Characterization of coenzyme binding and selectivity determinants in Mycobacterium tuberculosis flavoprotein reductase A: analysis of Arg199 and Arg200 mutants at the NADP(H) 2-phosphate binding site

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Mycobacterium tuberculosis FprA (flavoprotein reductase A) is an NAD(P)H- and FAD-binding reductase that is structurally/ evolutionarily related to adrenodoxin reductase. Structural analysis implicates Arg199 and Arg200 in interactions with the NADP(H) 2-phosphate group. R199A, R200A and R199A/ R200A mutants were characterized to explore the roles of these basic residues. All mutations abolished neutral FAD semiquinone stabilization in the NADPH-reduced enzyme, owing to weakened NADPH affinity. Instead, FAD hydroquinone was formed in all mutants, and each displayed substantially enhanced autooxidation rates (20–40-fold) compared with NADPH-reduced WT (wild-type) FprA. Steady-state ferricyanide reduction studies revealed diminished NADPH affinity (higher $K_m$ values), but lower NADH $K_m$ values. Despite a lowered $k_{cat}$, the R199A/R200A mutant exhibited a 200-fold coenzyme specificity switch towards NADH, although substrate inhibition was observed at high NADH concentrations ($K_i = 250 \mu$M). Stopped-flow FAD reduction studies confirmed substantially increased NADPH $K_d$ values, although the limiting flavin reduction rate constant was similar in all mutants. The R199A mutation abolished electron transfer between hydroquinone FprA and NADP+, while this reaction progressed (via an FADH2-NADP+ charge-transfer intermediate) for R200A FprA, albeit more slowly ($k_{lim} = 58.1 \text{s}^{-1}$ compared with $>300 \text{s}^{-1}$) than in WT. All mutations caused positive shifts in FAD potential ($\sim 40–65 \text{mV}$).

Binding of an NADPH analogue (tetrahydro-NADP) induced negative shifts in potential ($\sim 30–40 \text{mV}$) only for variants with the R200A mutation, indicating distinctive effects of Arg199/Arg200 on coenzyme binding mode and FAD potential. Collectively, these data reveal important roles for the phylogenetically conserved arginines in controlling FprA FAD environment, thermodynamics, coenzyme selectivity and reactivity.

Key words: adrenodoxin reductase, coenzyme binding, electron transfer, enzyme mechanism, flavoprotein reductase A, Mycobacterium tuberculosis.