

**Molecular Biophysics:**  
**Nucleotide interactions of the human  
voltage-dependent anion channel**

MOLECULAR  
BIOPHYSICS



Saskia Villinger, Karin Giller, Monika Bayrhuber, Adam Lange, Christian Griesinger, Stefan Becker and Markus Zweckstetter  
*J. Biol. Chem.* published online March 25, 2014

Access the most updated version of this article at doi: [10.1074/jbc.M113.524173](https://doi.org/10.1074/jbc.M113.524173)

Find articles, minireviews, Reflections and Classics on similar topics on the [JBC Affinity Sites](#).

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at  
<http://www.jbc.org/content/early/2014/03/25/jbc.M113.524173.full.html#ref-list-1>

Nucleotide interactions of the human voltage-dependent anion channel\*

Saskia Villinger<sup>1</sup>, Karin Giller<sup>1</sup>, Monika Bayrhuber<sup>1</sup>, Adam Lange<sup>1</sup>, Christian Griesinger<sup>1</sup>, Stefan Becker<sup>1</sup>, Markus Zweckstetter<sup>1,2,3</sup>

<sup>1</sup>Dept. of NMR-based Structural Biology, Max Planck Institute for Biophysical Chemistry,  
Am Fassberg 11, 37077 Göttingen, Germany

<sup>2</sup>German Center for Neurodegenerative Diseases (DZNE), Göttingen, 37077 Göttingen, Germany

<sup>3</sup>Center for the Molecular Physiology of the Brain, University Medicine Göttingen, 37073 Göttingen,  
Germany

\*Running title: *Nucleotide interactions of the human voltage-dependent anion channel*

To whom correspondence should be addressed: Markus Zweckstetter, German Center for Neurodegenerative Diseases (DZNE), Göttingen, Germany, Email: Markus.Zweckstetter@dzne.de

**Keywords:** NMR; Membrane proteins; ATP; Nucleotide; Anion transport; Biophysics; Ion channels; Mitochondrial metabolism; Mitochondrial transport

**Background:** Human VDAC1 mediates and controls the transport of metabolites across the outer mitochondrial membrane.

**Results:** The N-terminal helix of hVDAC1 is involved in binding to charged forms of ATP, UTP and GTP with an important contribution from lysine 20.

**Conclusion:** Weak binding of ATP confers specificity for ATP transport.

**Significance:** ATP interaction mapped at residue resolution supports metabolite selectivity of VDAC.

## ABSTRACT

The voltage-dependent anion channel (VDAC) mediates and gates the flux of metabolites and ions across the outer mitochondrial membrane (OMM) and is a key player in cellular metabolism and apoptosis. Here we characterized the binding of nucleotides to human VDAC1 (hVDAC1) on a single-residue level using NMR spectroscopy and site-directed mutagenesis. We find that hVDAC1 possesses one major binding region for ATP, UTP and GTP, which partially overlaps with a previously determined NADH-binding site. This nucleotide binding region is formed by the N-terminal  $\alpha$ -helix, the linker connecting the helix to the first  $\beta$ -strand and adjacent barrel residues. hVDAC1

**preferentially binds the charged forms of ATP, providing support for a mechanism of metabolite transport, in which direct binding to the charged form exerts selectivity, while at the same time permeation of the Mg<sup>2+</sup>-complexed ATP form is possible.**

Eukaryotic cell viability strongly depends on the function of mitochondria, the hosts of respiration and central metabolic pathways. A continuous exchange of ions and metabolites between mitochondria and the cytosol is essential for these processes. The OMM contains a single channel that performs this task: VDAC. This highly abundant protein is responsible for most of the flux of ions and metabolites across the OMM (1). The 19-stranded  $\beta$ -barrel of VDAC1 (2-4) forms a pore of ~3 nm diameter (5-7) that displays a large single-channel conductance (~4 nS in 1 M KCl) and is permeable for solutes up to 6 kDa (1). Charged residues in the pore lumen (8,9) ensure a mild anion-selectivity (10) and allow the passive permeation of various metabolites (most importantly ATP and ADP) through its 'open' state at low membrane potentials ( $\pm 10$  mV) (1,11). In the presence of membrane potentials above approximately  $\pm 30$  mV the channel can adopt a variety of 'closed' states that are characterized by a reduced ion conductance (~2 nS in 1 M KCl) (1,12) and a slight cation-selectivity (10,13). Channel closure, also known as VDAC gating, can

additionally be induced or modulated by a variety of small molecules and proteins (for a review see (14)). Voltage-induced channel closure and modulators of VDAC conductance (e.g. NADH, synthetic polyanions) strongly reduce nucleotide permeability and impair mitochondrial function, suggesting that gating enables VDAC to control metabolite flux between mitochondria and the cytosol (11,15-18). The central role in controlling metabolite flux and mitochondrial respiration granted VDAC the title of a “mitochondrial governor” (19).

The nature of VDAC gating as basis of the channel’s regulatory function is unknown. Electron microscopy and bilayer measurements suggest moderate to large structural changes during channel closure (5,9,20). Models for gating mechanisms include rearrangements of the N-terminal  $\alpha$ -helix, barrel deformations, a combination of helix and barrel motions to varying extent, and translocation of a membrane-embedded voltage-sensor out of the membrane (4,21-27). While NADH enhances the channel’s voltage-dependence and reduces nucleotide permeability of the OMM, ATP itself has no effect on VDAC gating (16,28). Instead, ATP passively permeates through VDAC that provides a diffusion pore for diverse metabolites. However, small non-physiological solutes of similar size and charge as ATP do not penetrate the channel, suggesting that VDAC achieves selectivity by specific interactions with permeating solutes (29). Indeed, specific nucleotide interactions with mammalian VDAC1 isoforms and an inhibitory effect of divalent ions have been demonstrated (30-33). These investigations identified three potential nucleotide binding sites (NBS) in mammalian VDAC1 (32). However, the applied methods did not allow determination of NBSs with single-residue resolution or on a structural level. In contrast, a solution NMR study reported weak interactions of human VDAC1 (hVDAC1) with ATP, which were not located to any specific site (3), contradicting earlier results. Therefore, a more detailed analysis of ATP binding is required to characterize VDAC’s specificity and resolve mechanisms of metabolite permeation.

With the 3D structure and NMR resonance assignments of hVDAC1 in solution available (2,22), interactions with transported substrates can be investigated in detail. Here we characterize the interaction sites of the major transported solute

ATP and investigate the effect of divalent ions and mutations on the interaction on a single-residue basis by solution NMR spectroscopy.

## EXPERIMENTAL PROCEDURES

*Protein expression and purification* – Expression, refolding and purification of WT, T60C/C127S/C232S, V87C/C127S/C232S and K20S hVDAC1 was done as described (2).

*NMR spectroscopy* – NMR spectra were recorded on  $^2\text{H}(75\%)\text{-}^{15}\text{N}$  or  $^2\text{H}(75\%)\text{-}^{13}\text{C}\text{-}^{15}\text{N}$  labeled samples containing 0.4-0.7 mM hVDAC1, 25 mM BisTris pH 6.8, approx. 250 mM lauryldimethylamine-oxide (LDAO, Fluka), and 5-10%  $\text{D}_2\text{O}$ . All spectra were measured at 37°C on Bruker 800 or 900 MHz spectrometers equipped with cryogenic probes. Using 2D  $^1\text{H},^{15}\text{N}$ -TROSY and 3D TROSY-based triple-resonance spectra we have previously assigned 84/97% (overall/barrel) of the backbone resonances of WT hVDAC1, as well as ~80% of  $^{15}\text{N}$  and  $^1\text{H}_\text{N}$  assignments of T60C/C127S/C232S and V87C/C127S/C232S hVDAC1 at these conditions (2,22). Backbone resonance assignment of K20S hVDAC1 was confirmed with  $^1\text{H},^{15}\text{N}$ -TROSY and 3D TROSY-HNCA spectra.

Interaction of hVDAC1 with nucleotides (Sigma-Aldrich) was investigated by titration of  $^2\text{H}(75\%)\text{-}^{15}\text{N}$  hVDAC1 from equimolar concentrations to 64-fold molar excess with respect to the protein:  $\text{Na}_2\text{ATP}$  and  $\text{MgATP}$  with WT,  $\text{Na}_2\text{ATP}$  with K20S,  $\text{Na}_2\text{GTP}$  with T60C/C127S/C232S, and  $\text{Li}_x\text{UTP}$  with V87C/C127S/C232S hVDAC1. A control with 50 mM NaCl was performed with WT hVDAC1. All mutants displayed similar NMR spectra, with shifted resonances observed only for residues close to the mutation sites. Thus, the mutants were assumed to be functional and to share a similar 3D structure as WT hVDAC1, in agreement with paramagnetic relaxation enhancement measurements (2). The pH of the nucleotide stock solutions (prepared in 25 mM BisTris pH 6.8, with 6% or without LDAO) was adjusted with NaOH, and when necessary the sample pH was kept constant during titrations by the addition of NaOH or HCl. The  $\text{MgATP}$  stock solution was prepared by mixing the  $\text{Na}_2\text{ATP}$  stock solution with an equimolar amount of  $\text{MgCl}_2$ . The reference sample for the  $\text{MgATP}$  titration contained 10 mM  $\text{MgCl}_2$ , such that a 10 mM  $\text{Mg}^{2+}$  excess over ATP was maintained at the initial titration steps (1-, 2-, 4-

16-, and 32-fold MgATP excess). For the last step (64-fold molar excess), the Mg<sup>2+</sup> excess was increased to 20 mM by further addition of MgCl<sub>2</sub>. According to calculations (34) > 99% of the ATP is thus found in complex with Mg<sup>2+</sup> at all titration steps.

<sup>1</sup>H,<sup>15</sup>N-TROSY spectra were recorded in order to follow <sup>15</sup>N and <sup>1</sup>H chemical shift changes and variations in peak intensities. Spectra were processed with nmrPipe (35) and analyzed with SPARKY (36). Normalized weighted average chemical shift differences (CSD), Δ<sub>HN</sub>, were calculated as:

$$\Delta_{HN} = \sqrt{\frac{(\Delta\delta_N/5)^2 + (\Delta\delta_H)^2}{2}}$$

Here, Δδ<sub>N</sub> and Δδ<sub>H</sub> are the chemical shift differences for the <sup>15</sup>N and <sup>1</sup>H dimensions, respectively. The digital resolution after zerofilling was defined as threshold for significant CSD.

An overall reduction in signal-to-noise was observed during titration steps due to an increase in salt concentration. Resonance intensities of the spectra upon ligand addition were scaled with respect to residues that exhibited no shifts and similar but small intensity changes. Unresolved resonances were excluded from the analysis of peak intensities (63 in WT, 76 in K20S, 54 in T60C/C127S/C232S, and 40 in V87C/C127S/C232S). Errors in resonance intensity ratios were calculated by error propagation using the median of spectral noise estimated by SPARKY.

Resonances that exhibited shifts but only small intensity changes (less than 20% in the final titration step) after intensity scaling were classified as resonances in fast exchange. For these resonances the variation of CSD versus ligand concentration was used to determine K<sub>D</sub> values by curve fitting to a single-site binding model (37) using Origin (OriginLab, Northampton, MA):

$$\Delta = \Delta_{\max} \left( \frac{[L]_T + [P]_T + K_D - \sqrt{([L]_T + [P]_T + K_D)^2 - 4[L]_T[P]_T}}{2[P]_T} \right)$$

Here, Δ is the CSD, Δ<sub>max</sub> the maximum CSD value at saturation, [L]<sub>T</sub> the total ligand concentration and [P]<sub>T</sub> the average protein concentration. Changes of [P]<sub>T</sub> were kept to a minimum during the titration. Δ<sub>max</sub> and K<sub>D</sub> were

simultaneously fit. Errors are given as standard deviations from an average of several residues.

## RESULTS

*ATP interacts with hVDAC1 at the N-terminal α-helix and linker* – Photoaffinity ATP labeling studies with rat VDAC1 (rVDAC1) in mitochondria or native rVDAC1 purified from mitochondria suggested the presence of three nucleotide binding sites (NBS): NBS1 in the α-helix/linker region (residues 13-28), NBS2 in β-strand 7 (residues 110-120) and NBS3 in several C-terminal β-strands (residues 257-274/283) (32). Further, magnesium was shown to reduce the affinity towards ATP (32). In order to determine ATP binding and Mg<sup>2+</sup> inhibition with recombinantly expressed and refolded hVDAC1, we titrated hVDAC1 with ATP in form of the disodium salt (Na<sub>2</sub>ATP) and the magnesium complex (MgATP) and followed changes in <sup>1</sup>H,<sup>15</sup>N-TROSY spectra (Fig. 1A). Upon Na<sub>2</sub>ATP addition, the largest signal shifts were observed for residues located in the N-terminal α-helix and in β-strands β1-β8 and β13-β19 (Fig. 1B). The shifts are small compared to commonly reported nucleotide interactions (see for example (38)), suggesting weak and potentially non-specific interactions distributed over the entire protein (3). However, many signals exhibited strong changes in intensity upon addition of Na<sub>2</sub>ATP (Fig. 1C). NMR line broadening in ligand titrations is a sign of exchange that is intermediate on the NMR time scale (i.e. the rate of binding exchange is of the same order as the difference in chemical shifts of the bound and free states). The degree of line broadening therefore depends on several factors such as differences in chemical environment induced by ligand binding, associated conformational changes as well as the kinetics of the exchange process. Since we did not observe any resonances corresponding to the bound state at higher ATP concentrations, it is likely that the signal broadening results from μs-ms time scale motions in the protein induced by the nucleotide. Notably, addition of either MgATP or NaCl at concentrations of similar ionic strength (Na<sub>2</sub>ATP at a protein-to-nucleotide ratio of 1:16 had an ionic strength of ~51 mM, MgATP (1:16) of ~53 mM, and NaCl of 50 mM) caused smaller CSD than Na<sub>2</sub>ATP (Fig. 1D) and did not induce significant resonance broadening (Fig. 1E). Hence, both CSD and resonance broadening are caused specifically

by uncomplexed ATP. We can therefore consider the resonance broadening as a result of ATP binding and define an ATP interaction region: residues in the  $\alpha$ -helix/linker and the C-terminal  $\beta$ -strands  $\beta$ 12- $\beta$ 19 were strongly broadened (Fig. 1B-D and Table 1). The two regions are in close proximity in the available 3D structures of VDAC1 (2,4). They can therefore be considered as one ATP interaction region located in the cytosolic (2) pore entrance (Fig. 1F). These residues overlap with residues exhibiting CSD (Fig. 1B), supporting the presence of a large ATP interaction region that contains the previously identified NBS1 and NBS3 (32).

The ATP interaction surface at the cytosolic pore entrance exhibits a positive electrostatic potential (Fig. 1G). Considering a  $pK_a$  of 6.76 for  $HATP^{3-}$  (39), approximately equal amounts of  $ATP^{4-}$  and  $HATP^{3-}$  are present at pH 6.8 in the absence of  $Mg^{2+}$ . In contrast, in the presence of a 10-20 mM excess of  $MgCl_2$  a  $MgATP^{2-}$  population >99% was present at all ATP concentrations used here (34). The positive electrostatic surface potential of the interaction region is in line with an electrostatically driven interaction with the negatively charged free  $ATP^{4-}$  /  $HATP^{3-}$ . In contrast, the small changes in CSD upon addition of  $MgATP$  may result from a reduced but not entirely inhibited interaction of the complexed form, traces of free ATP in equilibrium with the  $Mg$ -complexed form, or from sodium ions introduced by the stock solution.

Outside of the ATP interaction region described above we also detected changes in NMR signal intensity and position for residues located in loops on the opposite pore entrance facing the intermembrane space (Fig. 2A and Table 1). This includes D9 and K12 in the  $\alpha$ -helix, K34/S35 in loop L1 connecting  $\beta$ 1 and  $\beta$ 2, R93 in loop L5 and the C-terminal A283 (Fig. 2A). Residues 94-96 comprise a conserved glycine-leucine-lysine ( $^{94}GLK^{96}$ ) motif proposed to be important for ion selectivity, but dispensable for ATP binding (40) (Fig. 2A). R93 and the  $^{94}GLK^{96}$  motif are close to residues K119 and R120 in  $\beta$ 7 (Fig. 2A), which also exhibit the strongest CSD in this region (Fig. 1B) and are part of the previously assigned NBS2 ( $\beta$ 7) (32). The broadened residues largely overlap with regions exhibiting a positive electrostatic potential (Fig. 2B), in agreement with an interaction with the charged forms of ATP.

*Affinity of the ATP-hVDAC1 interaction* – For residues that are in the fast exchange regime on the NMR time scale – that is the rate of binding exchange is larger than the difference in chemical shifts of the bound and free states – dissociation constants ( $K_D$ ) can be estimated. This is achieved by fitting a two-state binding model to the variation of average CSD values with increasing ligand concentration (37). As outlined above, most residues in the two ATP binding regions of hVDAC1 exhibited shifts and broadening at the same time (Fig. 1B,C). Intermediate exchange contributions to chemical shifts introduce errors in  $K_D$  value determination, and might lead to an underestimation of the true value (41). Therefore only residues that exhibited signal broadening less than 20% after intensity scaling in the final titration step were included in the analysis. These resonances are located at the edges of the regions of strongest interaction (as defined by the resonance broadening) and showed only small shifts. The fits might therefore not yield very accurate  $K_D$  values, but provide a crude estimate for the affinity. Examples of shifting residues and calculated  $K_D$  values are shown in Fig. 3. Plots of average CSD versus ATP concentration demonstrate that saturation was nearly reached. Based on the analysis of the  $Na_2ATP$ -binding curves of 19 residues in the helix-comprising binding region an average  $K_D$  of  $(1.1 \pm 0.4)$  mM was determined. An interaction of hVDAC1 with  $MgATP$  was barely detectable: Only six out of the previously used 19 residues showed sufficient CSD for fitting, resulting in  $K_D$  values 8-25 times higher than for  $Na_2ATP$  (9-33 mM).

*A common binding site for ATP, GTP and UTP* – It was previously shown that VDAC1 is able to bind different nucleotides in the same binding region (32). Therefore we probed the interaction of GTP and UTP with hVDAC1 with single-residue resolution (Fig. 4A). Both UTP and GTP induced CSD and resonance broadening in similar regions as ATP: the linker,  $\alpha$ -helix residues, and  $\beta$ 12- $\beta$ 19 (ATP binding region), as well as charged loop residues (Fig. 4B). Thus, similar regions in hVDAC1 are involved in binding to different nucleotides. Both GTP and UTP induced weaker broadening than ATP (Fig. 4C), suggesting subtle differences for these two nucleotides in terms of their interaction with hVDAC1 and/or their influence on hVDAC1 dynamics.

*The K20S mutation modulates the interaction of hVDAC1 with ATP* – NMR signal changes suggest an important role of charges in the N-terminal  $\alpha$ -helix and the connecting linker for ATP-binding. To provide further support for their importance, we removed the positively charged side chain of K20 by mutation to serine. This mutation has been shown to reduce photo-affinity labeling with an ATP analog and to influence ATP transport and mitochondrial metabolism *in vivo* (31). The K20S variant was subjected to an NMR titration analysis with  $\text{Na}_2\text{ATP}$ . ATP-induced NMR signal changes occurred in regions of K20S hVDAC1 similar to the ones observed for the wild-type protein, indicating that K20S hVDAC1 is still able to interact with ATP. At the same time, however, many residues displayed decreased signal broadening and increased chemical shift changes in K20S hVDAC1 (Fig. 5A,B). An increase in intensity ratio of ATP-bound versus free form of more than 25% occurred for residues that are identical to or next to the residues strongly broadened by ATP in WT hVDAC1 (Table 1 and Fig. 5B). The strongest signal enhancement (increase by more than 75% upon mutation) was observed for T6, D9, L10, A14, V17, Y22, K34, S35, G38, K61, G94, S104, A209, N214, N216, F219, N239, S241, G244 and A283. Notably, introduction of the K20S mutation into the helix in the pore center influenced the interaction of ATP with the cytosolic pore entrance (Fig. 1F), as well as with residues facing the intermembrane space (Fig. 2A).

*K20S does not perturb the structure and dynamics of hVDAC1* – K20 is located at the C-terminal end of the  $\alpha$ -helix, which has been implicated in the regulation of VDAC function including gating (8,27,31,42). At the C-terminal end of the  $\alpha$ -helix we previously observed decreased NMR signal intensities (22), pointing to the presence of slow conformational exchange. In addition, ATP induced resonance broadening in WT hVDAC1, which is potentially connected to protein conformational exchange. Therefore, we asked the question if the K20S mutation not only influences the ATP-interaction but also the intrinsic structural and dynamic properties of the hVDAC1 architecture. Changes in amide proton and nitrogen chemical shifts, reporting on the chemical environment, were most pronounced (CSD > 0.03) for residues near the mutation site: in the C-terminal end of the  $\alpha$ -helix and the linker

(V17, Y22, F24), as well as in  $\beta$ -strands  $\beta$ 14- $\beta$ 18 (Fig. 5C,D). The location of the NMR signal changes support the 3-dimensional structure of hVDAC1/mVDAC1 (2,4). Amide proton and nitrogen chemical shift changes also occurred in several N-terminal barrel residues far from the mutation site. In addition, scattered changes in peak intensities were observed in several regions throughout the protein (data not shown). However, similar variations of peak intensities were observed between different WT samples with small pH differences. Despite the changes in amide proton and nitrogen chemical shifts,  $\text{C}\alpha$  resonances were very similar to those of the WT protein indicating that the structure of hVDAC1 was not perturbed by the K20S mutation (Fig. 5E). Thus, the mutation has at best a small influence on the dynamics of the channel.

## DISCUSSION

Regulated ATP permeation through VDAC plays a crucial role for the control of cellular metabolism (11,15-19), and biochemical experiments suggested the presence of several NBSs (30-33). In addition, an early NMR study on the interaction of VDAC with ATP detected a few residues potentially involved in ATP binding, such as F24 and G25, I114 and T116, and D264 and A283 (43), located in the three NBSs determined from cross-linking studies (32). However, due to the fact that the chemical shift changes were small and dispersed across the entire protein, the authors of this study concluded that ATP does not bind to a specific region of hVDAC1 (3). In the current study, we identified – based on an extensive backbone resonance assignment of hVDAC1 and a detailed analysis of NMR signal intensities – residues in the  $\alpha$ -helix/linker region and the neighboring C-terminal barrel part ( $\beta$ 12- $\beta$ 19) that form a single ATP interaction region in the cytosolic pore entrance of hVDAC1 (Fig. 1,6). The ATP-induced line broadening is caused by an exchange between free and bound ATP that is intermediate on the chemical shift scale. The broadening was specific for uncomplexed ATP and provides a reporter for nucleotide binding and induction of dynamics.

In line with an electrostatically driven interaction with the negatively charged free  $\text{ATP}^{4-}$  /  $\text{HATP}^{3-}$ , the identified ATP interaction region exhibits a positive electrostatic surface potential

(Fig. 1G). The dissociation constant of  $\text{ATP}^{4-}$  /  $\text{HATP}^{3-}$  to this region was found to be in the low millimolar range ( $K_D \sim 1.1$  mM, Fig. 3). In addition, more distributed NMR signal perturbations and resonance broadening was observed in the opposite pore entrance facing the intermembrane space, in support of an electrostatic interaction with positively charged side chains of loop residues (Fig. 2). Some of these residues (R93 and the  $^{94}\text{GLK}^{96}$  motif) are in proximity to residues K119 and R120, and an interaction with these residues could explain photo-affinity labeling of the previously assigned NBS2 ( $\beta 7$ ). Moreover, the cytosolic ATP interaction region comprises the previously proposed NBS1 and NBS3 (32). The shift between the region of strongest NMR line broadening ( $\beta 15$ - $\beta 17$ ) and the cross-linking region of the photoreactive ATP analogue ( $\beta 18$ - $\beta 19$ ) (Fig. 1B,C) might be due to a preferred orientation of the photoreactive group attached to the sugar of the modified ATP when bound to this ATP binding region (32). Alternatively, it might result from different susceptibility of these strands to ATP-induced conformational exchange.

The cytosolic ATP interaction region contains the linker that connects the  $\alpha$ -helix of VDAC1 to the channel's barrel. The linker sequence ( $^{19}\text{TKGYGFG}^{25}$ ) resembles an inverted Walker A motif (28,32,44), which on its own is sufficient to bind ATP (30). Moreover, K19 in *Saccharomyces cerevisiae* VDAC (homologous to K20 in hVDAC1) influences channel selectivity (8), and mutation of K20 to serine was found to strongly change channel properties, ATP binding and transport, and mitochondrial metabolism of mVDAC1 (31). Our NMR studies demonstrated that K20 contributes to the interaction of ATP with the ATP interaction region in the cytosolic pore entrance (Fig. 5), either because K20 might directly bind the negatively charged phosphate moiety of ATP or contribute to ATP interactions via long-range electrostatic effects. Both scenarios would result in a lower ATP affinity of K20S hVDAC1. The increased CSD and reduced broadening might thus be a result of a shift to a faster exchange regime of binding, in agreement with previous results from photo-crosslinking studies that indicated a reduced affinity of K20S VDAC1 for ATP analogs (31). In addition, a lower ATP affinity might result in a reduced ATP-induced conformational exchange in VDAC, potentially leading to less broadening but larger

CSD (due to the change in binding kinetics). The strong signal intensity changes observed for K34, S35, G38, G94, and A283 in the pore entrance facing the intermembrane space upon mutation of K20S (Fig. 5) further suggest that the regions, which interact with ATP at both pore entrances, i.e. below and above the  $\alpha$ -helix (Fig. 1,2), are not independent.

We found that hVDAC1 preferentially binds the free forms of ATP ( $\text{ATP}^{4-}$  /  $\text{HATP}^{3-}$ ) (Fig. 1) and that K20 modulates the ATP interaction (Fig. 5). Our observations are in line with an inhibitory effect of divalent metals found by photoaffinity ATP labeling studies (32) and a charge dependence of binding ( $\text{ATP}^{4-} > \text{ADP}^{3-} > \text{AMP}^{2-}$ ) observed in current noise measurements (33). The lower affinity of hVDAC1 for MgATP (9-33 mM), a faster permeation of succinate $^{2-}$  and citrate $^{3-}$  compared to  $\text{ATP}^{4-}$  in reconstituted bilayers (11), and the predominance of Mg $^{2+}$ -complexed ATP *in vivo* (45,46) suggest that MgATP is the major form transported by VDAC. At contact sites with the inner membrane, where the charged  $\text{ATP}^{4-}$  is exported by the adenine nucleotide translocase (ANT) (47), nucleotides might permeate through VDAC as the charged forms or form Mg-complexes prior to permeation. As ANT exchanges  $\text{ATP}^{4-}$  for  $\text{ADP}^{3-}$ , it is also conceivable that Mg $^{2+}$  is passed from  $\text{ADP}^{3-}$  to  $\text{ATP}^{4-}$ , before the latter permeates VDAC.

Since VDAC is a passive diffusion pore allowing permeation of various metabolites (1), we assume that free and complexed forms of ATP can permeate the channel in both directions, determined mainly by their concentration gradients across the mitochondrial membranes. The localization of the large cytosolic ATP interaction region might therefore not be connected to a directionality of transport. On the other hand, it might influence the consumption of mitochondrial ATP by hexokinase, an important cytosolic binding partner of VDAC1 (48).

Several studies on bacterial porins provided support for specific solute binding sites and a transversal electrostatic potential in the pore as requirements for selectivity and transport (49-55). For example, the selective OprP discriminates between organic phosphate and  $\text{Cl}^-$  by specific interactions with lysine residues (52), while a single lysine mutation inhibits phosphate flux (56). Although VDAC is a large diffusion pore for a variety of solutes (1), it has also been

demonstrated to discriminate between physiological and non-physiological charged molecules of similar size (29). The affinity of hVDAC1 for ATP is in line with the  $\mu\text{M}$ -mM solute affinities observed for various bacterial outer membrane proteins (57-61). This suggests that porins enable solute permeation by a sufficiently low affinity, but at the same time exert selectivity by specific interactions. Thus VDAC can bind to the charged form of ATP exerting selectivity towards this species (Fig. 6), while permeation of the  $\text{Mg}^{2+}$ -complex of ATP can still occur, supported by its high physiological concentration (2.5-3.3 mM cytosolic, 8-10 mM mitochondrial (45,46)). Charges in the ATP interaction regions facing the cytosol and the intermembrane space might together allow for a smooth gliding of both forms of ATP through the pore, similar to what has been observed for the selective maltoporin (50,51). Surprisingly, K20 has been found acetylated in mice liver and three human cell lines (62,63). The reduced affinity of K20S hVDAC1 for ATP as suggested by photo-crosslinking studies (31) and our study (Fig. 5)

therefore suggests that post-translational modifications might adjust ATP flux to cellular metabolite requirements *in vivo*.

The ATP binding region identified in this study partially overlaps with regions involved in  $\beta$ -NADH binding determined by solution NMR spectroscopy for hVDAC1, namely residues 242-244 ( $\beta$ 17) and 260-264 ( $\beta$ 18) (3). Preliminary NMR studies suggest that the same interaction interface is present in VDAC2 (64). In addition, GTP and UTP induced changes in NMR signal positions and intensities for the same residues as ATP (Fig. 4), suggesting that VDAC possesses a common nucleotide binding region that acts as a weak selectivity filter for nucleotides (Fig. 6). Given the discriminative properties of VDAC (29), this region therefore can serve as a general selectivity filter that influences transport of physiological metabolites. It is conceivable that this interaction contributes to NADH-induced inhibition of nucleotide permeation (16), for example by competition or by enhancing molecular crowding inside the pore.

## REFERENCES

1. Benz, R. (1994) Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. *Biochim Biophys Acta* **1197**, 167-196
2. Bayrhuber, M., Meins, T., Habeck, M., Becker, S., Giller, K., Villinger, S., Vonrhein, C., Griesinger, C., Zweckstetter, M., and Zeth, K. (2008) Structure of the human voltage-dependent anion channel. *Proc Natl Acad Sci U S A* **105**, 15370-15375
3. Hiller, S., Garces, R. G., Malia, T. J., Orekhov, V. Y., Colombini, M., and Wagner, G. (2008) Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. *Science* **321**, 1206-1210
4. Ujwal, R., Cascio, D., Colletier, J. P., Faham, S., Zhang, J., Toro, L., Ping, P., and Abramson, J. (2008) The crystal structure of mouse VDAC1 at 2.3 Å resolution reveals mechanistic insights into metabolite gating. *Proc Natl Acad Sci U S A* **105**, 17742-17747
5. Guo, X. W., and Mannella, C. A. (1993) Conformational change in the mitochondrial channel, VDAC, detected by electron cryo-microscopy. *Biophys J* **64**, 545-549
6. Goncalves, R. P., Buzhynskyy, N., Prima, V., Sturgis, J. N., and Scheuring, S. (2007) Supramolecular assembly of VDAC in native mitochondrial outer membranes. *J Mol Biol* **369**, 413-418
7. Mannella, C. A. (1982) Structure of the outer mitochondrial membrane: ordered arrays of porelike subunits in outer-membrane fractions from *Neurospora crassa* mitochondria. *J Cell Biol* **94**, 680-687
8. Blachly-Dyson, E., Peng, S., Colombini, M., and Forte, M. (1990) Selectivity changes in site-directed mutants of the VDAC ion channel: structural implications. *Science* **247**, 1233-1236
9. Peng, S., Blachly-Dyson, E., Forte, M., and Colombini, M. (1992) Large scale rearrangement of protein domains is associated with voltage gating of the VDAC channel. *Biophys J* **62**, 123-135
10. Colombini, M. (1989) Voltage gating in the mitochondrial channel, VDAC. *J Membr Biol* **111**, 103-111
11. Rostovtseva, T., and Colombini, M. (1997) VDAC channels mediate and gate the flow of ATP: implications for the regulation of mitochondrial function. *Biophys J* **72**, 1954-1962
12. Schein, S. J., Colombini, M., and Finkelstein, A. (1976) Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from paramecium mitochondria. *J Membr Biol* **30**, 99-120
13. Benz, R., Kottke, M., and Brdiczka, D. (1990) The cationically selective state of the mitochondrial outer membrane pore: a study with intact mitochondria and reconstituted mitochondrial porin. *Biochim Biophys Acta* **1022**, 311-318
14. Shoshan-Barmatz, V., Israelson, A., Brdiczka, D., and Sheu, S. S. (2006) The voltage-dependent anion channel (VDAC): function in intracellular signalling, cell life and cell death. *Curr Pharm Des* **12**, 2249-2270
15. Benz, R., Wojtczak, L., Bosch, W., and Brdiczka, D. (1988) Inhibition of adenine nucleotide transport through the mitochondrial porin by a synthetic polyanion. *FEBS Lett* **231**, 75-80
16. Lee, A. C., Zizi, M., and Colombini, M. (1994) Beta-NADH decreases the permeability of the mitochondrial outer membrane to ADP by a factor of 6. *J Biol Chem* **269**, 30974-30980

17. Liu, M. Y., and Colombini, M. (1992) Regulation of mitochondrial respiration by controlling the permeability of the outer membrane through the mitochondrial channel, VDAC. *Biochim Biophys Acta* **1098**, 255-260
18. Gellerich, F. N., Wagner, M., Kapischke, M., Wicker, U., and Brdiczka, D. (1993) Effect of macromolecules on the regulation of the mitochondrial outer membrane pore and the activity of adenylate kinase in the inter-membrane space. *Biochim Biophys Acta* **1142**, 217-227
19. Lemasters, J. J., and Holmuhamedov, E. (2006) Voltage-dependent anion channel (VDAC) as mitochondrial governor--thinking outside the box. *Biochim Biophys Acta* **1762**, 181-190
20. Zimmerberg, J., and Parsegian, V. A. (1986) Polymer inaccessible volume changes during opening and closing of a voltage-dependent ionic channel. *Nature* **323**, 36-39
21. Hiller, S., and Wagner, G. (2009) The role of solution NMR in the structure determinations of VDAC-1 and other membrane proteins. *Curr Opin Struct Biol* **19**, 396-401
22. Villinger, S., Briones, R., Giller, K., Zachariae, U., Lange, A., de Groot, B. L., Griesinger, C., Becker, S., and Zweckstetter, M. (2010) Functional dynamics in the voltage-dependent anion channel. *Proc Natl Acad Sci U S A* **107**, 22546-22551
23. Teijido, O., Ujwal, R., Hillerdal, C. O., Kullman, L., Rostovtseva, T. K., and Abramson, J. (2012) Affixing the N-terminal alpha helix of the voltage dependent anion channel to the channel's wall does not prevent its voltage gating. *J Biol Chem* **287**, 11437-11445
24. Zachariae, U., Schneider, R., Briones, R., Gattin, Z., Demers, J. P., Giller, K., Maier, E., Zweckstetter, M., Griesinger, C., Becker, S., Benz, R., de Groot, B. L., and Lange, A. (2010) beta-Barrel mobility underlies closure of the voltage-dependent anion channel. *Structure* **20**, 1540-1549
25. Mertins, B., Psakis, G., Grosse, W., Back, K. C., Salisowski, A., Reiss, P., Koert, U., and Essen, L. O. (2012) Flexibility of the N-terminal mVDAC1 segment controls the channel's gating behavior. *PLoS One* **7**, e47938
26. Song, J., Midson, C., Blachly-Dyson, E., Forte, M., and Colombini, M. (1998) The sensor regions of VDAC are translocated from within the membrane to the surface during the gating processes. *Biophys J* **74**, 2926-2944
27. Thomas, L., Blachly-Dyson, E., Colombini, M., and Forte, M. (1993) Mapping of residues forming the voltage sensor of the voltage-dependent anion-selective channel. *Proc Natl Acad Sci U S A* **90**, 5446-5449
28. Zizi, M., Forte, M., Blachly-Dyson, E., and Colombini, M. (1994) NADH regulates the gating of VDAC, the mitochondrial outer membrane channel. *J Biol Chem* **269**, 1614-1616
29. Rostovtseva, T. K., Komarov, A., Bezrukov, S. M., and Colombini, M. (2002) VDAC channels differentiate between natural metabolites and synthetic molecules. *J Membr Biol* **187**, 147-156
30. Florke, H., Thinnies, F. P., Winkelbach, H., Stadtmuller, U., Paetzold, G., Morys-Wortmann, C., Hesse, D., Sternbach, H., Zimmermann, B., Kaufmann-Kolle, P., and et al. (1994) Channel active mammalian porin, purified from crude membrane fractions of human B lymphocytes and bovine skeletal muscle, reversibly binds adenosine triphosphate (ATP). *Biol Chem Hoppe Seyler* **375**, 513-520
31. Yehezkel, G., Abu-Hamad, S., and Shoshan-Barmatz, V. (2007) An N-terminal nucleotide-binding site in VDAC1: involvement in regulating mitochondrial function. *J Cell Physiol* **212**, 551-561

32. Yehezkel, G., Hadad, N., Zaid, H., Sivan, S., and Shoshan-Barmatz, V. (2006) Nucleotide-binding sites in the voltage-dependent anion channel: characterization and localization. *J Biol Chem* **281**, 5938-5946
33. Rostovtseva, T. K., Komarov, A., Bezrukov, S. M., and Colombini, M. (2002) Dynamics of nucleotides in VDAC channels: structure-specific noise generation. *Biophys J* **82**, 193-205
34. Schoenmakers, T. J., Visser, G. J., Flik, G., and Theuvenet, A. P. (1992) CHELATOR: an improved method for computing metal ion concentrations in physiological solutions. *Biotechniques* **12**, 870-874, 876-879
35. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* **6**, 277-293
36. Goddard, T. D., and Kneller, D. G. (2006) *SPARKY 3*. In., University of California, San Francisco
37. Cavanagh, J., Fairbrother, W. J., Palmer III, A. G., Rance, M., and Skelton, N. J. (2007) *Protein NMR Spectroscopy*, second edition Ed., Elsevier Academic Press
38. Frank, F., Sonenberg, N., and Nagar, B. (2010) Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2. *Nature* **465**, 818-822
39. De Stefano, C., Milea, D., Pettignano, A., and Sammartano, S. (2006) Modeling ATP protonation and activity coefficients in NaClaq and KClaq by SIT and Pitzer equations. *Biophysical chemistry* **121**, 121-130
40. Runke, G., Maier, E., O'Neil, J. D., Benz, R., and Court, D. A. (2000) Functional characterization of the conserved "GLK" motif in mitochondrial porin from *Neurospora crassa*. *J Bioenerg Biomembr* **32**, 563-570
41. Feeney, J., Batchelor, J. G., Albrand, J. P., and Roberts, G. C. K. (1979) The effects of intermediate exchange processes on the estimation of equilibrium constants by NMR. *Journal of Magnetic Resonance* **33**, 519-529
42. Abu-Hamad, S., Arbel, N., Calo, D., Arzoine, L., Israelson, A., Keinan, N., Ben-Romano, R., Friedman, O., and Shoshan-Barmatz, V. (2009) The VDAC1 N-terminus is essential both for apoptosis and the protective effect of anti-apoptotic proteins. *J Cell Sci* **122**, 1906-1916
43. Malia, T. J., and Massachusetts Institute of Technology. Dept. of Chemistry. (2006) NMR structural and functional studies of the mitochondrial outer membrane protein VDAC / by Thomas J. Malia. In.
44. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *Embo J* **1**, 945-951
45. Akerboom, T. P., Bookelman, H., Zuurendonk, P. F., van der Meer, R., and Tager, J. M. (1978) Intramitochondrial and extramitochondrial concentrations of adenine nucleotides and inorganic phosphate in isolated hepatocytes from fasted rats. *Eur J Biochem* **84**, 413-420
46. Traut, T. W. (1994) Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem* **140**, 1-22
47. Klingenberg, M. (2008) The ADP and ATP transport in mitochondria and its carrier. *Biochim Biophys Acta* **1778**, 1978-2021
48. Abu-Hamad, S., Zaid, H., Israelson, A., Nahon, E., and Shoshan-Barmatz, V. (2008) Hexokinase-I protection against apoptotic cell death is mediated via interaction with the

- voltage-dependent anion channel-1: mapping the site of binding. *J Biol Chem* **283**, 13482-13490
49. Dutzler, R., Wang, Y. F., Rizkallah, P., Rosenbusch, J. P., and Schirmer, T. (1996) Crystal structures of various maltooligosaccharides bound to maltoporin reveal a specific sugar translocation pathway. *Structure* **4**, 127-134
  50. Schirmer, T., Keller, T. A., Wang, Y. F., and Rosenbusch, J. P. (1995) Structural basis for sugar translocation through maltoporin channels at 3.1 Å resolution. *Science* **267**, 512-514
  51. Meyer, J. E., and Schulz, G. E. (1997) Energy profile of maltooligosaccharide permeation through maltoporin as derived from the structure and from a statistical analysis of saccharide-protein interactions. *Protein Sci* **6**, 1084-1091
  52. Pongprayoon, P., Beckstein, O., Wee, C. L., and Sansom, M. S. (2009) Simulations of anion transport through OprP reveal the molecular basis for high affinity and selectivity for phosphate. *Proc Natl Acad Sci U S A* **106**, 21614-21618
  53. Zou, H., Zheng, M., Luo, X., Zhu, W., Chen, K., Shen, J., and Jiang, H. (2008) Dynamic mechanism of fatty acid transport across cellular membranes through FadL: molecular dynamics simulations. *J Phys Chem B* **112**, 13070-13078
  54. Nestorovich, E. M., Danelon, C., Winterhalter, M., and Bezrukov, S. M. (2002) Designed to penetrate: time-resolved interaction of single antibiotic molecules with bacterial pores. *Proc Natl Acad Sci U S A* **99**, 9789-9794
  55. Lou, H., Chen, M., Black, S. S., Bushell, S. R., Ceccarelli, M., Mach, T., Beis, K., Low, A. S., Bamford, V. A., Booth, I. R., Bayley, H., and Naismith, J. H. (2011) Altered Antibiotic Transport in OmpC Mutants Isolated from a Series of Clinical Strains of Multi-Drug Resistant E. coli. *PLoS One* **6**, e25825
  56. Sukhan, A., and Hancock, R. E. (1996) The role of specific lysine residues in the passage of anions through the Pseudomonas aeruginosa porin OprP. *J Biol Chem* **271**, 21239-21242
  57. Zachariae, U., Kluhspies, T., De, S., Engelhardt, H., and Zeth, K. (2006) High resolution crystal structures and molecular dynamics studies reveal substrate binding in the porin Omp32. *J Biol Chem* **281**, 7413-7420
  58. Kobayashi, Y., and Nakae, T. (1985) The mechanism of ion selectivity of OmpF-porin pores of Escherichia coli. *Eur J Biochem* **151**, 231-236
  59. Schulein, K., and Benz, R. (1990) LamB (maltoporin) of Salmonella typhimurium: isolation, purification and comparison of sugar binding with LamB of Escherichia coli. *Mol Microbiol* **4**, 625-632
  60. Kim, B. H., Andersen, C., Kreth, J., Ulmke, C., Schmid, K., and Benz, R. (2002) Site-directed mutagenesis within the central constriction site of ScrY (sucroseporin): effect on ion transport and comparison of maltooligosaccharide binding to LamB of Escherichia coli. *J Membr Biol* **187**, 239-253
  61. Benz, R., Schmid, A., Maier, C., and Bremer, E. (1988) Characterization of the nucleoside-binding site inside the Tsx channel of Escherichia coli outer membrane. Reconstitution experiments with lipid bilayer membranes. *Eur J Biochem* **176**, 699-705
  62. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* **325**, 834-840
  63. Kim, S. C., Sprung, R., Chen, Y., Xu, Y., Ball, H., Pei, J., Cheng, T., Kho, Y., Xiao, H., Xiao, L., Grishin, N. V., White, M., Yang, X. J., and Zhao, Y. (2006) Substrate and

- functional diversity of lysine acetylation revealed by a proteomics survey. *Mol Cell* **23**, 607-618
64. Yu, T. Y., Raschle, T., Hiller, S., and Wagner, G. (2012) Solution NMR spectroscopic characterization of human VDAC-2 in detergent micelles and lipid bilayer nanodiscs. *Biochim Biophys Acta* **1818**, 1562-1569

*Acknowledgements* – This work was supported by the Fonds der Chemischen Industrie (scholarship to S.V.), the DFG (Emmy Noether Fellowship to A.L.), the DFG collaborative research center SFB803 (to A.L., C.G. and M.Z.) and the ERC (ERC grant agreement number 282008 to M.Z.).

## FOOTNOTES

<sup>1</sup>The abbreviations used are: <sup>94</sup>GLK<sup>96</sup> motif, glycine-leucine-lysine motif; hVDAC1, human voltage dependent anion channel 1; LDAO, Lauryldimethylamine-N-oxide; mVDAC1, murine voltage dependent anion channel 1; NBS, nucleotide binding site; NMR, nuclear magnetic resonance; rVDAC1, rat voltage-dependent anion channel; TROSY, transverse relaxation optimized spectroscopy; VDAC, voltage-dependent anion channel; WT, wild type.

## FIGURE LEGENDS

**FIGURE 1.** *Mapping the ATP-hVDAC1 interaction with single-residue resolution.* (A) Regions of two-dimensional <sup>1</sup>H,<sup>15</sup>N-TROSY spectra before (black) and after addition of Na<sub>2</sub>ATP at a hVDAC1:ATP ratio of 1:16 (blue) and MgATP at a ratio of 1:64 (orange). (B, C) Average chemical shift deviations (CSD) (B) and scaled resonance intensity ratios  $I_{\text{ATP}}/I_{\text{ref}}$  (C) obtained from <sup>1</sup>H,<sup>15</sup>N-TROSY spectra after addition of Na<sub>2</sub>ATP (blue) or MgATP (orange) to hVDAC1 at a hVDAC1:ATP ratio of 1:16. The black line indicates the threshold due to digital resolution. Intensities were scaled relative to residues that were apparently unchanged by addition of ATP (residues 142-145, 152-156, 168-169, and 171-174). (D, E) Average chemical shift deviations (CSD) (D) and scaled resonance intensity ratios  $I_{\text{NaCl}}/I_{\text{ref}}$  (E) obtained from <sup>1</sup>H,<sup>15</sup>N-TROSY spectra after addition of 50 mM NaCl to hVDAC1. Intensities were scaled relative to the same residues as in (B). (B-E) Previously proposed nucleotide binding regions (32) are highlighted by dashed boxes, the secondary structure of hVDAC1 is indicated on top and highlighted with grey bars. (F) NMR resonance broadening induced by 16-fold excess of ATP mapped onto the crystal structure of mVDAC1 (PDB code: 3EMN) in cartoon representation viewed from the inside (left, intermembrane space (IMS) and cytosolic side (CYTO) are indicated (2)) or surface representation viewed from the cytosolic pore entrance (right). Resonance broadening is color-coded, with effects increasing from blue (not affected) to green (strongly broadened). Unresolved residues are colored in grey. Lysine and arginine side chains are presented as sticks (left) and affected residues are labeled (right). (G) Map of the electrostatic potential of mVDAC1 (PDB code: 3EMN) calculated using Delphi (Accelrys) with positive and negative potentials in blue and red, respectively. The orientation is the same as in (D, right).

**FIGURE 2.** *ATP interaction outside of the main interaction region.* (A) NMR signal broadening induced by 16-fold excess of ATP mapped onto the mVDAC1 structure (PDB code: 3EMN) viewed from the intermembrane space. Resonance broadening is color-coded, with effects increasing from blue (not affected) to green (strongly broadened). Unresolved residues are shown in grey. Strongly affected residues are labeled. \*I227 is V227 in mVDAC1. The last two residues of the previously proposed NBS2 (K119, R120) are encircled. (B) Map of electrostatic potential as in Fig. 1G, shown from the opposite pore entrance.

**FIGURE 3.** *Affinity of hVDAC1 for ATP.* (A) Spectral regions of <sup>1</sup>H,<sup>15</sup>N-TROSY spectra before (black) and after (grey) addition of a 32-fold excess of Na<sub>2</sub>ATP showing examples of residues shifting strongly in the presence of ATP. (B) Plot of averaged chemical shift deviation (CSD) values versus ATP concentration and curve fits for  $K_D$  determination using a one-site binding model. Resonance intensity ratios, scaled with respect to apparently unaffected residues (residues 142-145, 152-156, 168-169, and 171-174), were 0.82 (S193) and 1.0 (G213) for the last titration step, showing that these residues were mostly in the fast exchange regime on the NMR time scale. Due to potential contributions from intermediate exchange (41) these values provide lower limits of the  $K_D$  values.

**FIGURE 4.** *UTP and GTP binding to hVDAC1.* (A) Regions of  $^1\text{H}$ ,  $^{15}\text{N}$ -TROSY spectra before (black) and after addition of GTP (blue) or UTP (red) at hVDAC1:nucleotide ratios of 1:16 (GTP) or 1:4 (UTP). (B) Resonance broadening induced by 16-fold excess of GTP mapped onto the crystal structure of mVDAC1 (PDB code: 3EMN) with color-coding as in Fig. 1. Unresolved residues are colored in grey. Lysine and arginine side chains are presented as sticks. The intermembrane space (IMS) and cytosolic side (CYTO) are indicated. (C) Scaled resonance intensity ratios  $I_{\text{nucleotide}}/I_{\text{ref}}$  obtained from  $^1\text{H}$ ,  $^{15}\text{N}$ -TROSY spectra after addition of ATP (black), GTP (blue) or UTP (red) to hVDAC1 at indicated hVDAC1:nucleotide ratios. Intensities were scaled relative to residues that were apparently unchanged by addition of ATP (residues 142-145, 152-156, 168-169, and 171-174). The secondary structure of hVDAC1 is indicated on top and highlighted with grey bars.

**FIGURE 5.** *K20S hVDAC1 modulates the ATP interaction but leaves hVDAC1 structure unchanged.* (A) Difference in average chemical shift deviations (CSD) obtained from  $^1\text{H}$ ,  $^{15}\text{N}$ -TROSY spectra after addition of  $\text{Na}_2\text{ATP}$  to WT or K20S hVDAC1 at a ratio of 1:16 (hVDAC1:ATP). Blue lines indicate the threshold due to digital resolution. (B) Increase in peak intensity ratios of K20S vs. WT hVDAC1 ( $(I_{\text{ATP}}/I_{\text{ref}})_{\text{K20S}}/(I_{\text{ATP}}/I_{\text{ref}})_{\text{WT}}-1$ ). Intensities in each spectrum were scaled relative to residues that were apparently unchanged by ATP addition (residues 142, 144-145, 152-156, 168-169, and 171-174). The scaling of the y-axis was limited to a maximum value of 3.5. A283 showed a roughly 64-fold intensity increase (\*). (C) Average CSD in K20S hVDAC1 with respect to WT hVDAC1 extracted from  $^1\text{H}$ ,  $^{15}\text{N}$ -TROSY spectra at a  $^1\text{H}$  frequency of 800 MHz using the equation  $\Delta_{\text{HN}} = ((\Delta\delta_{\text{N}}/10)^2 + (\Delta\delta_{\text{H}})^2)^{0.5}$ . (D) Residues with CSD between 0.02 and 0.03 (cyan line in (C)) and larger than 0.03 (green line in (C)) are mapped onto the crystal structure of mVDAC1 (pdb id: 3EMN) in cyan and green, respectively. The K20 side chain is shown as red stick. The intermembrane space (IMS) and cytosolic side (CYTO) are indicated. (E) Difference in  $\text{C}\alpha$  chemical shifts of K20S compared to WT hVDAC1. The secondary structure of hVDAC1 in (A-C, E) is indicated on top and highlighted with grey bars.

**FIGURE 6.** *The hVDAC1 nucleotide binding region acts as a low affinity selectivity filter for metabolite permeation.* hVDAC1 is shown as surface representation viewed from the cytosolic pore entrance and colored as in Fig. 1F. Nucleotides are displayed as cartoons (red: phosphate groups; black: ribose, yellow: adenine base (A) / guanine base (G) / nicotinamide (NA)). Arrows indicate interactions of nucleotides with the nucleotide binding region (green surface, approximate location encircled by dashed line) and permeation through the pore.

## TABLES

**Table 1. Residue-specific broadening of  $^1\text{H}$ - $^{15}\text{N}$  NMR resonances of hVDAC1 by ATP.**

$\text{Na}_2\text{ATP}$  was added to WT or K20S hVDAC1 at a hVDAC1:ATP ratio of 1:16. Resonance intensity ratios ( $I_{\text{ATP}}/I_{\text{ref}}$ ) for WT and K20S hVDAC1 were obtained from  $^1\text{H}$ ,  $^{15}\text{N}$ -TROSY spectra and scaled relative to residues that were apparently unchanged by addition of ATP (residues 142-145, 152-156, 168-169, and 171-174). The listed residues showed intensity ratios ( $I_{\text{ATP}}/I_{\text{ref}}$ )  $< 0.75$  in WT hVDAC1 spectra (a) or an increase in intensity ratio ( $(I_{\text{ATP}}/I_{\text{ref}})_{\text{K20S}} / (I_{\text{ATP}}/I_{\text{ref}})_{\text{WT}} - 1$ )  $> 0.25$  for K20S vs. WT hVDAC1 (b), respectively.

Secondary structure element	Residues affected by ATP in WT hVDAC1 <sup>a</sup>	Strong changes in intensity ratio of K20S vs. WT hVDAC1 <sup>b</sup>
N-terminal $\alpha$ -helix	T6, D9, L10, K12, A14, R15, V17	T6, D9, L10, A14, V17
Linker	Y22, F24, G25	Y22, G25
$\beta$ 1	L26, D30	L26, K28
L1 ( $\beta$ 1- $\beta$ 2)	K34, S35	K34, S35
$\beta$ 2	G38, S43, N48	G38
$\beta$ 3	K61	K61, Y62
L4 ( $\beta$ 4- $\beta$ 5)	T77	D78
L5 ( $\beta$ 5- $\beta$ 6)	Q90, R93	-
$\beta$ 6	G94, L95, K96	G94, L95, K96, F103, S104
L6 ( $\beta$ 6- $\beta$ 7)	-	G108
$\beta$ 7	K110, K119	K113, K119
$\beta$ 8	-	D132
$\beta$ 12	H181	-
$\beta$ 13	F190, G192	-
L13 ( $\beta$ 13- $\beta$ 14)	N199	N199
$\beta$ 14	A209, W210	A209
L14 ( $\beta$ 14- $\beta$ 15)	T211	G213, N214, N216
$\beta$ 15	R218, F219, G220, A222, Y225, Q226, I227	F219, G220, A222, Q226, I227
L15 ( $\beta$ 15- $\beta$ 16)	D228, D230	D228, D230
$\beta$ 16	A231, C232, A235, K236	-
L16 ( $\beta$ 16- $\beta$ 17)	N239, S240, S241	N239, S241
$\beta$ 17	L242, G244, L245	G244
L17 ( $\beta$ 17- $\beta$ 18)	G254	-
$\beta$ 18	I255, L259, A261, L263	I255, L263
L18 ( $\beta$ 18- $\beta$ 19)	G265	V268, G272
$\beta$ 19	K274, A283	H273, L279, A283

Figure 1

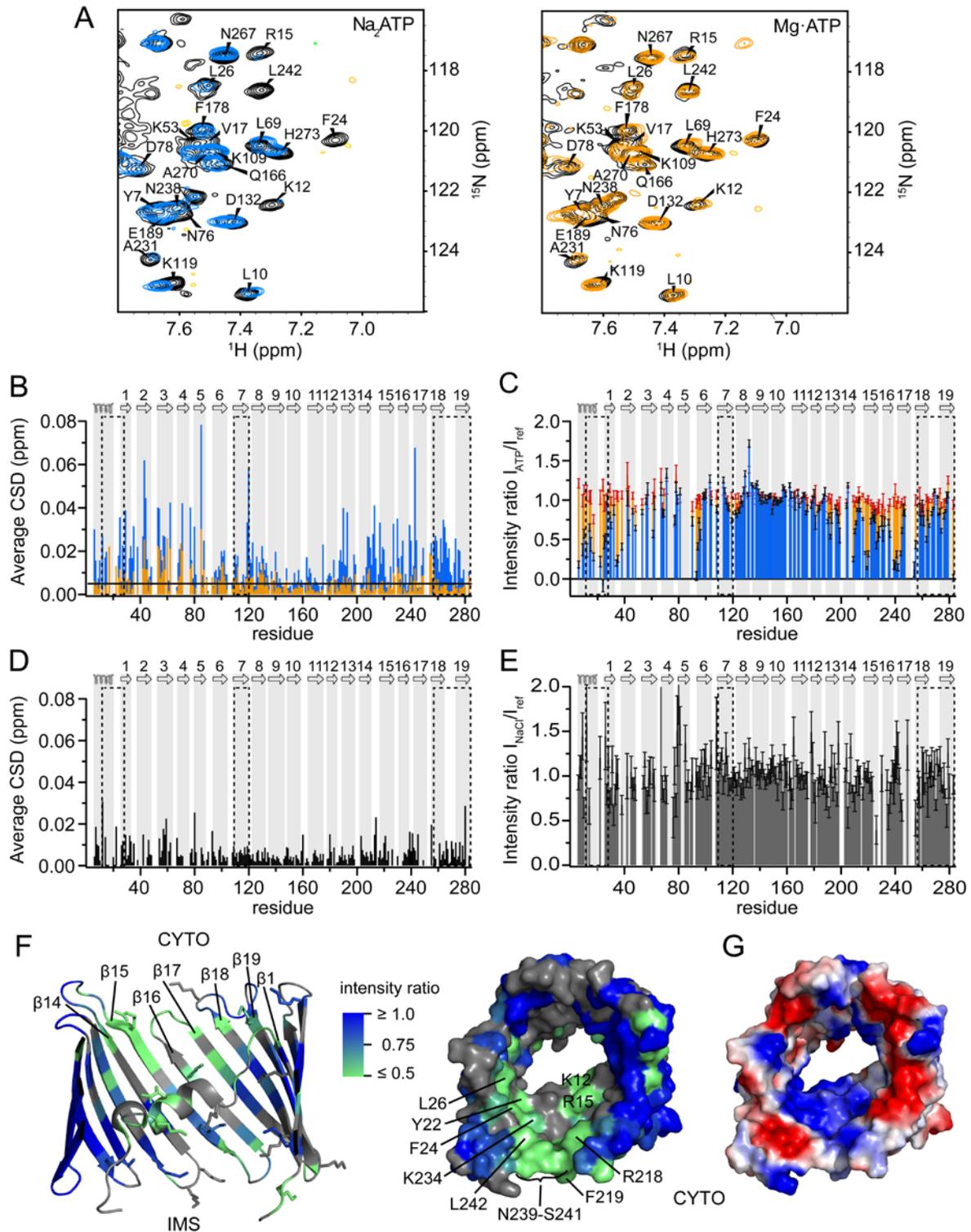


Figure 2

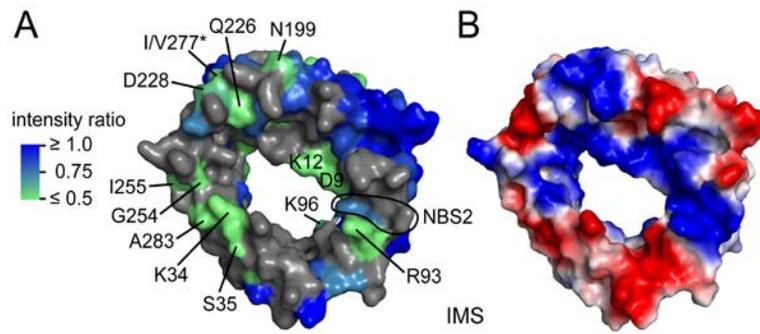


Figure 3

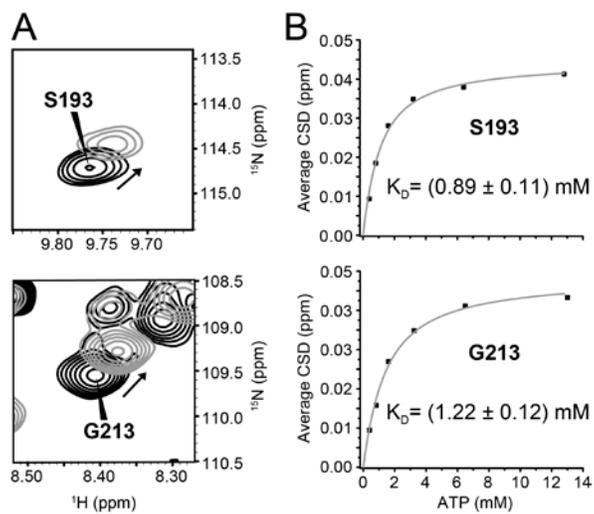


Figure 4

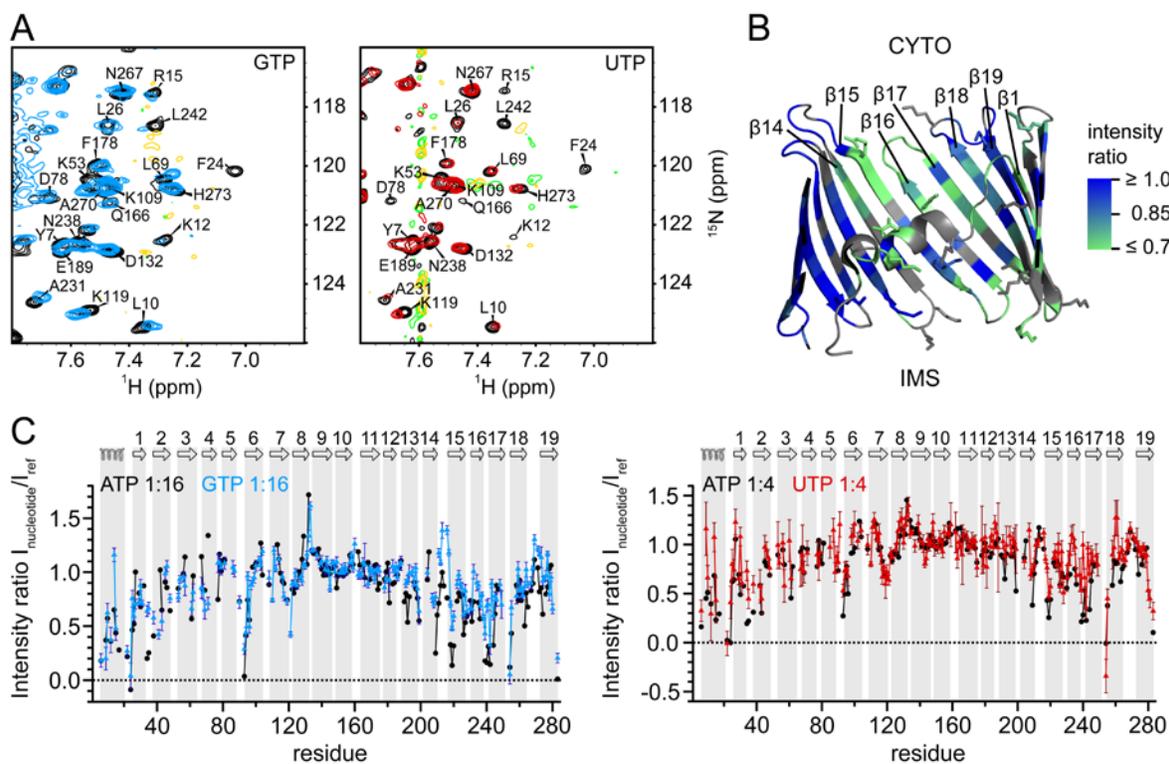


Figure 5

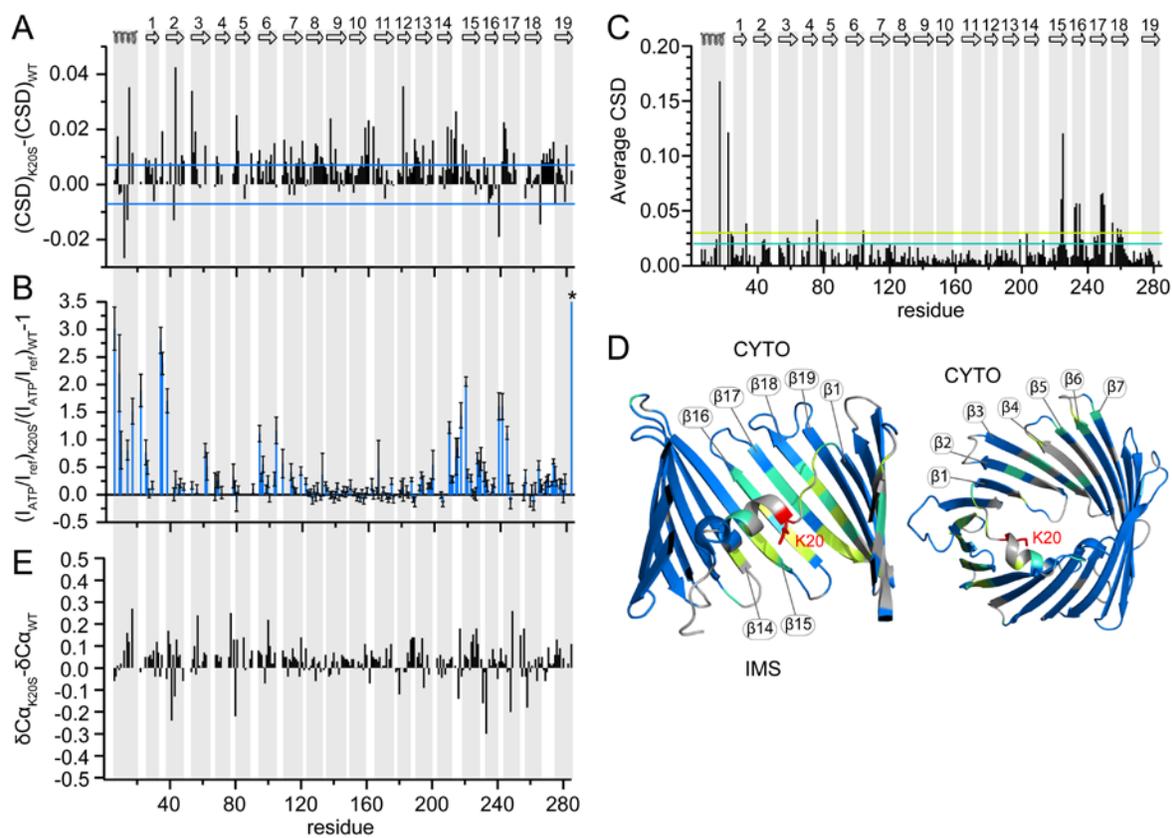


Figure 6

