Structure-based analysis of VDAC1: N-terminus location, translocation, channel gating and association with anti-apoptotic proteins

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Structural studies place the VDAC1 (voltage-dependent anion channel 1) N-terminal region within the channel pore. Biochemical and functional studies, however, reveal that the N-terminal domain is cytoplasmically exposed. In the present study, the location and translocation of the VDAC1 N-terminal domain, and its role in voltage-gating and as a target for anti-apoptotic proteins, were addressed. Site-directed mutagenesis and cysteine residue substitution, together with a thiol-specific cross-linker, served to show that the VDAC1 N-terminal region exists in a dynamic equilibrium, located within the pore or exposed outside the β-barrel. Using a single cysteine-residue-bearing VDAC1, we demonstrate that the N-terminal region lies inside the pore. However, the same region can be exposed outside the pore, where it dimerizes with the N-terminal domain of a second VDAC1 molecule. When the N-terminal region α-helix structure was perturbed, intra-molecular cross-linking was abolished and dimerization was enhanced. This mutant also displays reduced voltage-gating and reduced binding to hexokinase, but not to the anti-apoptotic proteins Bcl-2 and Bcl-xL. Replacing glycine residues in the N-terminal domain GRS (glycine-rich sequence) yielded less intra-molecular cross-linked product but more dimerization, suggesting that GRS provides the flexibility needed for N-terminal translocation from the internal pore to the channel face. N-terminal mobility may thus contribute to channel gating and interaction with anti-apoptotic proteins.

Key words: Bcl-2, Bcl-xL, channel conductance, glycine-rich sequence, hexokinase, voltage-dependent anion channel 1 (VDAC1).

INTRODUCTION

Previously, the 3D (three-dimensional) structure of VDAC1 (voltage-dependent anion channel 1) was solved using X-ray crystallography [1], NMR [2] and a combination of the two [3]. The three solved 3D structures of VDAC1 suggest that the N-terminal region of VDAC1 is located inside the pore and that a helical conformation is present within the N-terminal region, which is attached to the channel wall but is not a part of it [1–3]. The N-terminus interacts with the β-barrel wall and is stabilized by several hydrogen bonds [1]. The position of the α-helix, comprising residues Tyr7–Val17, within the barrel is approximately at the midpoint of the channel wall [3]. Similar positioning was seen in the crystal structure of mVDAC1 (murine VDAC1), with the helical region formed by amino acids 6–20, but being broken into two segments at Leu10 and Gly11. Residues 11–20 were difficult to observe by solution-state NMR, suggesting the dynamic behaviour of this segment [2]. Breaking the helix at Gly11 could increase the mobility in this region. Another structural element is the multiple glycine residues present in the sequence G2YGF25 [1] that connects the N-terminal domain to β-strand 1 of the barrel and is highly conserved among mammals [4].

In a lipid environment, the N-terminal region of VDAC1 is also helical [5]. In addition, CD and NMR studies showed that a synthetic peptide corresponding to VDAC1 residues 2–20 exists as an unstructured peptide in aqueous solvent, forming a well-ordered α-helix comprising residues 5–16 in SDS [6].

A wide range of biochemical and biophysical studies have addressed the structural arrangement of the N-terminal 25 residues of VDAC1, as well as the functional roles assumed by this domain. These studies suggest that the N-terminal domain of VDAC1 can be exposed to the cytoplasm [7], crosses the membrane [8] or lies on the membrane surface [9], as opposed to being located inside the pore [1–3]. Thus the accumulating evidence suggests that the N-terminal region of VDAC1 constitutes a mobile component of the channel.

Further evidence suggesting that the N-terminal region of VDAC1 in fact constitutes a mobile component of the protein comes from the observations that this protein segment exhibits motion during voltage gating [10–12], that anti-VDAC antibodies raised against the N-terminal region of the protein interact with membranal VDAC1, and that mobility of the N-terminal domain may modulate the accessibility of apoptosis-regulating proteins of the Bcl-2 family (i.e. Bax and Bcl-xL) to their binding sites on VDAC1 [13]. Furthermore, the VDAC1 N-terminus was shown to mediate the interaction of VDAC1 with the anti-apoptotic and pro-survival factors HK (hexokinase)-I, HK-II and Bcl2, suggesting it is exposed out of the pore [14].

The involvement of the N-terminal region of VDAC1 in the voltage gating of the channel has been demonstrated in various studies [1,2,14–17]. In its open conformation, VDAC1 shows a preference for transporting anions over cations. However, when high negative or positive voltage (>30 mV) is applied, VDAC1 conductance is significantly lowered and the pore becomes cationic-selective [18,19]. Such voltage-dependence of the conductance was not observed with N-terminal truncated Δ(26)mVDAC1, which exhibited high conductance at all of the voltages tested, in agreement with previous results [11,12,14,20].

Abbreviations used: 3D, three-dimensional; BMOE, bis(maleimido)ethane; CMC, carboxymethyl cellulose; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGS, ethylene glycol bis(succinimidyl succinate); GRS, glycine-rich sequence; HK, hexokinase; HRP, horseradish peroxidase; hVDAC, human VDAC; LDAO, N,N-dimethyldecylamonium-N-oxide; LDH, lactate dehydrogenase; mVDAC, murine VDAC; PK, pyruvate kinase; PLB, planar lipid bilayer; rVDAC, rat VDAC; siRNA, small interfering RNA; shRNA, short hairpin RNA; STS, staurosporine; VDAC, voltage-dependent anion channel; WT, wild-type.

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In addition, several mutations in the α-helix (conserved Asp\(^{16}\) and Lys\(^{20}\)) and in β-strands β1–β5 (Lys\(^{46}\), Lys\(^{61}\), Lys\(^{65}\) and Lys\(^{84}\)) affect the voltage-sensing mechanism of *Saccharomyces cerevisiae* VDAC [17].

Different models for voltage-dependent gating of the channel have been proposed. The first class of models emphasizes the blockage of the pore by a movement of the N-terminal helix from the barrel wall towards the centre of the channel. Such movement might involve the complete helix [1] or only residues 11–20 [2], which are difficult to observe in solution-state NMR. A slight movement of the helix was also seen when comparing the structure of hVDAC1 (human VDAC1) with mVDAC1. In addition, larger conformational re-arrangements upon voltage gating that also involve the β-barrel have been suggested, on the basis of electron microscopy and electrophysiological data [15,16]. Finally, solid-state NMR spectroscopy revealed that removal of the N-terminal helix induces a conformational change in the barrel of liposome-reconstituted hVDAC1 [5]. However, no experimental evidence unambiguously links any of these models to the voltage-gating process.

Finally, additional roles for the N-terminus of VDAC1 have been proposed, including serving as the voltage sensor, being an integral segment of the channel wall [11,21], regulating the conductance of ions and metabolites passing through the VDAC1 pore [1], regulating cytochrome c release and subsequent apoptosis [14] and interacting with apoptosis-regulating proteins of the Bcl-2 family (i.e. Bax and Bcl-xl) [13], Bcl-2 [22] and HK (hexokinase) [14,23]. In light of the above studies and the solved NMR and crystal 3D structures of VDAC1, we addressed the location of the N-terminal domain of VDAC1 within the pore, its translocation and function in voltage gating, and as the target for anti-apoptotic proteins. Using site-directed mutagenesis and cysteine residue substitution, in combination with a thiol-specific cross-linker, we demonstrate that the N-terminal region of VDAC1 exists in a dynamic equilibrium, either located within the pore or freely exposed to the cytosolic face of the β-barrel. Our results suggest that such translocation is responsible for VDAC1 gating and interaction with anti-apoptotic proteins. Additionally, our results suggest that the α-helix structure is involved in its positioning within the pore and that the glycine-rich G\(^2\)YGFG\(^2\) sequence provides the flexibility required for N-terminal region translocation out of the internal pore of the channel.

### MATERIALS AND METHODS

#### Materials

Cytoskeleton (carboxymethyl cellulose), DTT (dithiothreitol), EGTA, EDTA, leupeptin, PMSF, N-decane, soya bean aseolate, STS (staurosporine), sucrose and Tris were purchased from Sigma. LDAO (N,N-dimethyldecylamine-N-oxide) was obtained from Fluka. Hydroxyapatite (Bio-Gel G) was purchased from Bio-Rad Laboratories. Celite came from the British Drug Houses. Monoclonal anti-VDAC antibodies raised against the N-terminal region of VDAC1 were purchased from Calbiochem-Novabiochem. EGS [ethylene glycol bis(succinimidyl succinate)] and BMOE [bis(maleimido)ethane] were obtained from Pierce. HRP (horseradish peroxidase)-conjugated anti-mouse antibodies were obtained from Promega. Anti-actin antibodies were obtained from Santa Cruz Biotechnology. DMEM (Dulbecco’s modified Eagle’s medium), fetal bovine serum, L-glutamine and penicillin/streptomycin were purchased from Biological Industries. Blasticidin was purchased from InvivoGen. Puromycin was purchased from ICN Biomedicals.

#### Peptide synthesis

The synthetic water-soluble N-terminal (AVPPTYADLGKSAR-DWFKGYGFGGL) and G21W-N-terminal (AVPPTYADLGK-SARDVFKWTYGFGL) peptides were synthesized by GL Biochem.

#### Plasmids and site-directed mutagenesis

DNA encoding rVDAC1 (rat VDAC1) [24] was cloned into the BamHI/NotI sites of the tetracycline-inducible pcDNA4/TO vector (Invitrogen). VDAC1 mutations used in the present study were generated by PCR using the QuikChange\(^{TM}\) mutagenesis method, using rVDAC1 or ΔCys-rVDAC1 as cDNA templates (Table 1).

#### Cell lines and growth

T-REX-293 cells [HEK (human embryonic kidney)-293 cells that express the tetracycline repressor] were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and non-essential amino acids, and maintained in a humidified atmosphere at 37°C with 5% CO\(_2\). T-REX-293 cells stably expressing the pSUPERretro vector (OligoEngine) encoding shRNA (short hairpin RNA)-targeting hVDAC1 (hVDAC1-shRNA) and that showed a low (10–20%) level of endogenous hVDAC1 expression were grown with 0.5 μg/ml puromycin and 5 μg/ml blasticidin.

#### Cell transfection

T-REX-293 cells were transiently transfected with the plasmid pcDNA4/TO encoding native rVDAC1, mutated rVDAC1 or mutated ΔCys-rVDAC1 using calcium phosphate. Protein expression was induced by tetracycline (0.5–1.5 μg/ml) for 24–48 h.

For calcium phosphate-based transfection, cells (6×10\(^2\)) were seeded on to a 60-mm-diameter culture dish plate and grown in 3 ml of DMEM, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Plasmid DNA (0.2–1.0 μg) was added to 250 μl of sterile CaCl\(_2\) (240 mM) and mixed with 250 μl of sterile Hepes buffer (280 mM NaCl, 10 mM KCl, 1 mM Na\(_2\)HPO\(_4\), 1.5 mM Hepes, pH 7.05). The mixture was applied to the T-REX-293 cells. Cells were maintained in a humidified atmosphere at 37°C with 5% CO\(_2\) for 24 h, at which time the medium was replaced with 3 ml of fresh medium.

#### VDAC1 silencing and siRNA (small interfering RNA) transfection

Selective silencing of endogenous hVDAC1 was achieved using a shRNA-expressing vector. Nucleotides 337–355 of the hVDAC1 coding sequence were chosen as the target for shRNA specific to hVDAC1. The hVDAC1-shRNA-encoding sequence was created using the two complimentary oligonucleotides indicated below, each containing the 19 nucleotide target sequence and flanked with a non-specific sequence of 20 nucleotides: 5'-GATCCTTTCAAGAGATAATCTCGGTGCCTAGTGTG; oligo-antisense sequence of the target: oligonucleotide 1, AGCTGTTTTTTA. The hVDAC1-shRNA-encoding sequence was cloned into the BglII and HindIII sites of the pSUPERretro plasmid, containing a puromycin-resistance gene. Transcription of this sequence by RNA polymerase III produces a hairpin (hVDAC1-shRNA).
Table 1 Oligonucleotides used for VDAC1 mutagenesis

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Chemical cross-linking

Control T-REX-293 cells or T-REX-293 cells transfectected to express native rVDAC1, mutated rVDAC1 or mutated ΔCys-rVDAC1 (3 mg/ml), were cross-linked by incubation with different concentrations of EGS (50–300 μM) in PBS, pH 8.3 (15–30 min, 30°C) and the reaction was stopped by adding SDS/PAGE sample buffer. For cross-linking with BMOE, cells were incubated with the reagent (0.1 mM) in PBS, pH 7.2, at 30°C, and after 30 min the reaction was terminated by adding DTT (10 mM). Samples (30 μg of protein) were subjected to SDS/PAGE and immunoblotting using anti-VDAC antibodies. Protein concentrations were determined with the Lowry method [24a], using BSA as the standard.

Gel electrophoresis and immunoblot analyses

SDS/PAGE was performed according to Laemmli [25]. Gels were stained with Coomassie Brilliant Blue or transferred on to nitrocellulose membranes for immunostaining. Membranes containing the transferred proteins were blocked with 5% (w/v) non-fat dried skimmed milk powder and 0.1% Tween 20 in Tris-buffered saline (10 mM Tris/HCl and 150 mM NaCl, pH 7.8), then incubated with monoclonal anti-VDAC1 antibodies (1:10 000 dilution), followed by incubation with HRP-conjugated anti-mouse IgG secondary antibodies (1:10 000 dilution). An actin-specific polyclonal antibody (1:10 000 dilution) was used as a loading control. After treatment with the appropriate primary and secondary antibodies, enhanced chemiluminescence (Pierce) was performed. Quantitative analysis of the blotted membranes was carried out using EZQuant-Gel one-dimensional software analysis according to parameters defined by the software algorithms for band and lane depiction.

Purification of VDAC1

For recombinant VDAC1 purification, rVDAC1, R15P/D16P-rVDAC1 and G21V/G23V-rVDAC1 were expressed in the S. cerevisiae M22-2 por1-mutant strain as described previously [24]. VDAC1 was purified from yeast mitochondria as described previously for rat liver mitochondria [26]. Rats were anaesthetized with carbon dioxide and killed by decapitation. The work was approved by the Ben-Gurion University of the Negev and the National Animal Care and Use Committee. Rat liver mitochondria in 10 mM Tris/HCl, pH 7.2, were incubated with 2% (w/v) LDAO at 4°C for 30 min, followed by centrifugation (20 min, 20000 g) and the supernatant obtained was loaded on to a dry celite/hydroxyapatite (2:1) column. VDAC1 was eluted with a solution containing 2% (w/v) LDAO, 10 mM Tris/HCl, pH 7.2, 50 mM NaCl and 22 mM NaH2PO4. The VDAC1-containing fractions were dialysed against 10 mM Tris/HCl, pH 7.2, and subjected to a second chromatography step on a CMC column, from which VDAC1 was eluted with a solution containing 10 mM Tris/HCl, pH 7.2, 0.3% LDAO and 500 mM NaCl. The VDAC1-containing fractions were collected and used for VDAC1 analysis.

Bcl-2 and Bcl-xL expression and purification

Bcl-2(ΔC23) and Bcl-xL(ΔC21) were purified as described previously [22]. Briefly, DNA encoding Bcl-2(ΔC23) and Bcl-xL(ΔC21) were cloned into the pHis parallel and PET47b+ vector respectively and expression was induced with isopropyl β-D-thiogalactopyranoside in Escherichia coli BL21 cells. The proteins were purified from the soluble fraction by chromatography using Ni2+-ni-trilotriacetate resin and stored in aliquots at −80°C.

VDAC1 reconstitution, recording and analysis

The reconstitution of purified wild-type or mutated rVDAC1 into a PLB (planar lipid bilayer) and subsequent single and multiple channel current recordings and data analysis were carried out as described previously [26]. Briefly, the PLB was prepared from soya bean asolectin dissolved in n-decane (50 mg/ml). Purified VDAC1 was added to the chamber defined as the cis side containing 1 M NaCl. Currents were recorded under voltage-clamp using a Bilayer Clamp BC-535B amplifier (Warner Instruments). The currents, measured with respect to the trans side of the membrane (ground), were low-pass-filtered at 1 kHz and digitized online using a Digidata1440-interface board and pClampex 10.2 software (Axon Instruments).
Rat brain HK-I purification and assaying the detachment of mitochondria-bound HK

HK-I was purified from rat brain mitochondria as described previously [27], using a Cibacron Blue HP column in an AKTA basic purifier chromatography system (Amersham Biosciences). For mitochondria-bound HK detachment, isolated brain mitochondria (1.5–2 mg/ml) in buffer containing 2.5 mM MgCl₂, 100 mM KCl, 250 mM sucrose and 20 mM Heps, pH 7.5, were incubated with increasing amounts of synthetic peptides (0.0025–0.2 mM) for 60 min, followed by centrifugation at 10,000 g for 5 min at 4 °C. Released HK was measured spectrophotometrically at room temperature (25 °C) at 340 nm by assaying its activity in a coupled reaction of NADH oxidation by LDH (lactate dehydrogenase) with the production of ADP by HK, and its phosphorylation by PK (pyruvate kinase), with phosphoenolpyruvate (1 mM) as substrate. LDH and PK were added in excess, at a level of approximately 50 m-units of each enzyme per 5 m-units of HK.

RESULTS

In the present study, using site-directed mutagenesis and cross-linking approaches, we addressed the location and translocation of the VDAC1 N-terminal domain and the function of this domain in VDAC1 dimerization, the voltage-sensitivity of the channel and in the interaction with the anti-apoptotic proteins Bcl-2, Bcl-xL and HK.

Cysteine residue introduction into the VDAC1 N-terminal region reveals the location of this domain within the pore, its translocation and its involvement in oligomerization

To study the location and possible translocation of the VDAC1 N-terminal region, the ΔCys-rVDAC1 mutant was modified by replacing the threonine residue in the N-terminus with a cysteine residue to obtain T6C-ΔCys-rVDAC1. The protein was expressed in T-Rex cells silenced for hVDAC1 expression by siRNA specific for hVDAC1, which allows expression of rVDAC1, purified from these cells. Purified wild-type and mutant rVDAC1 were reconstituted into a PLB where VDAC1 activity was analysed. rVDAC1 mutant channel conduction and voltage dependence were similar to the wild-type protein (results not shown). The mutated protein was expressed in hVDAC1-shRNA cells, exposed to STS to induce apoptosis and then subjected to cross-linking with the thiol-specific reagent BMOE, followed by immunoblotting with anti-VDAC antibodies (Figure 1). Interestingly, BMOE cross-linking of cells expressing T6C-ΔCys-rVDAC1 resulted in both dimer formation (Figure 1A, solid arrow) and in the appearance of a band corresponding to monomeric T6C-ΔCys-rVDAC1 with modified electrophoretic mobility (Figure 1A, open arrow). This band most likely represents a monomer that underwent intra-molecular cross-linking that modified its electrophoretic mobility. Since T6C-ΔCys-rVDAC1 contains only a single cysteine residue, such intra-molecular cross-linking suggests that BMOE reacted with a non-thiol group in rVDAC1. As can be seen, the electrophoretic mobility of T6C-ΔCys-rVDAC1 with no cross-linking was identical with that of wild-type rVDAC1 (Figure 1A).

BMOE is highly specific to thiol residues, consistent with our finding that no rVDAC1-containing cross-linked products were formed in cells expressing ΔCys-rVDAC1 [28] (see also Figure 2A). Although we performed our cross-linking reactions at a physiological pH value (pH 7.4), it has been shown that, under alkaline conditions (pH>8.0), BMOE can react with primary amine groups [29]. Thus the monomeric T6C-ΔCys-rVDAC1 species presenting modified electrophoretic mobility may result from intra-molecular cross-linking of the introduced cysteine residue in the VDAC1 N-terminus with lysine residue(s) in the β-barrel facing the pore (see Figure 1B). This finding supports the previously published VDAC1 3D structure showing that the N-terminal region is located inside the β-barrel pore [1–3].

Analysing the three published VDAC1 3D structures revealed the location of several candidate lysine residues (Lys⁴⁰, Lys⁵⁰, Lys⁷⁰ and Lys⁹⁰), which can form a critical lysine cluster (Lys⁴⁰, Lys⁵⁰, Lys⁷⁰ and Lys⁹⁰) (Figure 1B, yellow cluster). This cluster is adjacent to the putative N-terminal lysine residues, which are involved in the formation of the β-strands of the β-barrel pore [1–3].
membrane-facing), formation of intra-molecular adducts was only linking. As with the single cysteine residue mutant, T6C-is no longer positioned at the required distance for BMOE cross-electrostatic repulsion in the pore. As such, the N-terminal domain the newly introduced cysteine residues (Figure 1C). The K197C-this mutant, formed upon cross-linking of VDAC1 monomers via /Delta1 rVDAC1, the double cysteine residue mutant K224C-T6C residue. Accordingly, these lysine residues were replaced with non-BMOE-reactive alanine residues. Cells expressing T6C-, T6C,R15P/D16P-[r15,D16]P]-, G21V/G23V-[G(21,23)V]- or native-rVDAC1. Cells were treated with STS (3.5 μM, 4 h) and then incubated with BMOE (100 μM, 30 min) and subjected to SDS/PAGE (11 % gel) and Western blotting using N-terminal specific anti-VDAC antibodies or anti-actin antibodies, as a loading control. The open arrow indicates an intra-molecular cross-linked band. The closed arrow indicates VDAC1 dimers. The relative amount (RU) of dimers and modified monomers formed upon cross-linking with BMOE are presented (n = 3). (B) T-Rex-293 cells stably expressing hVDAC1-shRNA were transfected to express ΔCys-rVDAC1 bearing T6C and either the R15P or D16P mutation was expressed in hVDAC1- ΔCys-rVDAC1. The VDAC1 N-terminal region can translocate between at least two positions and may participate in VDAC1 oligomerization.

The VDAC1 N-terminal α-helix and GR5 (glycine-rich sequence) are main factors in N-terminal translocation

The 25-amino-acid N-terminal region of VDAC1 comprises an amphipathic α-helix structure located between two unstructured regions [1–3]. To study the involvement of the α-helix domain in the location and translocation of the VDAC1 N-terminal region and in VDAC1 oligomerization, ΔCys-rVDAC1 was engineered to replace Thr8 with a cysteine residue, alone or with proline residue(s) also introduced in the α-helix domain to disturb the α-helix structure [30]. ΔCys-rVDAC1 bearing T6C and either the R15P or D16P mutation was expressed in hVDAC1-shRNA cells, exposed to STS to induce apoptosis, and then subjected to cross-linking with BMOE, followed by immunoblotting with anti-VDAC antibodies (Figure 2A). When the N-terminal α-helix domain of T6C-ΔCys-rVDAC1 was disrupted by substitution of Arg15 and Asp16 with proline residues, a 2–3-fold increase in the levels of T6C-ΔCys-rVDAC1 dimers and VDAC1-containing higher cross-linked oligomers was obtained (Figure 2A, closed arrow). These results suggest that interrupting the N-terminal α-helix structure increased the probability of the N-terminal region of VDAC1 molecules interacting with each other.

The translocation of the N-terminal region out of the channel pore is reflected in the high reduction or complete elimination of the appearance of the intra-molecular cross-linked monomeric T6C-ΔCys-rVDAC1 when Arg15 or Asp16 (results not shown), or both were replaced with proline residues (Figure 2A, open arrow). These findings indicate that the presence of a proline residue in the α-helix domain of the N-terminal region destabilizes the position of this domain within the pore and allows its movement toward the cytosolic face of the membrane.

Translocation of the VDAC1 N-terminal region requires a flexible hinge domain connecting this region to the β-barrel. Indeed, VDAC1 possesses a GRS within the β-strand 1 sequence G21YFG25 connected to the N-terminal domain. Accordingly, to alter the hydrophobicity and flexibility of this glycine-rich region in T6C-ΔCys-rVDAC1, we substituted Gly21 and Gly23 in the GRS with valine residues to yield a protein containing V21YYFG25. These substitutions led to a decrease in the level of the intra-molecular cross-linked product, with a concomitant increase in dimer formation (Figure 2A). It should be noted that other mutations in the N-terminal region (i.e. in the GRS) or in

![Figure 2](https://example.com/fig2.png)

Figure 2 The α-helix structure and the glycine-rich sequence affect the location of the VDAC1 N-terminal region

(A) T-Rex-293 cells stably expressing hVDAC1-shRNA were transfected to express ΔCys-rVDAC1 or T6C-ΔCys-rVDAC1. Cells were treated with STS (3.5 μM, 4 h) and then incubated with BMOE (100 μM, 30 min) and subjected to SDS/PAGE (11 % gel) and Western blotting using N-terminal specific anti-VDAC antibodies or anti-actin antibodies, as a loading control. The open arrow indicates an intra-molecular cross-linked band. The closed arrow indicates VDAC1 dimers. The relative amount (RU) of dimers and modified monomers formed upon cross-linking with BMOE are presented (n = 3). (B) T-Rex-293 cells stably expressing hVDAC1-shRNA were transfected to express ΔCys-rVDAC1 bearing T6C and either the R15P or D16P mutation was expressed in hVDAC1- ΔCys-rVDAC1. The VDAC1 N-terminal region can translocate between at least two positions and may participate in VDAC1 oligomerization.
the β-barrel, such as Y22A, Y22D, K119A or K174A, had no effect on the formation of either dimers or the intra-molecular cross-linked product (results not shown).

These results suggest that, when the flexible GRS serving as the N-terminal region linker to the β-barrel is modified, the N-terminal region favours its location out of the pore.

Finally, identical EGS cross-linking product profiles were obtained with either wild-type or mutated rVDAC1 (ΔCys-, R15P/D16P-rVDAC1 or G21V/G23V-rVDAC1), indicating that the mutations did not change the protein conformations, since the obtained oligomerization pattern is identical with the normal VDAC1 oligomerization pattern (Figure 2B).

The disrupted N-terminal α-helix in R15P/D16P-rVDAC1 displays low and asymmetric voltage-dependent conductance

The involvement of the N-terminus of VDAC1 in the voltage gating of the channel has been demonstrated in various studies [1,2,14–17]. Following the use of molecular biology and biochemical approaches to demonstrate the translocation of the N-terminal domain of VDAC1, we next asked whether this translocation affects the voltage gating of the channel. To address this question, we expressed rVDAC1 and the mutants R15P/D16P-rVDAC1 and G21V/G23V-rVDAC1 in the porin-deficient *S. cerevisiae* M22-2 por1-mutant strain and purified the various versions of VDAC1 from the mitochondria. Purified wild-type and mutated rVDAC1 were reconstituted into a PLB where VDAC1 activity was analysed (Figure 3). Wild-type VDAC1 activity is characterized by a maximal current conductance of 4 nS at moderate voltages (between −25 and +25 mV) and fluctuates between a high conductance state (open state) and low conductance sub-states (closed state) at high voltages (+25 to +80 mV).

At low voltage (−10 mV), a similar maximal conductance of 4 nS was obtained for both wild-type and mutated R15P/D16P-rVDAC1 proteins, suggesting that the mutation had no effect on either pore size or on other channel features that can affect conductance (Figure 3A, panel I, and Figure 3B, panel I). At high voltage (−50 mV), the bilayer-reconstituted wild-type rVDAC1 was stable mainly in the closed sub-states (see Figure 3A, panel II and Figure 3B, panel II). In contrast, the mutated R15P/D16P-rVDAC1 remains in the open high conducting state (Figure 3A, panel II and Figure 3B, panel II), suggesting that it has lost part of its voltage sensitivity. This behaviour was further demonstrated when channel conductance was analysed as a function of voltage (Figure 3C, black circles). Although the native protein exhibits a typical bell shape voltage-dependent conductance, with the highest conductance being obtained at membrane potentials between −25 and +25 mV, and decreasing at both high negative and positive potentials (Figure 3C), the mutant R15P/D16P-rVDAC1 showed asymmetric voltage-dependence conductance, with decreased voltage-dependent conductance being prominent at negative voltages (Figure 3C). The results suggest that disrupting the α-helix structure at the N-terminal region of VDAC1 led to its translocation out of the pore (see model in Figure 3D), resulting in a modified channel voltage-dependence, supporting the function of the VDAC1 N-terminal α-helical segment in voltage gating.

The inflexible VDAC1 N-terminal V21YVFG25 mutant shows various gating behaviours

Next, we examined the channel conductance of the inflexible VDAC1 N-terminal V21YVFG25 mutant channel, G21V/G23V-rVDAC1. In approximately 50% of the recordings, the bilayer-reconstituted G21V/G23V-rVDAC1 showed normal gating, as seen with the wild-type channel (Figure 4A). However, in other recordings, the same channel shifted from normal voltage-dependent conductance to the closed state (type I gating, see Figure 4B), the open state (type II gating, see Figure 4B) or a combination of the two gating types (Figure 4B). These results demonstrate the function of the GRS in conferring flexibility to the N-terminal domain.

The N-terminal region of VDAC1 interacts with anti-apoptotic proteins

The interaction of the N-terminal region of VDAC1 with the anti-apoptotic proteins HK, Bcl-2 and Bcl-xL is demonstrated in Figures 5 and 6. A synthetic VDAC1-based peptide corresponding to the N-terminal region of the protein was able to release mitochondria-bound HK-I from brain mitochondria expressing very high levels of HK-I [24]. Following incubation of isolated
Wild-type or G21V/G23V-rVDAC1 were expressed in porin-less yeast, purified and reconstituted into a PLB as described in the Materials and methods section. (A) Channel conductance of wild-type rVDAC1 (●) and G21V/G23V-rVDAC1 (□) as recorded in response to a voltage step from 0 mV to voltages between −60 and +60 mV. The relative conductance (G/Gmax) represents the ratio of conductance at a given voltage (G) to the maximal conductance (Gmax). The broken black line represents the average trend-line of native rVDAC1. G21V/G23V-rVDAC1 shows normal gating behaviour. (B) G21V/G23V-rVDAC1 conductance recording (□, ●) of the same channel shows various gating types that switch from closed in all voltages (□, type I) to open (●, type II) or gating only in positive or negative potentials, reflecting the function of the GRS in conferring flexibility to the N-terminal domain. (C) The inflexible N-terminal region of mutated G21V/G23V-rVDAC1 translocates to different positions during channel gating. Type I represents when the region is fixed inside the channel pore. Type II represents when the region is fixed on the cytosolic side of VDAC1.

Figure 5 Detachment of mitochondria-bound HK-I by VDAC1 N-terminal region-based peptides

(A) Brain mitochondria were incubated with synthetic N-terminal (N.T) or mutated G21W-N-terminal peptide (200 μM) and the released HK-I was analysed as described in the Materials and methods section. HK-I released by glucose 6-phosphate (G6P) (2 mM) is also presented. The results represent the net release after subtracting HK-I released in the absence of the peptides. (B) HK-I release as a function of the concentration of N-terminal or mutated G21W-N-terminal peptide (n = 2–5).

brain mitochondria with the peptide, HK activity appeared in the supernatant (Figure 5A). The G21W-mutated N-terminal region peptide, however, detached mitochondria-bound HK to a lesser extent than did the native N-terminal peptide (Figure 5A). HK was released from rat brain mitochondria by both N-terminal peptides in a dose-dependent manner. An apparent affinity (K0.5) of 20 μM was derived for the N-terminal peptides from the saturation curve fits, whereas over 10-fold decreased binding affinity was estimated for the mutated G21W N-terminal peptide (Figure 5B).

To demonstrate further the interaction of HK-I, Bcl-2(Δ21) and Bcl-xL(ΔC23) with the N-terminal region of VDAC1, we analysed the effect of all three proteins on the channel conductance of wild-type and mutated (R15P/D16P) rVDAC1 (Figure 6). Purified HK-I interacted with purified wild-type rVDAC1 reconstituted into a PLB and reduced its channel conductance by approximately 50% (Figure 6A). However, HK-I reduced the channel conductance of N-terminal-mutated R15P/D16P-rVDAC1 to a lesser extent (25%).

In contrast, recombinant purified truncated forms of Bcl-xL [Bcl-xL(ΔC23)] and Bcl-2 [Bcl-2(Δ21)], which do not include the hydrophobic C-terminal domain, reduced the conductance of bilayer-reconstituted wild-type and R15P/D16P-rVDAC1 to a similar extent (Figures 6B and 6C), with Bcl-2(Δ21) reducing the conductance of mutated VDAC1 more than that of the wild-type protein.

The weaker interaction of HK-I with the mutant R15P/D16P-rVDAC1 was observed at all voltages tested (+60 to −60 mV), some of which are shown in the present study, revealing that HK-I decreased wild-type channel conductance by 60%, but that of the mutated protein by only 30% (Figure 6D). As expected from the single-channel measurements (Figures 6B and 6C), Bcl-xL(ΔC23) similarly modified the conductance of the wild-type and R15P/D16P-rVDAC1, whereas Bcl-2(Δ21) decreased the conductance of the mutated protein by
approximately 1.5-fold more than the decrease observed with the wild-type channel (Figure 6D). These effects were obtained at all voltages tested (Figure 6D). The effect of mutation in the GRS on HK-I, Bel-xL and Bel-2 interactions was not evaluated due to the complex behaviour of this mutated channel (see Figure 4). These results indicate that the interactions of these anti-apoptotic proteins with the mutated VDAC1 protein are not identical, being weaker for HK-I, suggesting that the α-helix structure stabilizes its interaction with VDAC1.

**DISCUSSION**

In the present study, we addressed a major issue regarding the location and translocation of the VDAC1 N-terminal region and its function in channel voltage gating and apoptosis regulation by anti-apoptotic proteins. We were able to demonstrate that the N-terminal region of VDAC1 exists in a dynamic equilibrium, either located within the pore or freely exposed to the cytosolic face, is responsible for channel voltage-dependent gating and mediates the anti-apoptotic effect of Bcl-2, Bel-xL and HK.

**The N-terminal domain of VDAC1: location and translocation**

The results shown indicate that the N-terminal domain, a 25-residue-long sequence of VDAC1, is located within the pore, close to the β-barrel lysine residues Lys197 and Lys224. This is reflected in the appearance of faster migrating VDAC1 species in cells expressing T6C-ΔCys-rVDAC1 (Figure 1A), arising due to intra-molecular cross-linking by the thiol-specific reagent BMOE. Since this VDAC1 version contains only a single cysteine residue at position 6, the BMOE intra-molecular cross-linked product must involve this cysteine residue and another amino acid residue presenting a free amino group, such as primary amine residues [29].

Accordingly, we targeted lysine residues (i.e., Lys197, Lys199, Lys173, Lys178, Lys184, Lys212, Lys217) at a distance of approximately 8 Å (the length of BMOE) from the inserted cysteine residue at position 6 by replacing each of these lysine residues with alanine. In this manner, we identified Lys197 and Lys224 as being involved in the intra-molecular cross-linking (Figure 1D), Cys832, which faces the pore and is found in proximity to the same lysine cluster (Figure 1D) also produced intra-molecular cross-linking, thus strengthening our conclusion. Our findings support the published 3D structure of VDAC1 showing that the N-terminal region is located inside the β-barrel pore [1–3], but suggest that the N-terminal domain is, in fact, located deep in the pore (Figure 1D).

The results shown in the present study also indicate that the N-terminal region, with its α-helical moiety, does not constantly lie inside the pore but can translocate out of the pore. Once there, due to the amphipathic nature of the helix, it can associate with the β-barrel at its bilayer-facing surface or remain at the cytosolic face of the channel. When the T6C substitution was introduced into ΔCys-rVDAC1, BMOE-cross-linked dimers and higher oligomers were formed, suggesting that the N-terminal domain can be moved from its position within the pore to an exposed position outside of the pore, where it can associate with the N-terminal domain of another VDAC1 molecule (Figure 1A, solid arrow) or with other VDAC1-associated proteins.

Dimer formation was highly increased at the same time as the intra-molecular cross-linking product completely vanished when helix-disrupting proline residues were introduced into the predicted α-helix domain of the N-terminal region (Figure 2A). This implies that such a modification decreased N-terminal region interaction with the β-barrel and increased its exposure to the cytosolic surface, where it can interact with the N-terminal region of a neighbouring VDAC1 molecule to form a dimer, suggesting that the N-terminal domain can move out of the pore. In addition, the results indicate that the α-helix structure is not required for N-terminal region association with other N-terminal elements of VDAC1 molecules (during dimer formation) or with other associated proteins.

Oligomeric assembly of VDAC1 was shown to be coupled to apoptosis induction, with oligomerization increasing substantially upon apoptosis induction and being inhibited by apoptosis inhibitors [31]. In the present study, we demonstrated that two VDAC1 monomers can dimerize either through the pair of β-strands 13 or via the pair of β-strands 15 (Figure 1). In a previous study [32], β-strand 16 was found to be the dimerization interaction site. All of these findings together suggest that the dimerization interface involves more than a single β-strand.

The interaction of the N-terminal region with specific antibodies directed to this sequence [33–35] further supports the concept of the exposure of the N-terminal domain outside of the pore. We propose that the process of N-terminus movement is assisted by the conformational flexibility of glycine residues in the glycine-rich GXXXG sequence, which connects the VDAC1 N-terminal region to β-strand 1 of the barrel and is highly conserved among mammals [4]. We replaced glycine, the smallest and most flexible of the amino acids [36], with the less flexible valine. These substitutions led to a decrease in the amount of intra-molecular cross-linked product formed, with a concomitant increase in dimer formation (Figure 2A), suggesting that when the flexibility of the N-terminal region linker, GRS, is disrupted, the N-terminal region prefers to be outside the pore. Thus, in VDAC1, the GRS might be the motif that provides the flexibility required to allow the N-terminal region to move out of the pore lumen.

Interestingly, the GRS found in the N-terminal region of VDAC1 corresponds to the GXXXG motif that has been linked with dimerization in proteins such as glycoporin A [37], human carbonic anhydrase [38], yeast ATP synthase [39], carnitine palmitoyltransferase [40] and others. However, our results indicate that VDAC1 dimers can be formed via cross-linking of the N-terminal regions of two molecules even when this motif sequence is mutated (see Figures 2A and 2B), suggesting that this sequence does not serve as a dimerization motif in VDAC1. On the other hand, it might be involved in interaction with VDAC1-associated proteins, such as Bel-xL, as the GXXXXG motif of Bel-xL is proposed to mediate its dimerization [41]. Moreover, it was proposed that the interaction of plasmalemmal type-1 VDAC with amyloid β-peptides is mediated via GXXXG motifs, with such interactions resulting in apoptosis and inducing Alzheimer’s disease [42].

**The N-terminal region of VDAC1 interacts with HK and anti-apoptotic proteins**

The translocation of the VDAC1 N-terminal region from the pore to the cytosolic face of the protein is in accordance with the interaction of the N-terminal region with several cytosolic anti-apoptotic proteins, including HK-I, HK-II [14,23] and Bel-2 [13,14,22]. Purified Δ(26)mVDAC1 lost its capability to bind HK-I or Bel-2 [14]. Furthermore, an expressed and a synthetic VDAC1 N-terminal peptide was shown to interfere with the interaction of HK-I, HK-II and Bel-2 with VDAC1 and prevented the anti-apoptotic activities of these proteins [14,22].

Using bilayer-reconstituted purified wild-type or N-terminal region-mutated R15P/D16P-rVDAC1, with its enhanced exposure of the N-terminal domain to the cytosol (Figure 3D), we demonstrated that Bel-2(Δ21), Bel-xL(ΔC23) and HK-I interact
with VDAC1, as reflected in the decrease in channel conductance (Figure 6). C-terminal-region-truncated Bcl-xL(ΔC23) similarly decreased the channel conductance of wild-type and R15P/D16P-rVDAC1, suggesting that the disrupted α-helix structure does not affect its interaction with VDAC1. Moreover, this modified N-terminal structure resulted in increased inhibition of R15P/D16P-rVDAC1 conductance by Bcl2(Δ21) (Figure 6D), probably due to the movement of the N-terminal domain out of the pore (see Figure 3D). The C-terminal region is proposed to mediate the interaction of Bcl-2 and Bcl-xL with the membrane [43], yet the C-terminal-truncated protein binds to VDAC1, suggesting that this domain is not crucial for the interaction with VDAC1.

The interaction of HK-I with the N-terminal mutated R15P/D16P-rVDAC1 was modified, as reflected in a 50% decrease in its inhibition of the channel conductance (Figures 6A and 6D). This decrease was obtained regardless of the increased accessibility of the mutated N-terminus to the cytosolic face of the protein, suggesting that the mutation decreased the binding of HK-I to the N-terminal region. The importance of the N-terminal sequence for the interaction with HK-I is also reflected in the weak capability of the G21W-N-terminal peptide, in comparison with the wild-type peptide, to detach mitochondria-bound HK-I (Figures 5A and 5B).

If the N-terminal region of VDAC1 is located within the pore, the interactions of HK-I with the N-terminal domain [14,23] (Figures 5 and 6) suggest translocation of this VDAC1 region out of the pore, as demonstrated in the present study. However, it is also possible the N-terminal region of HK-I is inserted into the pore, where it interacts with the N-terminal region of VDAC1, as demonstrated for tubulin [44]. The N-terminal region of HK-I plays a crucial role in the HK-I interaction with VDAC1 [27]. The N-terminal region of human HK-I forms an 11-residue hydrophobic α-helix that was proposed to be inserted into the mitochondrial membrane [45], where it could interact with VDAC1. On the other hand, a computational model of the HK-I–VDAC1 complex suggests interaction of the first 15 N-terminal residues of HK-I with the inner part of the barrel of VDAC1 and not with the outside walls [46]. It has also been suggested that the position of the HK N-terminal helix within the VDAC1 could contribute to direct and ATP-mediated protein–protein interactions by stabilizing VDAC1 [46]. It was also suggested that interaction of the anti-apoptotic proteins with the N-terminal domain of VDAC1 may be coupled to conformational changes in VDAC1 [5] or to a partial or complete unfolding of its helical N-terminal domain [2,3].

An integrated model of N-terminal region translocation and VDAC1 gating

Various studies have demonstrated the importance of the N-terminal segment of VDAC1 in the voltage-gating behaviour of the channel [1,2,11,12,14–17]. For example, N-terminally-truncated mVDAC1 showed no voltage-dependence and exhibited high conductance at all membrane potentials tested [5,14]. In addition, larger conformational rearrangements upon voltage gating that also involve the β-barrel have been suggested, on the basis of electron microscopy and electrophysiological data [15,16,47]. Different models for voltage-dependent gating of VDAC1 via the N-terminal region have been proposed, including: (i) blockage of the pore by movement of the N-terminal domain within the lumen from the barrel wall towards the centre of the channel [1,2]; (ii) transition of the N-terminal region from an α-helical structure that aligns with the barrel wall to a less-structured unfolded helix element that interacts with the opposing barrel wall [2,3]; and (iii) movement of the N-terminal region into and out of the channel lumen.

In the present study, we addressed the possible mechanism for voltage gating of the channel by targeting two structural elements of the VDAC1 N-terminal region, namely the α-helix domain and the GRS. We made two major observations when a proline residue was introduced into the predicted α-helix domain of the N-terminal region to destabilize helix formation. First, it was noted that intra-molecular cross-linked product formation in T6C, R15P/D16P-ΔCys-rVDAC1 was abolished (Figure 2A). Secondly, the R15P/D16P-rVDAC1 mutant protein displayed modified voltage-dependent conductance (Figure 3A, Figure 3B, panel II, and Figure 3C). These observations suggest that the association and disassociation of the N-terminal region with the β-barrel inside the channel pore and its translocation out of the pore represent the mechanism of channel gating.

Our results also suggest that movement of the N-terminal region is facilitated by a molecular hinge, namely the GRS connecting the N-terminal region to the channel barrel. The GRS mutated channel, T6C, G21V/G23V-rVDAC1, disrupted in the flexible GRS, showed various abnormal conducting states and voltage-gating types (Figures 4B and 4C), suggesting that the GRS confers flexibility to the N-terminal domain and is involved in its ability to move. This is in agreement with the proposal that GRS could exist in different states and pivot at a point near the N-terminus of the β-strand 1 [4].

The contributions of the N-terminal region α-helix and GRS structural motifs in its association with the pore wall and translocation out of the pore are also reflected in the diminished formation of the intra-molecular cross-linked monomer and the enhanced dimer formation obtained with mutated VDAC1 proteins (Figures 3 and 4).

These functional studies, demonstrating the loss of the channel phenotype, i.e. modified voltage-dependent channel-gating, indicate that the α-helix domain of the N-terminal region is important for VDAC1 gating, most probably acting to stabilize the N-terminal region’s interaction with the channel wall. On the basis of increased dimer formation and decreased voltage sensitivity of the R15P/D16P-rVDAC1 mutant, we propose a model in which the N-terminal domain of VDAC1 is responsible for channel voltage gating. When the N-terminal region interacts via its amphipathic α-helix structure with the channel pore, as suggested by the VDAC1 3D structure [1–3] (Figure 1E, a), the flow of ions through the channel is disrupted, resulting in a sub-state with low conduction. When the N-terminal region detaches from the channel pore and translocates out of the pore (Figure 1E, c), the channel is in its ‘open’ state, allowing the passage of the conducting ion at the maximal level. Breaking the α-helix structure of the N-terminal region (Figure 3D, b) disrupts its interaction with the channel pore, resulting in its translocation out of the pore (Figure 3D, c).

Indeed, it has been shown previously that the N-terminus can adopt different conformations under different conditions [35,48]. Furthermore, in 2D (two-dimensional) crystals of Neurospora crassa VDAC1, oblique arrays of the N-terminal domain appear to extend laterally from the barrel into the aqueous phase [35]. In a recent publication [49], calculations using both Poisson–Boltzmann and Poisson–Nernst–Planck electrostatic equations agreed with the N-terminal region being involved in gating, although the precise mechanism is probably not as simple as a lateral or horizontal movement of the helix.

Further evidence suggesting that the N-terminal region of VDAC1 constitutes a mobile component of the protein comes from findings that the N-terminal domain exhibits motion during voltage gating [10] and that the N-terminus of the protein is

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accessible to anti-VDAC1 antibodies raised against this part of the protein [33–35], and exposed to kinases, as Thr13 at the N-terminus undergoes phosphorylation [50]. Finally, the mobility of the N-terminal domain out of the pore may modulate its accessibility to the anti-apoptotic proteins Bcl-2, Bcl-xl or HK, with such interaction mediating their anti-apoptotic activity [13,14,22,23].

Taken together, the electrophysiological data obtained in PLB experiments correlates well with the structural analysis pointing to the location of the N-terminal region within or outside of the VDAC1 pore. Accordingly, we suggest that a ‘paddle’ movement, namely a movement of the N-terminal region from the internal pore to the channel face, is associated with the voltage-dependent gating of the VDAC1. Applied voltage could alter the sensor regions such that the interaction of the N-terminal region with the barrel wall is destabilized and favors its location outside the pore. Since no major voltage changes occur in the outer mitochondrial membrane, the physiological signal modulating VDAC1 N-terminal region location and translocation has yet to be identified and could include modifications, such as phosphorylation of Ser13 [50].

Recently, using recombinant double-cysteine-residue-containing VDAC1 (L10C/A170C-ΔCys-rVDAC1) and addressing non- and cross-linked versions of the purified protein (using copper phenantroline), the intra-molecular proximity of the N-terminal region and the β-strand 11 cysteine residues was proposed [51]. In the present study, we demonstrated that a single cysteine residue is sufficient for formation of an intra-molecular cross-linking product (Figure 1A). Thus, in the study by Teijido et al. [51], one cannot rule out the formation of an intramolecular product involving a single cysteine in the N-terminal domain and another amino group in the lysine cluster presented above. Furthermore, Teijido et al. [51] reported that the double cysteine mutant with the N-terminal cross-linked to the β-barrel presented no differences in channel conductance, relative to the native protein. Their results, however, showed a 50% reduction in channel conductance at the positive potentials, suggesting that the location of the N-terminal region within the channel narrows the pore, resulting in decreased conductance. In addition, in contrast with the present study with cellular native membrane-located VDAC1, a recombinant protein in a non-membrane-embedded environment was exposed to cross-linking in the study by Teijido et al. [51].

In summary, we propose that the N-terminal region of VDAC1 is loosely attached to the barrel wall and can undergo translocation to become exposed at the membrane surface. This movement is responsible for VDAC1 gating, and also allows VDAC1 to interact with the anti-apoptotic proteins HK, Bcl-xl, and Bcl-2, and may participate in VDAC1 oligomerization. Precise targeted experiments will delineate the signal and nature of conformational changes induced in the VDAC1 N-terminal region that lead to its translocation, both under physiological and apoptotic conditions.

AUTHOR CONTRIBUTION
Shay Geula and Danya Ben-Hail designed the studies, performed the experiments and analysed the data. Shay Geula and Varda Shoshan-Barmatz wrote the paper.

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