider range than used to be recognized and can be very low under some physiological conditions, including stressed states of cells (41, 42). Therefore, membrane permeabilization by BNip3 can be of consequence for distribution of ions and electric potential across the outer membrane, which could trigger further events in the cell death scenario.

Taking into account the caveats made above, a speculation might be offered concerning the early stages of BNip3 cell death.
pathway in the case of hypoxia. Cellular response to hypoxia includes transition from respiration to anaerobic glycolysis, accompanied by acidification of cytosol and accumulation of NADH both in the cytosol and mitochondrial matrix (43). Accumulation of NADH inhibits mitochondrial metabolism; besides that, NADH was shown to inhibit mitochondrial outer membrane conductance acting as a VDAC ligand (44). Change of the outer membrane potential caused by altered fluxes of metabolites leads to VDAC closure (40, 45), isolating the mitochondria from the cytoplasm and effectively conserving them. Assuming that VDAC closure can restrict the outer membrane permeability to small cations, including protons, the intermembrane space pH would remain near its normal level. Cytosol acidification initiates expression of BNip3, which accumulates in the cytosol in the inactive form (10). This far, all the changes occurring with the cell are reversible in the sense that they do not kill the cell, as was demonstrated in the experiments with alkalization of cells under persisting hypoxia conditions (10). During hypoxia, cytosolic pH can rapidly drop to as low as 6.0 (43). At low pH the BNip3 becomes more hydrophobic, and acidification below 6.5 activates BNip3 by promoting translocation to the outer mitochondrial membrane (10). According to our hypothesis, incorporation of BNip3 increases permeability of the outer membrane, causing decrease of pH in the intermembrane space due to translocation of protons from cytosol and concomitant hyperpolarization of the inner membrane, which leads to permeability transition pore opening and ultimately to necrosis-like cell death.

The suggested mechanism of BNip3 activity does not rely upon the BNip3-induced conductivity being proton-specific; however, proton conductance alone is sufficient for it. It cannot be excluded that BNip3 can provide a conducting pathway for other ions (e.g. small metal cations), but in this case BNip3 would need to form oligomeric complexes or interact with some other mitochondrial membrane proteins, e.g. apoptotic Bcl-2 proteins or and VDAC. The polar pattern on the lipid-exposed surface of the BNip3 TM domain (Fig. 4C) provides a possibility for such intermolecular interactions. Another option is a specific interaction of BNip3 with lipids in mitochondrial membrane leading to formation of ion-conductive structures.

Hypoxia-induced cell death is not the only cell death pathway where the important role of BNip3 has been demonstrated. The possibility of the existence of other scenarios is evidenced by pro-apoptotic activity of the monomeric BNip3 His173-Ala mutant in human embryonic kidney 293 cells, in which BNip3 is known to induce significant cell death without hypoxia acidosis (46). Recently, homodimeric BNip3 was shown to induce ligand-dependent necrosis-like apoptosis in lymphocytes (12). The mitochondrial hyperpolarization was shown to be an essential early step of lymphocyte programmed death (47), and the identified conductive properties of BNip3 may prove important in this case.

Concluding Remarks—Formation of the right-handed parallel symmetric BNip3 tm dimer is made possible mainly due to intermonomeric helix–helix polar interactions of side chain and backbone atoms. Imidazole and hydroxyl groups of His173 and Ser172 serve as intra- and intermonomeric hydrogen bond donors and acceptors; Ala176, Gly180, and Gly184 forming two consecutive characteristic dimerization motifs are closely packed on the dimer interface. In addition, a group of aromatic rings of Phe157, Phe161, and Phe165 on the N-terminal juxtamembrane side of the BNip3 TM domain forms a hydrophobic cluster stabilized by intra- and intermonomeric stacking interactions.

The structural dynamic properties of the TM domain of human protein BNip3 with a flexible network of hydrogen bonds and water accessibility up to the middle of the membrane appear to enable the protein to form an ion-conducting pathway across the membranes. Indeed, the TM domain was shown to induce conductivity of an artificial bilayer lipid membrane in a pH-dependent manner. These findings and currently available information about the phenomenology of programmed cell death allow us to propose a mechanism of triggering necrosis-like cell death by BNip3 in the case of hypoxia acidosis of human tissues. Because the direct relation of the conductive properties of the BNip3 TM domain with pro-apoptotic activity requires further investigation, these results can stimulate further functional studies of this protein with the unique cell death pathway.

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