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REVIEW

The Sodium Cycle in *Vibrio cholerae*: Riddles in the Dark

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Abstract—Twenty years ago, V. P. Skulachev put forward the revolutionary concept of the chemiosmotic sodium cycle which is an integral part of the paradigm of modern bioenergetics. This fundamental concept stimulated studies in many areas and yielded plenty of sometimes quite unexpected (and thus most valuable) discoveries. In particular, variations of the sodium cycle have been found in a surprisingly large number of pathogenic microorganisms, raising the question about the possible link of sodium energetics and virulence. This brief review discusses some paradoxes related to the Na^+ cycle in an important human pathogen, *Vibrio cholerae*.

Key words: sodium cycle, *Vibrio cholerae*, oxidative phosphorylation, Na^+/H^+ antiport, NhaD, arsenate resistance

"Does it guess easy? It must have a competition with us, my precious! If precious asks, and it doesn't answer, we eats it, my precious. If it asks us, and we doesn't answer, then we does what it wants, eh? We shows it the way out, yes!"

J. R. R. Tolkien "The Hobbit or There and Back Again"

In 1984, V. P. Skulachev put forward the revolutionary concept of a sodium cycle in membrane energetics [1], which is now widely accepted as a natural generalization of Mitchellian proton cycle. Recognition of Na^+ as a "twin" coupling ion, which complements or, in some cases, substitutes for proton in the reactions of energy conversion in biomembranes, reshaped many areas of modern bioenergetics. It opened new avenues of research and yielded plenty of (sometimes quite unexpected and thus most valuable) discoveries. In this brief review, I shall discuss some paradoxes related to the Na^+ cycle in an important human pathogen, *Vibrio cholerae*, and recent developments in the field.

In our 2001 review [2], we attempted to summarize the current state of knowledge about the role of the Na^+ cycle in pathogenic microorganisms. It turned out that the transmembrane Na^+ circulation plays a significant role in the physiology of very different pathogens, from the obligate intracellular parasite *Chlamydia* to *V. cholerae*, a common component of some aquatic ecosystems. The latter case is especially interesting. Almost 20 years ago, V. P. Skulachev hypothesized that *V. cholerae* possessed a Na^+ cycle that plays a key role in the colonization of the small intestine [3]. The hypothesis implied that the cholerae toxin-induced $[Na^+]$ increase in the intestinal lumen is in fact needed to maintain the *V. cholerae* sodium cycle operative in the relatively alkaline intestinal environment where the "classic" proton cycle is less effective [3]. In accordance with this hypothesis, the cell membrane of *V. cholerae* contains a primary Na^+ pump, Na^+-translocating NADH:ubiquinone oxidoreductase (NQR), a battery of Na^+/H^+ antiporters, and various consumers of the sodium motive force (smf), such as the Na^+-dependent flagellar motor, Na^+-dependent multidrug efflux pump, as well as Na^+ symporters for alanine, glutamate, proline, serine, citrate, and inorganic phosphate. Some of these constituents of the Na^+ cycle have been characterized experimentally (including NQR, two of at least six Na^+/H^+ antiporters, Na^+-P_i symporter of the NtpA-type), while others are still just putative proteins whose genes are identified in the *V. cholerae* genome. Furthermore, an apparent influence of Na^+ cir-

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culation on the expression of virulence factors in *V. cholerae* was reported in 1999 by Häse and Mekalanos [4]. However, despite progress made in cataloging and primary characterization of the components of Na^+ cycle in *V. cholerae*, Na^+ circulation in this bacterium remains a bottomless source of riddles and paradoxes.

1. The riddle of oxidative phosphorylation. The general bioenergetic question here is the one concerning the nature of coupling ion (H^+ and/or Na^+) energizing oxidative phosphorylation. The issue of energy requirements of the oxidative phosphorylation in halotolerant *Vibrio* species is still somewhat controversial. Previous studies suggested that the free-living marine bacterium *V. alginolyticus* can use the smf to energize ATP synthesis [5]. Na^+-coupled ATP synthesis driven by respiration or an artificial sodium ion gradient has been also reported in closely related *V. parahaemolyticus* [6, 7]. In such organisms as *Propionigenum modestum* and *Acetobacterium woodii*, F_oF_1-type ATPases were shown to transport Na^+ [6-8], which led to the suggestion that the vibrional enzyme, too, might be Na^+-translocating [9].

However, the sequence of the *c* subunit of *V. cholerae* F_oF_1-ATPase suggests that this enzyme should translocate H^+ ions. More specifically, it lacks the characteristic Na^+-binding motif $Px_3Qx_{32}ET$, which is present in *c* subunit of all sodium-motive ATPases of F_oF_1-type experimentally studied so far. Essentially the same motif ($Sx_3Qx_{28}ET$) could be identified in the K subunit of A/V-type Na^+ ATPases [10]. Indeed, we found that the oxidative phosphorylation in *V. cholerae* is mediated by a H^+-translocating, not Na^+-translocating F_oF_1-ATPase [10]. In particular, we showed that irrespective of the presence of Na^+ ions, ATP hydrolysis by inside-out membrane vesicles of *V. cholerae* results in generation of both transmembrane pH gradient (ΔpH, acid inside the vesicles) and membrane electric potential ($\Delta\psi$, positive inside). The protonophore CCCP completely collapsed both ATP-dependent $\Delta\psi$ and ΔpH. *V. cholerae atpE* mutants with deleted subunit *c* of the F_oF_1-ATPase were unable to use succinate or glycerol as sole carbon and energy source. Vesicles isolated from mutant cells were unable to generate either $\Delta\psi$ or ΔpH at the expense of ATP hydrolysis.

One can ague that an alternative Na^+-ATP synthase possibly exists in *V. cholerae*, an enzyme which could be repressed under the growth conditions used in our work. However, the analysis of the *V. cholerae* genome seemingly does not support such a possibility. There is only one *atp* operon encoding the F_oF_1-type ATPase. In addition, the genome of *V. cholerae* contains three open reading frames (VC1033, VC1437, and VC2215) encoding P-type cation transport ATPases (E_1-E_2 ATPases). Judging by the sequence similarity of VC1033 to the *E. coli* Zn^{2+} transporter ZntA, and VC1437 as well as VC2215 to the *E. coli* Cu^+ transporter CopA, they most probably transport divalent cations, which makes their involvement in oxidative phosphorylation highly unlikely. An inducible, two-gene ABC-type system extruding Na^+ ions, NatAB, has been reported in *Bacillus subtilis* [11]. This transport system supposedly expels toxic Na^+ from the cytoplasm and stimulates K^+ uptake when the barrier function of cytoplasmic membrane is affected by uncouplers or alcohols [11]. A number of genes encoding putative ABC-type transporters can be found in the *V. cholerae* genome, but neither of them shows significant similarity to the bacillar *natAB* genes. Therefore, it seems that the *V. cholerae* genome does not contain an operon encoding putative Na^+ ATPase which could mediate oxidative phosphorylation, and the membrane energetics of *V. cholerae* is essentially "dualistic", relying on both proton motive and sodium motive force. Why *V. cholerae* disobeys the "molecular logic" of the *complete* Na^+ cycle (including, by definition, the Na^+ dependent oxidative phosphorylation) remains mysterious.

2. The riddle of the "Silver Bullet". The antibacterial effects of silver salts have been noticed since ancient times and today silver is used to control bacterial growth in a variety of applications, including dental work, catheters, and burn wounds. Added at millimolar concentrations, Ag^+ inhibits a number of enzymatic activities, reacting with electron donor groups, especially sulfhydryl groups [12]. However, the molecular mechanism of the bactericidal action of low (sub-micromolar to micromolar) concentrations of Ag^+ has not been elucidated. The Na^+-translocating NADH:ubiquinone oxidoreductase (NQR) has been recognized as one of the primary targets for Ag^+ in some bacteria. In two independent studies, sub-micromolar concentrations of Ag^+ were shown to inhibit energy-dependent Na^+ transport in inside-out vesicles of alkalophilic *Bacillus* sp. FTU [13] and to inhibit purified NQR of *Vibrio alginolyticus* [14]. Later, in experiments with purified protein, Ag^+ was shown to irreversibly bind to the β-subunit of NQR (NqrF or Nqr6), causing enzyme denaturation and the loss of its FAD cofactor (see [15] for review). These observations suggested that the specific binding to NQR may be responsible for the bactericidal effect of low concentrations of Ag^+. However, no evidence that NQR can serve as a target for the bactericidal action of Ag^+ *in vivo* has been presented.

Experimenting with *V. cholerae*, we found that the NQR enzyme is not crucial for the survival of *V. cholerae*. Indeed, mutants of the *V. cholerae* wild-type strain O395N1 carrying a transposon insertion or a deletion of the entire *nqr* operon were able to grow in LB and in mineral media supplemented with glucose at neutral pH [16]. Nevertheless, growth in all three strains was completely arrested by 1.25 μM $AgNO_3$ added to the minimal growth medium. Therefore, the action of silver on growth of *V. cholerae* could not be attributed to the specific binding to NQR.

In a hallmark study published in *Nature* 50 years ago, the uncoupler-like effects (stimulation of respiration and

adenosine triphosphatase activity) of micromolar concentrations of Ag^+ added to isolated mitochondria were documented by Chappell and Greville [17]. This was done well before P. Mitchell formulated the chemiosmotic hypothesis revealing the role of the proton motive force (pmf) in oxidative phosphorylation [18, 19]. In the context of Mitchell's concept, the observation by Chappell and Greville suggests an ability of Ag^+ to collapse the proton motive force on the membrane. Amazingly, no direct experimental evidence for the effect of Ag^+ on the proton motive force has been published since then. In 1982, Schreurs and Rosenberg mentioned (as an unpublished observation) that Ag^+ apparently collapses the proton motive force on the membrane [20]. However, neither the effective concentration of Ag^+ nor the experimental model used was specified in that communication.

To investigate this long-standing issue, we decided to measure directly the effect of Ag^+ on the proton motive force on the membrane of the wild-type and ΔNQR strains of *V. cholerae*. It turned out that the addition of sub-micromolar to low-micromolar concentrations of Ag^+ to inside-out membrane vesicles of either strain of *V. cholerae* induced a total collapse of the respiration-generated transmembrane pH gradient, ΔpH, as well as the membrane electric potential, Δψ, in the absence of added Na^+ [16]. These data clearly demonstrated that the Ag^+-modified membrane was leaky for protons and that loss of NQR did not alter the sensitivity of the mutant *V. cholerae* membrane to Ag^+ compared to the wild-type. Thus, in accordance with our growth experiments, the presence of NQR in the membrane was not required for the effect of Ag^+. Therefore, the bactericidal action of low concentrations of Ag^+ in *V. cholerae* is due to proton leakage through Ag^+-modified (probably, partially denatured) membrane protein(s), which results in complete de-energization of the membrane and inevitable cell death. The identity of the membrane protein targeted by micromolar silver remains mysterious. One could expect that such a protein (or proteins) should be common for both bacterial and mitochondrial coupling membranes.

3. Paradoxes related to Vc-NhaD, the specific Na^+/H^+ antiporter of *V. cholerae*. Vc-NhaD is a specific Na^+/H^+ antiporter from *V. cholerae* showing an unusual pH profile of activity with sharp maximum at pH 7.75 [21]. Vc-NhaD homologs are also present in pathogenic *V. vulnificus* and *V. parahaemolyticus* but not in very similar free-living *V. alginolyticus*, suggesting that it may be a "bioenergetic pathogenicity marker" in vibrios. Unexpectedly enough, chromosomal deletion of *VcnhaD* does not result in higher sensitivity of *V. cholerae* to Na^+ or Li^+; overall Na^+/H^+ antiport measured in inside-out membrane vesicles is unaffected by the deletion (J. Dzioba and P. Dibrov, unpublished). Moreover, experimental data collected in our laboratory over the last few years indicate that Vc-NhaD is most probably an electroneutral exchanger, which mediates import of Na^+ rather than its export at external pH > 8.0. For example, being introduced into the antiporter-less strain of *E. coli*, Vc-NhaD efficiently protects cells from external Li^+ only in acidic or neutral but not alkaline growth medium, despite the fact that the antiporter has its pH optimum close to 8.0 [21]. Thus, under the conditions when the proton motive force is represented by sole Δψ (and ΔpH on the membrane is of opposite orientation, i.e., more acidic inside the cell), Vc-NhaD is physiologically "silent". On the other hand, the ΔNhaD mutant of *V. cholerae* grows better in alkaline media supplemented with 200 mM Li^+ compared to the wild type parental strain; overexpression of Vc-NhaD from a multicopy plasmid clearly inhibits the growth under these conditions (J. Dzioba and P. Dibrov, unpublished). These observations can be easily explained assuming the electroneutral ion exchange via Vc-NhaD. This phenomenology is in sharp contrast to that of "regular" bacterial Na^+/H^+ antiporters, which are typically electrogenic (exchanging more than 1 H^+ per each Na^+) and are involved in the export of toxic Na^+ and Li^+ ions from the bacterial cytoplasm. It should be noted also that coexistence in the same membrane of an electrogenic Na^+/H^+ antiport system (represented in *V. cholerae* by NhaA and NhaB) and an electroneutral one (Vc-NhaD) could apparently result in complete dissipation of both pmf and smf. It seems, however, that the presence of primary Na^+ pump (NQR) and the difference in pH profiles of NhaA (which is shut off below pH 6.5 and reaches its maximal activity at pH 8.5, see [22]) and NhaD makes such arrangement quite possible.

A paradoxical mode of operation of Vc-NhaD may serve two physiological functions: (i) allowing the return of Na^+ into the cytoplasm at alkaline pH, where NQR is most active, Vc-NhaD maintains sufficiently high concentration of the substrate cation for actively operating the respiratory Na^+ pump; on the other hand, removing protons it prevents possible over acidification of the cytoplasm due to the activity of NhaA. In acidic media, where NQR (as well as the major electrogenic Na^+/H^+ antiporter, NhaA) is downregulated, Vc-NhaD substitutes for it, using the ΔpH of normal orientation to expel sodium ions from the cell. (ii) By changing intracellular $[Na^+]$, Vc-NhaD could modulate the expression of genes having "sodium-sensitive" promoters, perhaps including those encoding some virulence determinants. This possibility is currently under investigation in our laboratory.

Studying physiological consequences of the chromosomal deletion of Vc-NhaD, J. Dzioba in our laboratory recently found that mutant *V. cholerae* cells devoid of this antiporter (ΔNhaD) show surprisingly high resistance to millimolar concentrations of added arsenate compared to the wild type cells. While the aerobic growth of the wild type was completely arrested by 7.5 mM arsenate at pH below 7.6, the ΔNhaD strain showed robust growth (A_{600}

about 2.1-2.4 after overnight growth at 37°C). Introduction of the intact *nhaD* gene (under its natural promoter) on multicopy plasmid restored the arsenate sensitivity in ΔNhaD cells to the wild type level. However, when a number of mutant forms of *nhaD* (encoding inactive variants of the antiporter) were introduced into ΔNhaD cells, they failed to restore the wild type phenotype, indicating that activity of Vc-NhaD is required for the effect. Since arsenate is a structural analog of phosphate, these observations suggested an intriguing possibility of a modulation of the inorganic phosphate import in *V. cholerae* by Vc-NhaD. Indeed, the addition of phosphate to the arsenate-containing growth medium mimicked the ΔNhaD phenotype. Furthermore, direct measurements of $^{32}P_i$ import in the wild type and ΔNhaD cells confirmed that ΔNhaD cells have somewhat impaired P_i uptake (J. Dzioba and P. Dibrov, manuscript in preparation).

Genomic analysis provides additional support for the possible linkage of Vc-NhaD to phosphate/arsenate transport. Thus, the promoter region of Vc-*nhaD* gene contains the sequence motif AAGTCACAACTTCAAAT reminiscent of the *E. coli* Pho box consensus, a standard regulatory element characteristic for bacterial genes that form the phosphate regulon and are induced by binding of the transcriptional activator PhoB to the Pho box under conditions of phosphate deprivation [23, 24]. This is in agreement with the idea that Vc-NhaD is somehow linked to the phosphate metabolism in *V. cholerae*. In addition, the sequence comparison immediately reveals that Vc-NhaD belongs to the Cluster of Orthologous Groups of proteins COG1055 named "Na^+/H^+ antiporter NhaD and related arsenite permeases", sharing surprisingly high level of overall identity/similarity with ArsB, the major prokaryotic arsenite permease which by itself mediates electrogenic antiport of external protons with cytoplasmic arsenite or, in complex with ArsA, pumps out arsenite in an ATP-dependent manner [25]. We found, however, that the deletion of Vc-NhaD does not affect the resistance of *V. cholerae* to arsenite. Being functionally expressed in antiporter-less *E. coli*, Vc-NhaD displays Na^+/H^+ antiport in inside-out membrane vesicles, but, in contrast to ArsB, shows no arsenite/H^+ exchange (J. Dzioba and P. Dibrov, unpublished). The molecular mechanism of the modulation of phosphate transport by Vc-NhaD remains obscure at the moment. It seems unlikely that Vc-NhaD transports P_i by itself, because its expression in different strains of *E. coli* does not affect arsenate sensitivity (J. Dzioba and P. Dibrov, unpublished). Most probably, it interacts with a yet unidentified partner protein residing in the *V. cholerae* membrane to perform this function. We are currently trying to solve this puzzle.

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