Membrane Transport

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The Tricky Task of Nitrate/Nitrite Antiport**

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Microorganisms often show substantial metabolic diversity to cope with fundamental changes in energy sources and nutrients. This is particularly critical for the essential element nitrogen, whose availability is commonly a growth-limiting factor.[1] In its fully reduced form, NH_4^+, nitrogen can be assimilated into amino acids for biosynthetic purposes, and a network of redox reactions serves to interconvert different modifications of the element, preserving free enthalpy in a proton motive force that drives adenosine triphosphate (ATP) synthesis.[2]

The enteric bacterium *Escherichia coli*, a laboratory paradigm and common inhabitant of the mammalian gut, contains three different nitrate reductases (Nars) able to reduce nitrate to nitrite, and two nitrite reductases (Nirs) converting nitrite to bioavailable ammonium (Figure 1).[3] Nars and Nirs are large enzymes containing intricate metal centers, and their arrangement in or around the cytoplasmic membrane raises a functional issue. The enzymes are located in the cytoplasm, as the reaction consumes protons (by H_2O formation) and thus contributes to the generation of proton motive force. However, their product, nitrite, is a potent cytotoxin that must not accumulate inside the cell. *E. coli* uses a constitutive housekeeping enzyme, NarZYW, and switches to the high-level expression of a second enzyme, NarGHI, for respiratory nitrate reduction. Since the ionic species NO_3^-, NO_2^-, and NH_4^+ cannot permeate biological membranes, dedicated transport systems are required. These have to be highly selective to prevent the passage of water and protons and yet be able to transport ions even against a concentration gradient. This is achieved by the nitrate/nitrite exchangers NarK (for NarGHI) and NarU (for NarZYW; Figure 2), members of the nitrate/nitrite porter (NNP) family within the major facilitator superfamily (MFS) of transporters, by the NO_2^- channel NirC, a member of the formate/nitrite transporter family, and by the NH_4^+ transporters of the Amt/Rh family.[3,4]

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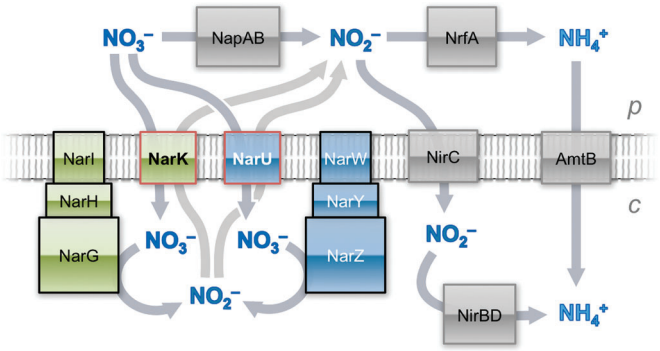

Figure 1. Nitrogen metabolism in *Escherichia coli*. Nitrate reductases NarGHI and NarZYW are oriented towards the cytoplasm. Nitrate is imported and the toxic product, nitrite, is exported by the exchangers NarK and NarU. The periplasmic nitrate and nitrite reductases NapAB and NrfA serve in detoxification, while the NH_4^+ transporter AmtB and the cytoplasmic nitrite reductase NirBD, with the corresponding nitrite channel NirC, are required for nitrogen assimilation for biosyntheses.

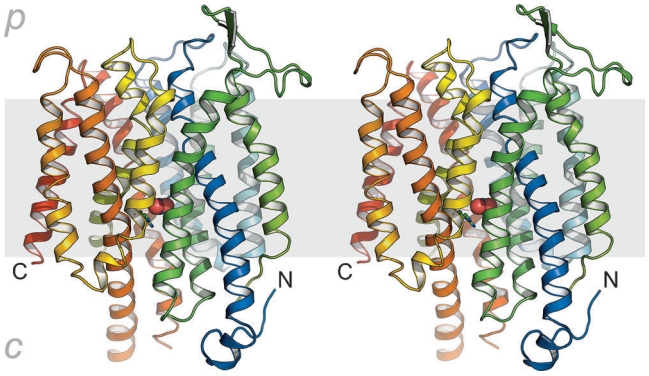

Figure 2. Three-dimensional structure of the nitrate/nitrite antiporter NarU from *E. coli* (PDB ID: 4IU8).[5] The stereo image shows a ribbon representation colored blue at the N-terminus and red at the C-terminus. NarU operates by a statistical "rocker-switch" mechanism, where a binding site for the cargo molecules nitrate and nitrite is alternatingly exposed to either side of the membrane. *p* denotes the periplasm, the "out" side, while *c* stands for the cytoplasm, the "in" side.

A wealth of information has been gathered on Amt proteins[4] and on NirC,[6] but until recently no structural information was available for a nitrate/nitrite exchanger. This has changed with the recent crystal structures of NarU[5] and

NarK[7] from *E. coli* which were determined independently by the Shi and Gonen groups, respectively. Interestingly, the structure of NarK was solved with bound nitrite, while that of NarU contained nitrate. These combined results can be analyzed to determine how the transporters achieve their most outstanding capability: the reliable distinction between two cargo molecules as similar as NO_3^- and NO_2^-.

Nitrate/nitrite exchangers are typical MFS proteins, in that an inverted repeat of five transmembrane α-helices creates two domains connected by a pseudo-twofold rotation axis in the membrane plane (Figure 3 A). Transport involves

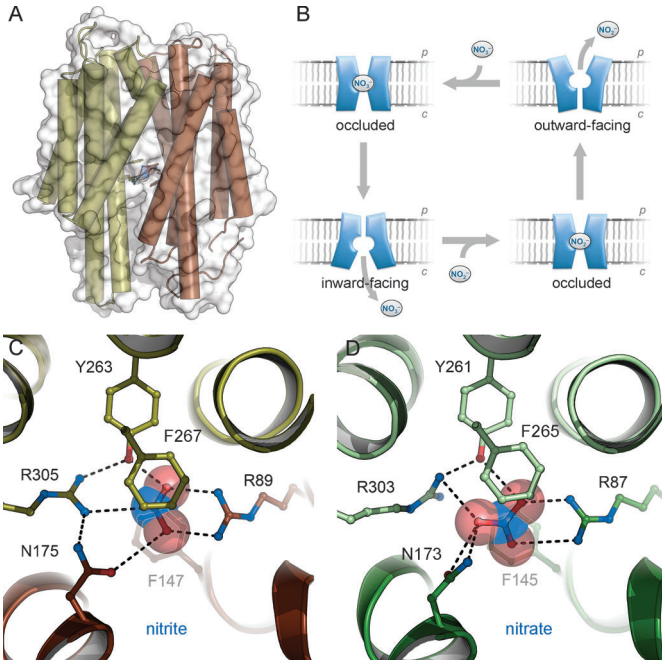

Figure 3. Transport mechanism of nitrate/nitrite exchangers. A) *E. coli* NarK, like many other transporters, is constructed from two homologous inverted repeats. B) The alternating-access ("rocker-switch") model of membrane transport is based on a conformational change of two rigid half-channels. C) The NarK structure, obtained from co-crystals with an antibody F_{ab} fragment and a nitrite molecule bound. A short (2.9 Å) hydrogen bond connects N175 to R305 from the two halves of the protein. D) Binding of nitrate in the structure of NarU. The additional oxygen atom of NO_3^- interferes with the surrounding ligands and breaks the hydrogen bond between R303 and N173.

a relative movement of the two halves of the protein which exposes a central binding site for the cargo molecule either on the "out" side (the bacterial periplasm) or on the "in" side (the cytoplasm). These conformations are connected through "occluded" states in which the anion is locked within the transporter. Figure 3 B shows the simplest scheme for such an alternating-access principle, known as the "rocker-switch" mechanism. Notably, in order to function as strict nitrate/nitrite exchangers and exclude the uniport of either ion, NarK/U must not allow a transition from the outward-facing to the inward-facing state without a bound ion. NO_3^- and NO_2^- are equal in charge and differ merely by one oxygen atom. It was difficult to envision how a binding site that specifically accommodates nitrate can distinguish the smaller nitrite molecule. The structures of NarK and NarU now provide the answer to this question in striking clarity. Key to ion binding in the NarK/U proteins are two arginine residues that form part of a conserved nitrate signature motif. In the nitrite-bound structure of NarK (Figure 3 C), two arginine residues, R89 and R305, form short hydrogen bonds to the anion that resides in a cavity formed by the aromatic side chains of F147 and F267. Two other relevant residues in this binding pocket are Y263 and N175. Of the six amino acid side chains surrounding the ion, three belong to one half of the transporter (R89, F147, N175), and three to the other half (F267, Y263, R305). Nitrite binding facilitates the formation of a hydrogen bond from N175 to R305 that connects the two halves of NarK. In the highly homologous NarU, a nitrate molecule fits into the very same binding pocket, and it can only do so by displacing some of the ligands in order to accommodate its additional oxygen atom. Indeed, while the interactions with one arginine (R87 in NarU) and with tyrosine Y261 remain unchanged, the oxygen atom breaks the H-bonding interaction between R303 and N173. The solution to specifically distinguishing nitrate from nitrite thus lies not in a rigid binding pocket, but rather in a flexible one that rearranges to fit the ions precisely. The region surrounding the binding pocket forms the pivot point around which the transporter swings from one state to another, and it is well conceivable that the breaking of a single hydrogen bond, corresponding to an enthalpy change of approximately 20 kJ mol^{-1}, might be the deciding factor.

Although the structures of NarK and NarU combine well to describe ion binding in a central pocket, they do not represent the same protein, and—more importantly—both show an inward-facing state. Crystals of NarU contained two monomers in the asymmetric unit that differed in structure, with the second showing a substrate-bound occluded state. Binding affinities determined by isothermal titration calorimetry were ten times higher for nitrate ($K_D = 33$ μM) than for nitrite ($K_D = 373$ μM). While this corresponds to the finding that nitrate bound to NarU while nitrite did not, one might expect that the conformation with high affinity for nitrate would be the one facing outward, not inward. The structure of NarK, on the other hand, was obtained in complex with the F_{ab} fragment of a monoclonal antibody to assist crystallization. It shows an inward-facing state as well, but the strong interaction of transporter and antibody may limit the conformational flexibility of NarK. Questions concerning the mechanism of the NNP family of nitrate/nitrite exchangers thus remain unanswered, and it will be of particular importance crystallize the remaining conformational states of NarK and NarU to assess the extent of rearrangements that take place. Although structures of MFS transporters in different conformations are available,[8] the structural variability within the superfamily forbids drawing general conclusions without addressing other family members individually.

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