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Ion selectivity and gating mechanisms of FNT channels

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Abstract

The phospholipid bilayer has evolved to be a protective and selective barrier by which the cell maintains high concentrations of life sustaining organic and inorganic material. As gatekeepers responsible for an immense amount of bidirectional chemical traffic between the cytoplasm and extracellular milieu, ion channels have been studied in detail since their postulated existence nearly three-quarters of a century ago. Over the past fifteen years, we have begun to understand how selective permeability can be achieved for both cationic and anionic ions. Our mechanistic knowledge has expanded recently with studies of a large family of anion channels, the Formate Nitrite Transport (FNT) family. This family has proven amenable to structural studies at a resolution high enough to reveal intimate details of ion selectivity and gating. With five representative members having yielded a total of 15 crystal structures, this family represents one of the richest sources of structural information for anion channels.

Introduction

Cellular respiration consists of a series of reduction/oxidation (redox) reactions, which ultimately generate the proton-motive force necessary for energetic processes such as ATP synthesis to occur. The conserved process of aerobic respiration taking place in the mitochondria of eukaryotes uses oxygen as the sole terminal electron acceptor. In contrast, prokaryotic organisms perform respiration using a wide range of terminal electron acceptors including carbon dioxide, oxidized sulfur and nitrogen compounds [1,2] and the concentration of the reactants and products of these metabolic processes are highly regulated by a number of different enzymes, transporters and ion channels. Under anaerobic conditions, a significant accumulation of formate ions (HCOO^-) arises from glucose mixed-acid fermentation (Figure 1a) [3,4]. Similar to formate, the anions nitrite (NO_2^-) and hydrosulfide (HS^-) are produced via the reduction of nitrate and the dissociation of hydrogen sulfide, respectively. While potentially detrimental at high concentrations [5], the

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often useful redox potential of these ions is tightly regulated by the anaerobically respiring cell.

The intracellular concentrations of HCOO^- , NO_2^- and HS^- are largely regulated via direct transport across the cellular membrane through formate nitrite transport (FNT) channels [6,7]. This family of proteins is further divided into at least three subfamilies: the formate channels (FocA) [6], the nitrite channels (NirC) [8] and the hydrosulfide channels (HSC) (Figure 1b) [9]. In addition to those found in prokaryotes, FNT family members have been discovered in eukaryotes, specifically fungi, in which they seem to play a role in nitrite and nitrate transport [10].

FNT proteins are also believed to play an important role in pathogen-host interactions. The nitrite channel NirC from *Salmonella typhimurium* has recently been implicated as a potential pathogenesis factor, with ΔnirC strains showing significantly decreased levels of virulence in mouse models [11]. Additionally, the newly-discovered hydrosulfide channel HSC from *Clostridium difficile* [9] identified a long-sought after anion channel [12–15] that was first suggested by the iron-sulphur theory of the origin of life [16]. Recent progress in the structural, biochemical and biophysical analysis of this family of anion channels has provided considerable insight into their cellular function and transport mechanism.

Overall Structure

Over the past few years, crystal structures of five FNT proteins have been reported from the three subfamilies identified thus far (Figures 1–3): FocA from *E. coli*, *Vibrio cholera* and *S. typhimurium* [17–19], HSC from *C. difficile* [9] and NirC from *S. typhimurium* [20]. All of these structures are highly similar, showing a homopentamer, and the fold of each protomer resembles that of the tetrameric aquaporin and glyceroporin channels [21–24], likely indicating a common evolutionary history (Figure 1c–f). The five protomers interact tightly with one another, with $\sim 1,600 \text{ \AA}^2$ of buried surface area shared with each neighbor [17], yielding a stable pentamer in agreement with biochemical observations [18,25]. The protomers are each composed of six tilted transmembrane α -helices forming a right-handed bundle that surrounds a 15- $\text{\AA}$ long narrow pore, which is believed to be the ion permeation pathway (Figure 2). The N-terminal (TMs1–3) and C-terminal halves (TMs4–6) of the bundle are related by a pseudo two-fold symmetry axis parallel to the membrane plane. In addition, helices TM2 and TM5 are broken in the middle of the membrane into 2a/2b and 5a/5b, and the loop connecting TM2a and TM2b is termed the Ω loop [18].

As a result of structural conservation, the essential aspects of the pore in the center of the FNT protomer are very similar among the five FocA, HSC and NirC proteins [9,17–20]. In all structures, the proposed translocation pathway is defined by a narrow central pore region that opens into a funnel on both the cytoplasmic and periplasmic sides (Figure 2e). From inside the cell, the cytoplasmic funnel gradually narrows to a slit of $\sim 2 \times 4 \text{ \AA}$, which is also the site of a highly-conserved histidine residue (vcFocA:H208) in the loop between TM5a and TM5b in the membrane region. From the central His residue, the pore then passes a $\sim 2.3 \text{ \AA}$ periplasmic constriction ring composed of four conserved residues before finally opening up into the periplasmic funnel and the extracellular environment.

Variations between the FNT structures are found in the position of the TM2b helix and the Ω -loop relative to the ion conducting pore (Figure 3). In the three FocA proteins, the region encompassing the TM2b helix and the connecting loop of TM3 is highly flexible as evidenced by the high crystallographic B-factors and disordered loop segments in the refined models [17–19]. This labile region forms the outer perimeter of the cytoplasmic funnel and contains an absolutely conserved threonine residue (vcFocA:T90) in the Ω loop [18]. In each protomer of the *ecFocA* structure, the TM2b helix is in the same position in the

middle of the membrane lining the pore (named the UP position), while the conserved threonine residue makes a hydrogen bond with the central histidine residue [17]. Because access to the histidine in cytoplasmic slit is blocked from the cytosol, this structure was proposed to represent a closed state [18]. In contrast, while in three of the protomers the *vcFocA* structure TM2b is found in the same UP position as in *ecFocA*, in the other two protomers (protomers D and E) TM2b along with the Ω loop is moved in the direction of the cytoplasm by ~ 5 Å (DOWN position) [18]. The DOWN position removes the threonine from the slit and breaks its interaction with the central histidine, and critically allows for contiguous opening from the narrow pore region into the cytosol, yielding an open state [18]. In the *sfFocA* structure, the position of TM2b and the Ω -loop also varies between protomers, and their position correlates with the rearrangements of an N-terminal helix [19]. However, any assignment of closed/open states should be regarded as tentative at this point. As the field is new, more structural and activity experiments are needed before the conducting states of the FNT proteins can be fully defined.

The structures of *cdHSC* and the *snirC* also vary from those of *vcFocA* by the presence of a ~ 20 residue α -helix of the cytosolic N-terminus along one side of the intracellular funnel (Figure 3) [9,20]. This helix is suggested to provide support for the TM2b helix in the UP position helping to keep the channel closed [9].

Ion selectivity

FNT channels are permeable to monovalent anions. Although each subfamily is named after its own predominant anion, strict selectivity does not appear to be a feature of the FNT family. Competition assays and electrophysiological measurements have shown that FNT channels are polyspecific, permeating small, monovalent anionic ions. Both *cdHSC* and *snirC* are permeable to formate [9,20], and *sfFocA* is able to conduct additional ions, including larger organic anions such as lactate and acetate [26]. However, FocA channels do not permeate cations or multivalent anions. In contrast to the high specificity displayed by many cation channels, univalent polyspecificity is often observed for anion channels [27–29] and is probably due to selection based on the diffuse anionic electron clouds rather than the point-like cationic charges [9,30].

The 15 Å-long narrow pore in the center of each protomer encompasses two constriction sites and is the most attractive candidate for the selectivity filter (Figure 1e) [17,18]. The 2.3 Å periplasmic constriction ring has been hypothesized to prevent the passage of water and hydrated ions into the cell, and to possibly impose a size limit on translocating ions [9]. However the cytoplasmic constriction site, termed the cytoplasmic slit, is the location of a membrane buried histidine residue which represents the only charged side chain throughout the pore and implicates this site as a critical element of the selectivity filter. The combination of size limitation by the selectivity filter and the specific interaction observed between the translocating formate ion with the histidine residues [18] provides an attractive mechanism for ion selectivity of the FocA channel.

In the *vcFocA* structures, crystallization with 120 mM formate shows a formate ion (FMT2) located directly at the cytoplasmic slit in the DOWN conformation (putative open state) (Figures 3b & 4) [18]. At present, this represents the sole observation of a coordinated permeating anion for the FNT family. This formate is oriented endlong to the pore and forms a bidentate hydrogen bond with the N_ϵ of the central histidine (*vcFocA*:H208). This ionizable histidine residue is located in the low dielectric of the membrane and represents a single polar side chain in an otherwise hydrophobic narrow extended pore. The positioning of FMT2 by the histidine residue at the cytoplasmic slit is reminiscent of a coin in a slot, except that the coin is unable to rotate [18]. Mutation of this histidine eliminates

conductance [26], in agreement with its importance for the selectivity towards monovalent anions. Thus, the selectivity for the FNT family appears to arise from surface electrostatics, geometric constraints and interaction with the central histidine.

The pentameric organization is a general property of FNT proteins, which is reminiscent of the tetrameric aquaporins (Figure 1). For the aquaporin family member, GlpF, the monomeric 6-helix bundle alone has been shown to have poorer conductivity than the tetrameric species [31], and such oligomerization is often required to achieve the rigidity needed for the selectivity filter of ion channels [32]. At the same time, it is expected that the mechanism of interactions for the other permeating ions of the FNT family will be similar to that seen for *vcFocA* despite the differences in preference for permeating ions. Observed differences in permeability for various ions between the FNT family members are probably due to small structural variations in the selectivity filter, particularly at the cytoplasmic slit.

Gating mechanisms

In the above model derived from various protomer structures of *vcFocA* [18], channel gating is realized by limiting access to the central histidine, a function likely provided by the TM2b helix and the Ω -loop and supported to varying degrees by the rigidity of the N-terminal helix (Figure 4). In TM2b conformations congruous to the “UP” conformation in the cytosolic funnel, access to the central histidine is blocked, representing a closed pore. In other observed conformations, TM2b is more flexible and represents an intermediate probability, with the “DOWN” conformation of *vcFocA* representing a completely accessible histidine and an open pore. Blocking of translocation from the cytosolic side resembles what has been observed for the aquaporins, where a plug blocks the pore from the cytosolic side [33]. Whereas the location of the blockage is similar among different aquaporins, the plug itself varies and the mechanism that controls its movement changes. In the spinach aquaporin SoPIP2, for example, the plug consists of a long loop between TM4 and TM5 and its movement is controlled by amino acid phosphorylation or deprotonation [33]. Similarly, in the mechanosensitive aquaporin Aqp1 from yeast, channel closure is achieved by an N-terminal helix plug from the cytoplasm, whereby a tyrosine residue occludes the entrance to the selectivity filter [34].

What controls the opening of an FNT channel? A number of gating mechanisms may exist that affect the open probability of an ion channel, and evidence for several variations has been explored functionally and structurally for the FNT family, including gating by cytosolic formate concentration and by pH. The observation, in the high-formate *vcFocA* structure, of specific interactions between the central histidine and a permeating formate, which replaces the previously interacting threonine in the UP configuration, led to a proposal of *FocA* gating being controlled by the cytosolic formate ions (Figure 4) [18]. In addition, electrophysiological experiments have identified a ‘fast gating’ mode of transport for *stFocA*, indicative of a single flexible side chain in the transport pathway [26]. Such a simple gating mechanism could allow the cell to maintain a reducing environment in the cytoplasm under anaerobic conditions by keeping the channel closed when the formate concentration is low.

Another gating mechanism described in the literature is gating by pH. In fermenting *E. coli* cultures, formate initially accumulates in the culture medium, with levels diminishing after the media reaches a pH of 6.8 [6]. This suggests that *FocA* could be stimulated by low pH to provide reuptake of formate for reduction in the cytoplasm by an intracellular reductase. Based on structural and electrophysiological characterization of *FocA* from *Salmonella typhimurium*, a pH gating mechanism was proposed (Figure 3c) [19]. *stFocA* was crystallized at a pH of 4.0, and ~10 residues at the N-terminus in various protomers are

found to adopt different conformations in the cytosolic funnel. Two protomers are described as “closed”, and show a partially occluded intracellular funnel that still maintains a large continuous pathway from the cytoplasm to the central histidine. Electrophysiology experiments show attenuated formate charge movement at acidic ranges that drops steeply at pH 5.6, which is interpreted as pH-dependent closing of the channel. It was therefore proposed that the N-terminal conformations seen in the *stFocA* structure represent relevant conformational changes responsible for pH gating in the *stFocA* channel [19]. However, under mixed-acid fermentation conditions the pH decreases significantly, and more undissociated formic acid ($pK_a = 3.8$) will consequently become available. Since formate and other organic acids exhibit high permeation coefficients, such circumstances could result in free diffusion through the lipid bilayer making this the dominant mode of reuptake back into the cell [9,35].

It is unclear how *stNirC* or *cdHSC* could be gated [36]. The electrophysiological studies of *stNirC* did not show a pH-dependent current [20], and the well-structured N-terminal helix of both *stNirC* and *cdHSC* at various pHs suggests that these proteins may not be gated by pH. The *cdHSC* protein structure was solved under the varied pH conditions of 9.0, 7.5 and 4.5, and it exhibits a nearly identical structure at each pH (Figure 3d) [9]. Mutations designed to disrupt glutamate salt bridges did not result in an altered pore diameter at the cytoplasmic slit region. Importantly, mutations of these residues also did not increase the *cdHSC* channel permeability as measured by concentrative uptake assays, which suggests that the glutamate salt bridge interactions in the pore are not likely to be responsible for the modulation of channel gating. These results suggest that yet another mechanism exists for FNT proteins to gate or regulate the cytoplasmic entrance of the ions [37].

Conclusions

Up until recently it was uncertain whether FNT proteins could be classified as transporters or channels [6,8,24]. While the surmounting biochemical and biophysical evidence has begun to reveal that FocA, NirC and HSC are likely to be channels, there is experimental evidence that some FNT proteins may operate as transporters [38], which is reminiscent of the ClC chloride channel/transporter family [39,40]. It would be interesting to explore the fine details of ion passage through these channels with electrophysiological measurements in conjunction with mutagenesis experiments and meaningful molecular dynamics simulations [41] and to integrate such data into our current framework of structural information.

The structural and biochemical characterization of the FNT channel family has yielded a unique mechanism of monovalent anion permeation through biological membranes. The pentameric variation on the fold of the aquaporins has been a startling discovery [24], and it elucidates a remarkable evolutionary process of ion selectivity via oligomerization geometry. Several key mechanistic questions remain for the FNT family including pH gating and proton coupling mechanisms. Using the consolidated available data, this review attempts to provide a comprehensive framework for the general mechanism by which members of the FNT family operate, the more precise details of which will require further investigation.

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Highlights

Formate-nitrite channel (FNT) family is a novel group of channels for monovalent anions

FNT channels are polyselective and there is much selectivity overlap between the three channel subfamilies

FNT proteins have an aquaporin fold but form a pentamer, with the ion conducting pore being located at the center of a monomer

Selectivity of FNT is achieved by size restriction and by specific interaction between the permeating ion and the selectivity filter

There are probably several mechanisms of gating for various FNT channels

Figure 1.

Formate nitrite transport family of anion channels. (a) FNT members are central to anaerobic membrane bioenergetics. FocA regulates formate levels and NirC controls nitrite availability (along with the nitrate/nitrite transporters NarU and NarK) to membrane bound reductases (FDH-O,N,H, and NirBD), thus conserving the reducing power in the membrane quinone pool. Furthermore, HSC provides a crucial pathway for hydrosulfide efflux generated during sulfite assimilation and under sulfur rich anaerobic conditions. (b) Phylogenetic tree of 474 bacterial and archaeal members of the FNT family. Branches are colorized based on genetic linkage to metabolic enzymes: *FocA* genes are linked to pyruvate formate lyase (*pflAB*) or formate dehydrogenase (*fdhAB*) and are colored pink, *NirC* genes are linked to nitrite reductase (*nirBD*) and are colored blue, and *HSC* genes are linked to sulfite reductase (*asrABC*) and are colored green. The FocA protein in archaea is encoded by the *fdhC* gene. The gray areas represent FNT family members with no assigned function based on genetic linkage. (c) Pentameric structure of FocA from *Vibrio cholerae* (PDB ID: 3KLY) [18]. (d) Tetrameric structure of bovine AQP1 water channel (PDB ID: 1J4N) [23]. (e) Superposition of FocA and AQP1 protomers, top view from the extracellular side. (f) Superposition of FocA and AQP1 protomers, side view from within the membrane. The r.m.s.d for α -carbon between FocA and AQP1 protomers is 7 Å for the 212 amino acids in the middle of the sequence.

Figure 2.

FNT protein fold and topology. Overall structure and transmembrane topology of FocA protomer in the low-formate crystal form (PDB ID: 3KLY), represented by Protomer A. Transmembrane α -helices are numbered sequentially from TM1 to TM6. Helices in the N- (TMs1–3) and C- (TMs4–6) terminal halves are colored in red and orange, respectively, highlighting the structurally related inverted repeats. (a) Top view from the periplasmic side. (b) Side view from within the membrane plane along the pseudo-twofold axis. (c) Side view from within the membrane plane as in (b) and rotated 90° counterclockwise along the membrane normal. (d) Topology diagram with sequence colored as described above. Helices TM2 and TM5 are broken in the middle of the membrane. The segment that connects TM2b and TM3 is designated as the Ω loop. The positions of two key residues, T90 and H208, are indicated. (e) The pore structure in the middle of a *vcFocA* protomer in the UP conformation, superimposed with the pore structure calculated by HOLE [42]. The green section of the pore represents the putative ion selectivity filter, in which the central restriction ring and the cytoplasmic slit are indicated. The protomer structure and transmembrane topology of other known FNT proteins, including those of *sdHSC* and *sNirC*, are similar to *vcFocA* as shown here.

Figure 3.

Pore structure and the electrostatic properties of various FNT proteins. **(a)** FocA from *E. coli* (PDB ID 3KCU) [17]. Instead of forming a hydrogen bond with the central histidine H209, the threonine residue from the Ω loop forms a hydrogen bond with N172. **(b)** FocA from *V. cholerae* (PDB ID 3KLY). Orange structure represents UP conformation and purple overlay represents DOWN conformation. The surface representation is of the DOWN conformation [18]. **(c)** FocA from *S. typhimurium* (PDB ID 3Q7K). Orange structure represents the "CLOSED" conformation and purple structure represents the "INTERMEDIATE" conformation. The surface representation is of the CLOSED conformation [19]. **(d)** HSC from *C. difficile* (PDB ID 3TDO) [9]. **(e)** NirC from *S. typhimurium* (PDB ID 4FC4)[20]. In (a — e), TMs 4 and 6 have been removed for clarity. All of the known FNT protein structures are similar. The surface electrostatics were calculated with APBS [43].

Figure 4.

Ion recognition by the selectivity filter in *vcFocA*. (a) Detailed view of a proposed closed pore structure (Protomer A), with TM2b and the Ω loop in the UP position. (b) Detailed view of the selectivity filter in a proposed closed pore. In this UP configuration, T90 of the Ω loop makes a hydrogen bond with the central histidine H208. (c) Schematic drawing showing the proposed ion selectivity filter in a closed state. (d) Detailed view of the pore structure (Protomer E), with TM2b and the Ω loop in the DOWN position. (e) Detailed view of the selectivity filter in a proposed open pore. In this DOWN configuration, the Ω loop moves toward the cytoplasmic direction and the hydrogen bond between T90 and H208 is broken. Two formate ions are resolved at the entrance to the pore. Formate ion number 2 (FMT2) forms a bidentate hydrogen bond with H208, an interaction believed to be critical for ion selection. (f) Schematic drawing showing the *vcFocA* ion selectivity filter in a proposed open state.