Modulation of Human Mitochondrial Voltage-dependent Anion Channel 2 (hVDAC-2) Structural Stability by Cysteine-assisted Barrel-lipid Interactions

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Background: Human VDAC isoform 2 is crucial for apoptosis regulation and cell survival.

Results: Cys-less mutant displays significantly lowered unfolding free energy despite greater barrel rigidity.

Conclusion: Cysteine residues are key contributors of strong barrel-lipid interactions; hVDAC-2 also requires packing defects in the lipid system to remain stably refolded.

Significance: Cys-mediated VDAC-2-lipid interactions may contribute to its anti-apoptotic function.

Human mitochondrial voltage-dependent anion channel 2 (hVDAC-2), the most predominant isoform seen in brain mitochondria, is not only crucial for cell survival but is also implicated in Alzheimer disease. The abundance of cysteines in this isoform is particularly fascinating, as hVDAC-1 cysteines have no associated functional role. We report a detailed biophysical examination of a Cys-less mutant of hVDAC-2, and its behavioral comparison with the wild type protein. Our findings suggest that cysteine mutation results in the formation of a better barrel at the expense of weakened protein-lipid interactions. The wild type protein displays stronger lipid association, despite being less structured. A reversal in behavior of both proteins is observed in the case of chemical denaturation, with the Cys-less mutant exhibiting lowered unfolding free energies. In bicellar systems comprising 14-C phosphocholines, we observe that protein-lipid interactions are weakened in both constructs, resulting in barrel structure destabilization. Our biochemical and biophysical studies together reveal key structural roles for the cysteine residues. We find that minor conformational variations in local residues are sufficient to define the membrane protein dynamics in hVDAC-2. Such subtle sequence variations contribute to differential stability of VDACs and may have implications in their in vivo regulation and recycling.

Voltage-dependent anion channel or VDAC,3 initially identified as the most abundant mitochondrial “porin,” is a highly conserved 19-stranded β-barrel. It is involved primarily in conductance of ions and molecules up to ~5 kDa across the outer mitochondrial membrane (1–3). Interest in the function and regulation of this protein, initiated by its involvement in mitochondrial-mediated apoptosis, has expanded further due to its remarkable conservation across eukaryotes (4). Furthermore, it plays a key role in regulating conductance of calcium, ATP, NADH, metabolites etc., voltage-dependent switch in ion conductance, and hexokinase binding (5–7). Owing to its major function, VDAC is now being referred to as the “governor of mitochondrial bioenergetics” (8). The wealth of structural and functional information on VDACs has primarily been obtained from the isoform 1 from human and murine sources (1, 2, 5, 6, 8–12).

In mammalian systems, three VDAC isoforms have been observed (supplemental Fig. 1A) (4, 13, 14). Although hVDAC-1 is the primary channel protein involved in anion transport and cellular apoptosis (10), knock-out strains of hVDAC-2 are not viable (15), indicating other critical roles for the isoform 2 that is present only in mammals (15). Very little study has been carried out on hVDAC-2, which may primarily be due to amounts of sample that can be isolated from natural sources. It has been examined that the hVDAC-2 isoform shows 10-fold less mRNA levels than hVDAC-1 (16), with correspondingly lower protein levels (17). Moreover, our limited knowledge of this molecule is due to difficulties in artificial protein reconstitution in vitro and achieving prolonged sample stability (18).

Our current knowledge of hVDAC-2 indicates that the protein primarily possesses anti-apoptotic properties (4, 15), unlike hVDAC-1, which is proapoptotic (2, 6, 19) and is therefore critical for cell survival (15). hVDAC-2 suppresses apoptosis by specifically binding to the proapoptotic protein BAK and inhibiting BAK-mediated mitochondrial apoptosis (15). hVDAC-2 levels are also known to be elevated in bovine brain mitochondria (20), as well as in Alzheimer disease (7, 21). This protein has

lauryl dimethylamine oxide; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; LPR, lipid-to-protein ratio; KSV, Stern-Volmer constant; Tm, thermal denaturation mid-point; Cm, chemical denaturation mid-point; ∆GU, unfolding free energy; GdnHCl, guanidine hydrochloride.

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3 The abbreviations used are: VDAC, voltage-dependent anion channel; WT, wild-type hVDAC-2 protein; C0, Cys-less mutant of hVDAC-2; LDAO, lauryldimethylamine oxide; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; LPR, lipid-to-protein ratio; KSV, Stern-Volmer constant; Tm, thermal denaturation mid-point; Cm, chemical denaturation mid-point; ∆GU, unfolding free energy; GdnHCl, guanidine hydrochloride.

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also been shown to bind erasin, which subsequently modulates VDAC function, leading to oxidative cell death, using a non-apoptotic mechanism (22).

Biochemically, hVDAC-2 has an additional 11 amino acids at the N terminus that is unique to this protein (4, 8) and may be required for its function and regulation (8, 22). Channels formed by mouse VDAC-2 are noisier than VDAC-1 (4, 23), suggesting alternate functions for the less abundant hVDAC-2 and hVDAC-3 isoforms other than the “ion conductance” usually associated with these proteins. The abundance of cysteine residues in hVDAC-2 is particularly intriguing (supplemental Fig. 1A). It has been speculated that these cysteines may play some role in oxidative stress response and reactive oxygen species control, by acting as molecular buffers in mitochondria (4, 8). Hence, despite the 72% sequence identity between hVDAC-1 and hVDAC-2, and the overall functional similarities (7), major behavioral differences exist between these isoforms.

Earlier reports have suggested no functional role for cysteine residues in hVDAC-1 (24). We therefore speculated that the nine cysteine residues of hVDAC-2 may have a more structural role. By comparing the biophysical properties and thermodynamic stability of hVDAC-2 with its Cys-less mutant, we observe that cysteine residues are involved in anchoring the barrel to its lipid environment and allow for stabilization of hVDAC-2 structure. Additionally, although the Cys-less mutant gains more structure compared with the wild type, our results indicate that this structural stabilization occurs with the concomitant loss of strong protein-lipid interactions. The implications of these observations on the function and regulation of VDAC have been discussed.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Refolding—**hVDAC-2 WT and C0 were overexpressed as inclusion bodies in Escherichia coli BL21(DE3) cells and purified by ion exchange under denaturing conditions. For refolding experiments, 250 μM hVDAC-2 in 6 M guanidine hydrochloride (GdnHCl) was rapidly 10-fold diluted in the refolding mixture containing 65 mM lauryldimethylamine oxide (LDAO), 10 mM DTT (for WT), 100 mM NaCl in 50 mM phosphate buffer, pH 7.2, at 4 °C. Residual GdnHCl was removed by dialysis. Details are provided in the *supplemental data.* All studies were carried out in 50 mM phosphate buffer, pH 7.2, and 100 mM NaCl (and 2 mM DTT for WT) supplemented with required LDAO and 5 μM protein, at 20 °C, unless otherwise stated.

**Disulfide Mapping and Mass Spectrometry—**In-gel tryptic digestion of refolded hVDAC-2 was carried out with or without prior iodoacetamide treatment, and the extracted fragments were subjected to peptide mass fingerprinting by MALDI-ToF/ToF. Peak identification was carried out using Mascot and is described in the *supplemental data.*

**Folding Kinetics—**Steady state fluorescence measurements were obtained by excitation at 295 nm, and emission scans were recorded between 310 – 400 nm using slit widths of 3 and 5 nm, respectively, and a data integration time of 0.1 s. Average wavelengths were calculated from this data, as reported previously (25). Steady state anisotropy was measured using λ<sub>ex</sub> of 295 nm and λ<sub>em</sub> of 340 nm. All data were fit to a double exponent with a constant, using SigmaPlot (version 11.0; Systat Software, Inc.), to obtain folding rates.

**Monitoring Trp Environment—**Acrylamide-quenching measurements were carried out between 10 mM and 0.5 M acrylamide. Stern-Volmer plots generated from this data were fit to a linear equation to provide K<sub>SV</sub> values. Lifetime measurements were carried out using the time correlated single photon counting method using λ<sub>ex</sub> of 295 nm and a fixed λ<sub>em</sub> for each sample, which corresponded to their respective emission maxima. Data were fit to an exponential decay function to obtain lifetimes and populations. See *supplemental data* for details.

**Thermal Denaturation Measurements—**Far-UV CD thermal denaturation measurements were carried out between 4 – 95 °C, at ramp rates of 1 °C/min (unless otherwise specified) and monitored at 215 nm. Unfolded fractions were calculated, and T<sub>M</sub> values were obtained from fits to a two-state equation. For thermal denaturation in the presence of urea and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), the pre- and post-transition baselines of the 0 M sample were used for normalizing all other data. Details are provided in the *supplemental data.*

**Chemical Denaturation and Measurement of Unfolding Free Energy—**Fluorescence measurements for refolded protein incubated in increasing GdnHCl for at least 1 h at 20 °C were carried out using λ<sub>ex</sub> of 295 nm. Thermodynamic parameters (ΔG<sub>U</sub>, m value, C<sub>m</sub>) were obtained by fits of averaged wavelengths and raw anisotropy data to a two-state equation. Details are available in the *supplemental data.*

**RESULTS**

**hVDAC-2 Cysteines Are in Reduced Form in the in Vitro Refolded Protein—**Cysteine residues of hVDAC-2 populate loop segments that are oriented toward the inter-membrane space, in the native state (see Fig. 2A). Although the high redox potential in this region would prevent disulfide bond formation, the latter may not hold true during *in vitro* refolding of hVDAC-2. Using mass spectrometric analysis of in-gel tryptic digests, we probed the occurrence of disulfide patterns in hVDAC-2 refolded in LDAO micelles. Our data indicate preponderance of all Cys residues in their reduced form (Fig. 1), suggesting that *in vitro* refolded protein maintains a reduced state for the nine Cys residues. Examination of hVDAC-2 structure (Fig. 2A) modeled on the basis of hVDAC-1 using I-TASSER (26), also suggests that upon adopting a 19-stranded form, cysteines are unlikely to be spatially proximal for disulfide bond formation.

**Cysteine Residues Do Not Substantially Influence hVDAC-2 Folding Rates—**Of its nine cysteines, Cys-103, Cys-210, and Cys-227 are unique to the isoform 2 (Fig. 2B). Although the high redox potential in this region would prevent disulfide bond formation, we generated a Cys-less mutant of hVDAC-2 (henceforth referred to as C0) and studied the refolding kinetics of this protein against the wild type hVDAC-2 (henceforth labeled WT) in LDAO micelles. Unlike the slow multi-step refolding process observed in other β-barrel membrane proteins (27), our steady state fluorescence (hVDAC-2 has four Trp residues; Fig. 2A) and anisotropy measurements indicate a rapid refolding event for both WT and C0 constructs in LDAO micelles (Fig. 2B). In particu-
lar, the burst phase, which reflects the initial protein-lipid association event (28, 29), occurs within the experiment dead time of 1 min and can be captured only with anisotropy measurements (Fig. 2B). Interestingly, apart from minor differences in the folding rates, which may arise from local structural rearrangements in both proteins, both WT and C0 exhibit a largely similar refolding behavior. Furthermore, black lipid membrane experiments suggest that the deletion of cysteines does not affect the activity of the protein.4

We probed the effect of lipid-to-protein ratio (LPR), on the folding efficiency of WT and C0. In our experiments, both proteins required a minimum lipid concentration of ~65 mM LDAO and a minimum LPR of 2600:1 in the refolding reaction, to prevent manifestation of aggregation pathways during folding. This behavior indicates that during the initial refolding event, presence of sufficient lipid amount (and not LPR alone) is necessary to drive the equilibrium favorably toward folding. Further assessment of the differences in lipid-protein interactions between WT and C0 were made by choosing two different LPRs, namely, 2600:1 (low LPR) and 13,000:1 (high LPR). The

4 M. K. Mathew, personal communication.

FIGURE 1. Mass spectrometric analysis of refolded hVDAC-2 WT to determine the oxidation states of the nine cysteines. A and B, representative mass spectrometric profiles of in-gel trypsin digested refolded samples without (A) and with (B) iodoacetamide treatment. Marked in red are the regions of the protein corresponding to peaks identified using BioTools (version 3.2). Observed peak intensities were generally lower than that usually obtained for soluble proteins, which may be because of low ionizing ability or greater apolar content of the transmembrane region. In the untreated sample (A), we observed the presence of three peaks (although at negligible intensities) that corresponded to two disulfide bonds involving residue Cys-76 (inset). One of these disulfides is formed between Cys-47–Cys-76 and the other between Cys-76–Cys-103, which may arise from the refolded protein or from a minor population of the unfolded protein present in the refolding reaction. Notably, Cys-47 and Cys-76 are in close proximity if hVDAC-2 also adopts the 19-stranded barrel, similar to isoform 1. These peaks disappear when the refolded protein is first treated extensively with iodoacetamide before trypsinization (B, inset), leading us to speculate that these disulfides are generated only during sample processing for mass spectrometric analysis and are inherently not present in the refolded protein in appreciable amounts. C, hVDAC-2 WT sequence indicating the disulfide bond connectivity detected in our experiments (dotted yellow lines). Segments of the protein identified in mass spectrometric profile have been marked in red, and cysteine residues were highlighted in yellow. D, ribbon diagram of hVDAC-2 WT barrel highlighting cysteine residues generated using PyMOL, the three cysteines that underwent disulfide bond formation during our sample processing have been labeled.
Cys-modulated Barrel-lipid Interaction of hVDAC-2

**TABLE 1**

| LPR        | hVDAC-2 WT       | hVDAC-2 C0       |
|------------|------------------|------------------|
|            | r (nm)           | $\chi_{a b}$     |
| 2600:1     | 0.147            | 1.13             |
|            | 0.140            | 1.18             |
| 2600:1 (D)$^c$ | 0.075         | 1.10             |
|            | 0.071            | 1.16             |
| 13,000:1   | 0.148            | 1.27             |
|            | 0.140            | 1.11             |
| 13,000:1 (D)$^c$ | 0.085         | 1.02             |
|            | 0.075            | 1.09             |
| 0.1 (D)$^c$ | 0.070            | 1.06             |
|            | 0.065            | 1.07             |
| 0.1 (Buffer) | 0.177          | 0.015            |
|            | 0.169            | 0.020            |

$^a$ Average lifetime derived from fits of data to a three exponential function.

$^b$ Goodness of the fits for lifetime measurements.

$^c$ Denatured (D) using 6 M GdnHCl.

$^d$ Could not be determined.

**FIGURE 2.** A, ribbon diagram of 1-TASSER modeled structure of hVDAC-2 generated using PyMOL. The left panel highlights all of the cysteines as spheres; those unique to hVDAC-2 are colored in orange, and others are in yellow. The right panel highlights the four Trp residues. B, folding kinetics of hVDAC-2 monitored using fluorescence intensity at 340 nm, change in $\lambda_{\text{em,max}}$ and anisotropy (measured in arbitrary units, A, U) at 4°C. Data were fit to two exponential functions; fits are indicated as solid lines. Only the slow folding rates for both WT and C0 derived from anisotropy measurements provided meaningful rates and S.D. of 0.385 ± 0.037 min$^{-1}$ for WT and 0.185 ± 0.015 min$^{-1}$ for C0. Trp fluorescence suggests that most of the refolding is completed within the dead time of the experiment, indicating that the indole rings are buried. The 0 min data were obtained from the unfolded protein and is connected to the refolding data by exponential functions; fits are indicated as dashed lines.

**FIGURE 3.** A, thermal denaturation of hVDAC-2 under various conditions. A, thermal denaturation of hVDAC-2 WT in low (○) and high LPR (□) monitored using far-UV CD measurements at a scan rate of 1 °C/min; shown is the mean of three independent experiments. Solid lines represent fits to a two-state equation. Inset shows the $T_m$ values derived from thermal denaturation carried out at different scan rates from 0.5–7.5 °C/min, which were used to generate a modified Arrhenius plot depicting the scan rate dependence of WT in low (○) and high LPR (□), and C0 in low (●) and high LPR (▲). Because there was no linear correlation in these graphs, activation energies could not be determined. B, change in fluorescence anisotropy as a function of temperature, to monitor whether hVDAC-2 (WT, top graph; C0, bottom graph) denaturation and aggregation in low (○) and high LPR (▼) are simultaneous processes. Anisotropy of unfolded protein in 6 M GdnHCl is shown as a solid line. Similar transition temperatures obtained from both CD and anisotropy measurements with concomitant increase in anisotropy indicate that the unfolding and aggregation processes are simultaneous events. C, variation in $T_m$ as the ramp rate is increased from 0.5–7.5 °C/min. Irreversible thermal denaturation of soluble proteins shows strong ramp rate dependence; however, in the case of hVDAC-2, the increase in $T_m$ becomes prominent only at very high ramp rates. This data suggests the lack of a prominent kinetically driven unfolding process for the VDAC barrel. D, dependence of $T_m$ on protein concentrations. Concentrations ≤ 5 μM were possible for low LPR, whereas concentrations up to 25 μM were achieved in high LPR. A marginal decrease in $T_m$ is seen only when very high protein concentrations are used. Both experiments suggest that the thermal transition occurs before aggregation.

hVDAC-2 Exhibits Surprisingly High $T_m$ but Undergoes Irreversible Thermodynamically Controlled Heat Denaturation—We tested the effect of thermal denaturation on both proteins, in the two LPRs, using CD. WT and C0 show irreversible thermal denaturation (Fig. 3, A and B), indicating that protein unfolding and aggregation are synergistic events. The aggregation event is largely concentration-independent (Fig. 3D), suggesting that direct conversion of native to aggregated protein occurs, and an unfolded intermediate, if present, is short-lived. Additionally, it has been observed that irreversible thermal denaturation of soluble proteins is a kinetically driven process with only a marginal thermodynamic contribution (30). However, the absence of a direct correlation between denaturation...
rates and $T_m$ (thermal denaturation mid-point) in our studies with membrane proteins suggests that hVDAC-2 heat denaturation is primarily a thermodynamically driven process, with the thermal transition preceding the aggregation event (Fig. 3C) (30).

When we compare the response of WT and C0 to thermal denaturation, three interesting observations are apparent: (i) high LPR shows comparable stability of both proteins; (ii) a 5-fold reduction in LPR does not result in a proportionate decrease in the $T_{m}$, indicating that low lipid concentrations are sufficient for refolded hVDAC-2; (iii) in low LPR, C0 exhibits a marginally greater thermal stability compared with the WT protein (Fig. 3A). Results from anisotropy measurements (Fig. 3B) provide similar $T_{m}$ values as that of CD measurements. We speculate that upon successful refolding, hVDAC-2 barrel stays stable by retention of a minimal lipid film that sufficiently shields the hydrophobic patches from the aqueous environment. Additionally, in C0, strand dissociation is driven only at higher temperatures; therefore, cysteine mutations may result in greater strand rigidity or stronger hydrogen bonding, leading to stability of this mutant even in low LPR. Although the increase in secondary structure content of C0 supports this argument (Fig. 4A, inset and supplemental Fig. 2, A and C), it must be noted that this stabilization is dependent on the ramp rate (Fig. 3C), indicating that aggregation rates influence thermal stability measurements.

High LPR Affects Thermal Stability of C0 in the Presence of Urea, whereas WT Is Affected by Hydrophobic Mismatch—Contrary to what is generally observed for membrane proteins (31), it is intriguing that hVDAC-2 exhibits comparable stability even when lipid concentrations are lowered. We therefore evaluated the strength of the barrel-lipid interaction by probing the influence of denaturants such as urea, on the $T_m$ of both proteins. We observe maximum decrease in secondary structure in C0 at LPR of 13,000:1 (Fig. 4A). This is also accompanied by a significant reduction in the $T_{m}$ with increasing urea, whereas no appreciable change in the WT protein, under identical conditions, is noted (Fig. 4C and supplemental Fig. 2, A and B). Notably, under similar experimental conditions, C0 exhibits greater thermal stability compared with WT (Fig. 3A) in the absence of urea. Both refolded WT and C0 are not affected by increasing urea concentrations in low LPR (Fig. 4C and supplemental Fig. 2, A and B). Hence, the destabilizing effect in high LPR, in the presence of a denaturant, is likely to arise due to differences in protein-lipid interactions.

Structural characterization of hVDAC-2 using NMR has been attempted in DMPC nanodiscs with promising results (18). Therefore, we addressed the effect of protein-lipid interactions on barrel stability by monitoring thermal denaturation profiles of both proteins in mixtures of LDAO and DMPC. Titrating LDAO into DMPC converts vesicles of the latter into bicellar forms of varied sizes, which, we hypothesized, would adopt a micellar behavior with further LDAO addition. Phase change of the lipid milieu would impose varied curvature stress on refolded hVDAC-2. Indeed, our attempts at direct refolding of both WT and C0 in DMPC vesicles did yield sufficient amount of folded protein; such poor refolding efficiency due to
absence of packing defects in homogeneous preparations of long chain lipids was reported previously (32, 33).

Both WT and C0 exhibit notable sensitivity to curvature stress exerted by DMPC, in the form of loss in secondary structure (supplemental Fig. 2C). A closer examination of barrel dimensions reveals a marginal hydrophobic mismatch between the transmembrane region of hVDAC-2 (~22 Å, based on hVDAC-1 structures) and DMPC (~23 Å). Although it is believed that such minor differences are well accommodated with negligible loss in free energy (32), in hVDAC-2, however, we speculate that curvature stress dramatically destabilizes the barrel structure, due to lipid deformation forces (33, 34). Arguably, the change toward lowered free energy of unfolding due to hydrophobic mismatch is alleviated when proteins with a longer transmembrane segment is folded into thinner bilayers (32).

This explains the observed loss in barrel structure and reduced protein stability in our experiments with DMPC (supplemental Fig. 2D). Stern-Volmer constants ($K_{SV}$) from acrylamide quenching measurements (Fig. 4, E and F) further indicate barrel destabilization and increased formation of either adsorbed protein or soluble aggregates in high DMPC; such unfavorable interactions are possibly alleviated in the presence of a more dynamic system such as LDAO.

The drastic reduction in the $T_m$ values upon DMPC addition follows a similar profile for both WT and C0 (Fig. 4, B and D, supplemental Fig. 2D); a low LPR system is affected to a greater extent compared with that of high LPR. Remarkably, a difference in $T_{m}$ of ~30 °C is obtained between 13 mM LDAO (+4 mM DMPC) and 65 mM LDAO (+20 mM DMPC), despite the LDAO:DMPC ratio of 13:4 being attained in both systems. It is presently unclear whether this dramatic difference in thermal stability is due to variations in lipidic phases, hydration levels or whether the apparent $T_m$ shift is due to differences in the rate of formation of protein aggregates in both systems.

In these data, what is indeed noteworthy is the persistence of secondary structure content in C0, whereas WT experiences rapid loss in structure (~40% higher β-sheet content in C0 in high LPR of 13,000:1 and 20 mM DMPC; supplemental Fig. 2, C and D) and concomitant increase in aggregation, coupled with a marginally inferior $T_{m}$ (Fig. 4D). The thermal stability and secondary structure content of C0 indicates that the barrel structure may indeed be more rigid compared with its WT counterpart. Structuring of the barrel at the solvent-lipid interface upon mutation of the cysteine residues could account for the increased tolerance of C0 to DMPC; the WT, on the contrary, is better “matched” to LDAO and exhibits greater stability even when thermal denaturation is carried out in the presence of urea (Fig. 4C).

We also recognize that our results contradict previous observations that report greater stability for hVDAC-2 in DMPC nanodiscs and poor stability in LDAO (18). Our hVDAC-2 preparations in LDAO exhibit stability for up to 50 h at 40 °C (data not shown) and up to 1 week at 4 °C, with no indication of sample precipitation. The preparations in DMPC were, however, found to be short-lived. We believe that these differences may arise due to different sample production and preparation methods, absence of a His$_6$ tag, and other currently unknown factors that may serve as key contributors in dictating the overall barrel stability in artificially reconstituted systems.

Cysteines Increase Equilibrium $\Delta G^0_{CL}$ whereas the Barrel Loses Cooperativity while Unfolding in High LPR—To obtain better estimates on the unfolding free energies of both proteins, we carried out chemical denaturation of WT and C0. Urea served as a poor denaturant in these experiments, as fluorescence studies, provided only a marginal change in the Trp fluorescence intensity, suggesting that the unfolded protein retained lipid-binding ability, especially at the aromatic girdle. Indeed, earlier studies on E. coli PagP indicate GdnHCl to be a superior denaturant (35).

In GdnHCl, hVDAC-2 exhibits cooperative and reversible unfolding, allowing for free energy estimations (see supplemental “Materials and Methods”). We observe a lowering of $\Delta G^0_{CL}$ in high LPR (13,000:1) with some loss of cooperativity, as opposed to low LPR, for both proteins (Fig. 5). In line with our observations from thermal denaturation, these data also suggest that the barrel possibly undergoes moderate destabilization in high LDAO. Low $\Delta G^0_{CL}$ is accompanied by the concomitant reduction in $m$ values during denaturation, reflecting a possible increase in the solvent-accessible surface area of the refolded protein in high LPR. Although our data are in agreement with a two-state unfolding model, we do not rule out the occurrence of multiple intermediates, which could also account for the low $\Delta G^0_{CL}$ and $m$ values, as described previously (35). However, higher $K_{SV}$ (Fig. 2C) obtained in 65 mM LDAO, which is suggestive of exposed interfacial Trp residues, and reduction...
Cys-modulated Barrel-lipid Interaction of hVDAC-2

Figure 6. Schematic showing the effect of temperature and chemical denaturant on hVDAC-2. A, hVDAC-2 WT exhibits greater protein-lipid interactions in the refolded form as compared with C0 (shown in black as a representative image) but displays a lower secondary structure content possibly due to highly mobile loops and shortened strands. Hence, WT unfolds faster with temperature than C0. The relative stabilities observed for both proteins in high LPR is illustrated using a representative activation energy barrier ($E_a$) that is not very different from the low LPR system. B, in contrast to heat-mediated unfolding, C0 rapidly unfolds under chemical denaturation in GdnHCl, exhibiting lowered unfolding free energies ($\Delta G_U$) compared with WT. Sensitivity to LPR, however, is similar in all cases. Color scheme was as follows: green and black, WT and C0 in low LPR of 2600:1; red and blue, WT and C0 in high LPR of 13,000:1. GdnHCl is displayed as red dots.

in secondary structure content (Fig. 4A, inset; supplemental Fig. 2, A and C) seen in high LPR point to an increase in the available surface area of the refolded protein in these conditions. With these findings, we presume a three-state unfolding process to be less likely in hVDAC-2.

Despite the observed low $\Delta G_U^0$, our data still allow for a comparison between WT and C0. The results, summarized in Fig. 5B, indicate that C0 is more susceptible to chemical denaturation compared with WT. Indeed, whereas thermal denaturation points to a greater stability for C0, the effect is reversed in the presence of GdnHCl. Thermal unfolding in the presence of urea (Fig. 4C) and GdnHCl denaturation studies together suggest that C0, although more structured, is destabilized in high LDAO. Therefore, C0 rapidly loses affinity to its surrounding lipid upon addition of a chemical denaturant. The WT protein, however, shows higher affinity toward LDAO micelles, and the Trp residues therefore stay more "shielded" from the denaturing effect of GdnHCl. The $\Delta G_U^0$ of unfolding (difference in unfolding free energies of WT and C0) in LPR 2600:1 is 0.77 kcal/mol, whereas in LPR, 13,000:1 is 1.92 kcal/mol, reflecting the destabilizing effect of high LPR on C0.

We also measured the tryptophan lifetimes of both proteins to gain insight on the effect of high versus low LPR on the folded barrel (Table 1). Anisotropy values of WT and C0 are only marginally different, indicating the formation of comparable protein-lipid complexes. Change in anisotropy values monitored with increasing GdnHCl (Fig. 5), provides us with similar conclusion as the total fluorescence measurements. Notably, the lifetimes are different for both proteins, with Trp residues in the WT protein showing shorter lifetime (Table 1). Upon closer examination of the structure of hVDAC-2, the proximity of cysteines to some of the indole rings become apparent. Indeed, a single Trp construct (Trp-75) of WT exhibits a dramatic difference between the folded (1.05 ns) versus unfolded states (2.33 ns), due to the presence of Cys-76 in the vicinity (36). In WT, increase in LPR is accompanied by an increase in lifetime, which indicates removal of the local quencher due to increased loop dynamicity. We speculate that this occurs due to protein unfolding in high LPR and is supported by the CD and acrylamide quenching data discussed earlier. In C0, difference in observed lifetimes between low and high LPR is only marginal, whereas there is a larger change in $K_{SV}$ (Fig. 2C), suggesting that the indole rings of the Cys-less hVDAC-2, although solvent-exposed, possibly retain local conformational rigidity.

DISCUSSION

Our results shed light on some unique behavioral features of hVDAC-2. Although the protein has nine cysteines, our data indicate that the thiols are in the reduced form upon protein folding. Considering the intracellular reducing environment, it is likely that disulfide bonds, if formed in vitro, are transient. Although these cysteines may contribute to protection from ROS (16, 24), it can be argued that they are likely to have a more structural role under normal physiological conditions. Our experiments indicate that association energy of the barrel surface with the lipid is dictated by the rigidity of the native structure, which in turn, is likely to be decided by the cysteines. In the native protein, when all cysteines are present in the reduced state, the overall barrel structure is less defined and consequently leads to stronger protein-lipid interactions in vitro. When one considers the distribution of cysteines in hVDAC-2, it is evident that this residue is preferentially populated in loops. Substitution of cysteines with the corresponding amino acid(s) in hVDAC-1 and hVDAC-3, introduces changes in the local secondary structure, leading to strand extension and a better structural scaffold.

The Cys-less mutant of hVDAC-2 exhibits marginal thermal stability, albeit a pronounced susceptibility to chemical denaturation. Notably, although both proteins exhibit sensitivity to increased lipid, particularly DMPC, the Cys-less mutant retains structure over WT. Curvature stress caused by hydrophobic mismatch, coupled with the inherently low stability of the folded protein ($\Delta G_U^0 \sim 2–5$ kcal/mol obtained for hVDAC-2, compared with the observed $\Delta G_U^0 \sim 10–32$ kcal/mol for pro-
karyotic barrel proteins (for examples, see Refs. 28 and 35 and references therein) result in barrel destabilization and protein aggregation. Lipid packing defects are required to lower the free energy of folding in other transmembrane barrels (32); hVDAC-2 may require such packing defects to stay folded, which in vivo, may be provided by cardiolipin and similar lipids in the mitochondrial outer membrane (37).

We have summarized our observations of the behavior and thermodynamic stability of hVDAC-2 WT and C0 in Fig. 6. We found only marginal differences in the overall barrel structure and the surrounding micellar environment, when hVDAC-2 WT and C0 were simulated in η-dodecylphosphocholine micelles (data not shown). The behavioral differences we observe could therefore be due to subtle structural changes that we cannot presently capture using simulations. Analogies can be drawn between the changes that the structure of hVDAC-2 WT undergoes after being converted to C0, to the subtle differences that are generally observed (in terms of strand lengths), in the structures derived using NMR and crystallographic methods for hVDAC-1 (9, 10). Increased ordering of residues at the solvent-lipid interface observed in long chain lipids (38) can be brought about by cysteine substitutions in hVDAC-2.

Marginal differences in structural rigidity may play a key role in defining the regulation and recycling of both isoforms in the mitochondrial outer membrane and its ability to interact with other regulatory proteins involved in apoptosis. Indeed, recent reports that Bcl-2 family proteins are recruited to mitochondria by interacting with loop regions of hVDAC-1 (39) suggest that loop length and strand registry is vital for VDAC function. It would be of great interest to examine the effect of the three unique cysteines (Cys-103, Cys-210, Cys-227) on the overall structure of the barrel as well as monitor the folding and unfolding pathways adopted by this barrel using strategically positioned reporter molecules.

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