

## Chemiosmotic Mechanism of Antimicrobial Activity of $\text{Ag}^+$ in *Vibrio cholerae*

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**Although the antimicrobial effects of silver salts were noticed long ago, the molecular mechanism of the bactericidal action of  $\text{Ag}^+$  in low concentrations has not been elucidated. Here, we show that low concentrations of  $\text{Ag}^+$  induce a massive proton leakage through the *Vibrio cholerae* membrane, which results in complete deenergization and, with a high degree of probability, cell death.**

The antibacterial effects of silver salts have been noticed since ancient times (for a review, see references 5, 11, and 12), and today, silver is used to control bacterial growth in a variety of applications, including dental work, catheters, and burn wounds. Added at high (i.e., millimolar) concentrations,  $\text{Ag}^+$  ions inhibit a number of enzymatic activities, reacting with electron donor groups, especially sulfhydryl groups (12). However, the molecular mechanism of the bactericidal effect of much lower, i.e., micromolar, concentrations of  $\text{Ag}^+$  ions remains somewhat controversial.

The  $\text{Na}^+$ -translocating NADH:ubiquinone oxidoreductase (NQR) has been recognized as one of the primary targets for  $\text{Ag}^+$  ions. In two independent studies, submicromolar concentrations of  $\text{Ag}^+$  ions were shown to inhibit energy-dependent  $\text{Na}^+$  transport in membrane vesicles of the NQR-possessing alkalophilic *Bacillus* sp. strain FTU (10) and to inhibit purified NQR of *Vibrio alginolyticus* (4). These observations suggested that the specific binding to NQR may be responsible for the bactericidal effect of low concentrations of  $\text{Ag}^+$ . However, we found that, like in *V. alginolyticus* (13), the NQR enzyme is not crucial for the survival of *Vibrio cholerae* (3). Indeed, a mutant of the *V. cholerae* wild-type strain O395N1, carrying a deletion of the entire *nqr* operon, was able to grow in Luria broth and in mineral medium supplemented with glucose at a neutral pH (data not shown). Nevertheless, growth in both strains was completely arrested by the addition of 1.25  $\mu\text{M}$   $\text{AgNO}_3$  to the minimal growth medium (Table 1). Therefore, the action of low concentrations of silver on the growth of *V. cholerae* could not be attributed to a specific binding to NQR.

In a study published almost 50 years ago, the uncoupler-like effects (stimulation of respiration and ATPase activity) of micromolar concentrations of  $\text{Ag}^+$  added to isolated mitochondria were documented by Chappell and Greville (2). This was done well before P. Mitchell formulated the chemiosmotic hypothesis revealing the role of the proton motive force in oxidative phosphorylation (7, 8). In the context of Mitchell's concept, the observation by Chappell and Greville suggests an

ability of  $\text{Ag}^+$  ions to collapse the proton motive force on the membrane. Paradoxically, to our knowledge, no direct experimental evidence for the effect of  $\text{Ag}^+$  on the proton motive force has been published since then. In 1982, Schreurs and Rosenberg mentioned (as an unpublished observation) that  $\text{Ag}^+$  collapses the proton motive force on the membrane (9). However, neither the effective concentration of  $\text{Ag}^+$  nor the experimental model used was specified in that communication.

To investigate this long-standing issue, we decided to measure directly the effect of  $\text{Ag}^+$  ions on the proton motive force on the membranes of the wild-type *V. cholerae* strain O395N1 (6) and its isogenic  $\Delta\text{NQR}$  derivative (1). The formation and dissipation of the respiration-generated transmembrane pH gradient ( $\Delta\text{pH}$ ) was measured by acridine orange (AO) fluorescence quenching and dequenching in inside-out membrane vesicles prepared from both strains. Vesicles were obtained as described in reference 14 with some modifications. Cells were grown aerobically in standard Luria broth medium at 37°C to mid-log phase, cooled in an ice bath for 30 min, and harvested by centrifugation. Cells were washed once and resuspended in buffer containing 10 mM MOPS (morpholinepropanesulfonic acid)-Tris (pH 7.5), 10% (wt/vol) glycerol, 0.2 M  $\text{K}_2\text{SO}_4$ , 25 mM  $\text{MgSO}_4$ , 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.2  $\mu\text{g}$  of pepstatin A/ml. The obtained suspension was passed twice through a French press at 1,000  $\text{kg}/\text{m}^2$ . After removal of the cell debris by low-speed centrifugation (25,000  $\times g$  for 10 min), vesicles were collected by ultracentrifugation (250,000  $\times g$  for 90 min) and washed once with and resuspended in the same buffer. Aliquots of vesicles were resuspended in the above-mentioned buffer supplemented with 0.5  $\mu\text{M}$  AO.

Here, we show that the addition of submicromolar to low micromolar concentrations of  $\text{Ag}^+$  to inside-out membrane vesicles of *V. cholerae* induced a total collapse of  $\Delta\text{pH}$  irrespective of the presence of NQR in the membrane (Fig. 1A). Preincubation with  $\text{Ag}^+$  completely prevented the formation of the  $\Delta\text{pH}$  in vesicles (Fig. 2B). It is worth noting that, in the presence of valinomycin, when the respiration-generated membrane electric potential ( $\Delta\psi$ ) did not limit the transmembrane ion flow, added  $\text{Ag}^+$  provoked the very fast dissipation of  $\Delta\text{pH}$ , indicating that the resulting  $\text{H}^+$  leakage is massive (Fig. 1C). Figure 2 shows that the addition of  $\text{Ag}^+$  also col-

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TABLE 1. Inhibition of bacterial growth by silver in M9 mineral medium

AgNO <sub>3</sub> (μM)	OD <sub>600</sub> <sup>a</sup>	
	O395N1	O395N1Δnqr
0	0.814	0.713
0.625	0.218	0.121
1.25	0.017	0.017
2.5	0.017	0.016
5	0.018	0.019
10	0.014	0.015

<sup>a</sup> OD<sub>600</sub>, optical density at 600 nm.

lapses  $\Delta\psi$  in the absence of added Na<sup>+</sup> ions. These data clearly demonstrate that the Ag<sup>+</sup>-modified membrane is indeed leaky for protons and that the loss of NQR does not alter the sensitivity of the mutant *V. cholerae* membrane to Ag<sup>+</sup> ions compared to that of the wild type. To demonstrate that NQR does not contribute significantly to the overall H<sup>+</sup> leakage induced by Ag<sup>+</sup> ions, we measured the initial rate of  $\Delta$ pH dissipation at

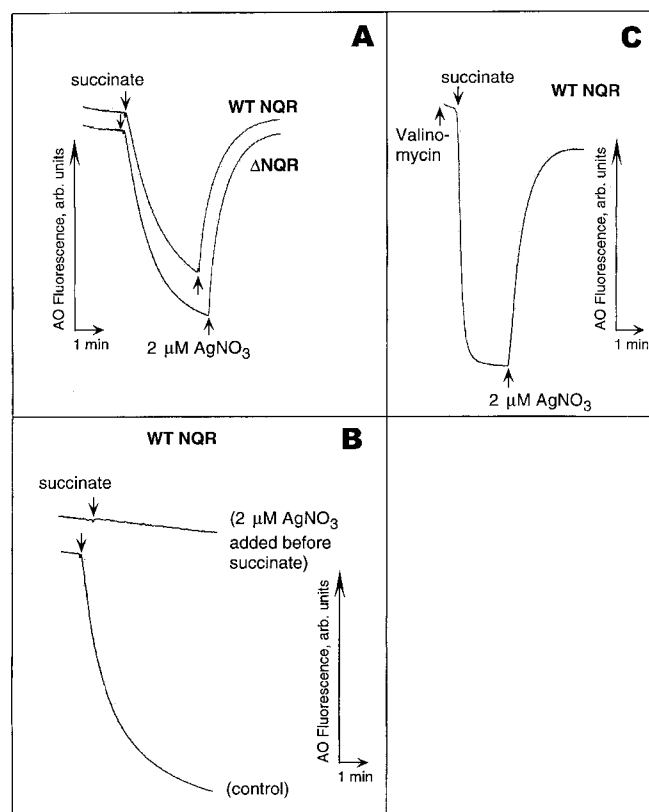


FIG. 1. Effects of Ag<sup>+</sup> on the H<sup>+</sup> permeability of the membrane measured in the inside-out membrane vesicles prepared from *V. cholerae*. Respiration-dependent formation of  $\Delta$ pH was initiated by the addition of 10 mM Tris-succinate (down arrows). Where indicated, 2.0 μM AgNO<sub>3</sub> was added to the vesicles (up arrows). (A) Addition of Ag<sup>+</sup> after succinate collapses  $\Delta$ pH generated in wild-type (WT) NQR (upper trek) as well as  $\Delta$ NQR (lower trek) membranes. (B) Ag<sup>+</sup> prevents the formation of the respiration-dependent  $\Delta$ pH. (C) Ag<sup>+</sup>-induced  $\Delta$ pH collapse is very fast in the presence of 0.2 μM valinomycin. In each case, typical treks from five independent experiments are shown.

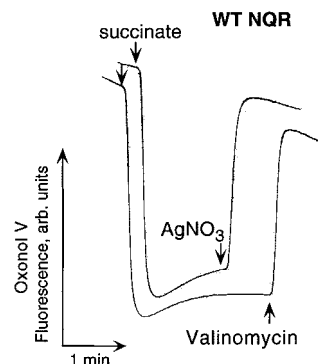


FIG. 2. Effects of Ag<sup>+</sup> on  $\Delta\psi$  in subbacterial vesicles of *V. cholerae*. The  $\Delta\psi$ -sensitive dye Oxonol V (1.0 μM) was used instead of AO. Ag<sup>+</sup> (4.0 μM) completely dissipates  $\Delta\psi$  generated by respiration in vesicles derived from the strain possessing the wild-type (WT) NQR (upper trek). Nearly identical results were obtained with  $\Delta$ NQR vesicles (data not shown). In the control experiment (lower trek), 0.2 μM valinomycin was added instead of AgNO<sub>3</sub>. Shown are typical treks from five independent experiments.

different concentrations of added Ag<sup>+</sup> in vesicles isolated from either wild-type or  $\Delta$ NQR cells (Fig. 3). We found that, in accordance with our growth experiments, the presence of NQR in the membrane is not required for the effect of Ag<sup>+</sup> ions (Fig. 3).

In summary, the addition of low micromolar concentrations of Ag<sup>+</sup> to inside-out membrane vesicles of *V. cholerae* induced a total collapse of both  $\Delta$ pH and  $\Delta\psi$  irrespective of the presence of Na<sup>+</sup> ions. This effect of Ag<sup>+</sup> was independent of the presence of the Na<sup>+</sup>-translocating NQR, known as a specific target for submicromolar Ag<sup>+</sup>, suggesting that the other Ag<sup>+</sup>-modified membrane proteins (or perhaps the Ag<sup>+</sup>-modified phospholipid bilayer itself) can cause the H<sup>+</sup> leakage, thus explaining the broad spectrum of the antimicrobial activity of Ag<sup>+</sup> ions. The two most significant results of this study are (i) the first (to our knowledge) direct experimental demonstration of the ability of Ag<sup>+</sup> ions to collapse the proton motive force and (ii) the irrelevance of NQR as a specific target for such a protonophore-like action of low micromolar concentrations of

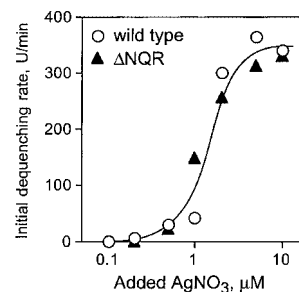


FIG. 3. The initial rate of  $\Delta$ pH dissipation in the wild-type and  $\Delta$ NQR membrane vesicles as a function of the AgNO<sub>3</sub> added. A transmembrane pH gradient was generated by the addition of 10 mM Tris-succinate to the vesicles at pH 7.5, and varying concentrations of AgNO<sub>3</sub> were added after a steady-state  $\Delta$ pH was reached. In each case, changes in AO fluorescence were monitored for 20 s after Ag<sup>+</sup> addition. Initial dequenching rates are expressed in arbitrary fluorescence units per minute.

Ag<sup>+</sup>. It is conceivable that the bactericidal action of these concentrations of Ag<sup>+</sup> in *V. cholerae* is not mediated by a specific target but is due to the H<sup>+</sup> leakage occurring through virtually any Ag<sup>+</sup>-modified membrane protein or perhaps through the Ag<sup>+</sup>-modified phospholipid bilayer itself. In the absence of Ag<sup>+</sup> resistance determinants (encoding pumps capable of efficient expelling of the Ag<sup>+</sup> ion), this would result in a complete deenergization of the membrane. Taking into account the well-documented crucial importance of the transmembrane proton gradient in overall microbial metabolism, it seems inevitable that the protonophore-like effect of Ag<sup>+</sup> described here should result in cell death. Thus, finally, the controversy over the mechanism of the bactericidal activity of low concentrations of Ag<sup>+</sup> ions has been clarified.

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