D397 IN SUBUNIT B OF THE *VIBRIO CHOLERAE* NA^+-PUMPING NADH: QUINONE OXIDOREDUCTASE IS INVOLVED IN CATION UPTAKE - AN ANALYSIS BY STEADY STATE / TRANSIENT KINETICS AND NUCLEAR MAGNETIC RESONANCE

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ABSTRACT

The Na^+-pumping NADH: quinone oxidoreductase (Na^+-NQR) is a membrane-bound enzyme complex present in *Vibrio cholerae* and other marine and pathogenic bacteria. Similar to the prokaryotic and mitochondrial H^+-pumping NADH: quinone oxidoreductase (Complex I), Na^+-NQR oxidizes NADH and reduces quinone, but pumps sodium ions instead of protons. The mechanism of sodium ion uptake and release is of significant importance as this represents one of the main functional differences between Na^+-NQR and Complex I. Though Na^+-NQR and Complex I share no sequence homology nor possess similar prosthetic content, both derive energy for cation pumping from redox substrates, and are therefore classified as primary ion pumps; Na^+-NQR is one of only two documented complexes to function as a primary sodium pump.

A homolog of Na^+-NQR, *Rhodobacter* nitrogen fixation protein (Rnf), has been discovered to be widely distributed among *Prokarya*, and its diversity of biological roles is striking. In addition to sequence homology, Na^+-NQR and Rnf share various enzyme cofactors in common, and alignments between these two proteins have revealed a series of conserved acidic residues within Rnf that correspond to those of Na^+-NQR that have recently been identified as participating in Na^+ uptake / conduction across the membrane. These observations suggest that Rnf may participate in the sodium cycle of organisms that rely on its activity. The Rnf of *Acetobacterium woodii* has been recently shown to be a sodium pump; this observation was instrumental as it was the first to conclusively demonstrate sodium pumping for an Rnf. Though little is currently known about Rnf as compared to Na^+-NQR, our increasing knowledge about Na^+-NQR serves to better inform hypotheses in regard to the biochemical repertoire of Rnf.

In the current investigation, a conserved aspartic acid residue that is likely part of a sodium ion binding site on the cytosolic face of NqrB subunit at position 397 has been investigated. Mutation of this residue to alanine reduced the ability of the enzyme to bind sodium (K_m^{Na+} = >100 mM). When point mutations altered the identity of this residue to glutamic acid or cysteine, the K_m^{Na+} (1.7 and 1.1 mM NaCl, respectively) was not significantly different from that of wild-type Na^+-NQR (1.6 mM NaCl); each mutant conserved the negative charge of this residue. When the negative charge was sequentially removed by introducing serine and asparagine, the K_m^{Na+} increased accordingly: 5.8 mM and 14.2 mM NaCl, respectively. Presteady-state fast kinetics of enzyme reduction by NADH using stopped flow revealed that the rate constants of redox transitions between Na^+-NQR cofactors that are sodium dependent (2Fe-2S → FMN_C and FMN_B → riboflavin) in wild-type enzyme become insensitive to sodium in the NqrB-D397A mutant; an attenuated degree of sodium sensitivity at these steps is recovered in NqrB-D397E and NqrB-D397C. ^{23}Na solution NMR was used to measure pre-equilibrium accessibility of sodium to reduced and oxidized samples of: wild-type Na^+-NQR, NqrB-D397A, NqrB-D397E, and NqrB-D397C. The results suggest that there is a greater accessibility for reduced wild-type Na^+-NQR and NqrB-D397C as compared to oxidized, however NqrB-D397E and NqrB-D397A did not appear to present such a degree of redox-state dependence on accessibility level. Taken all together, these data are consistent with the hypothesis that Na^+-NqrB-D397 participates in at least part of the sodium ion binding site and a gating mechanism that likely relies on conformational changes in the protein. This thesis demonstrates quite conclusively that conservation of aspartic acid at NqrB-D397 is an absolute requirement for maximal sodium binding.