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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

Muna SABRI, Adrian J. DUNFORD, Kirsty J. McLEAN, Rajasekhar NEELI, Nigel S. SCRUTTON, David LEYS and Andrew W. MUNRO[1]

[1]Manchester Interdisciplinary Biocentre, The University of Manchester, Faculty of Life Sciences, 131 Princess Street, Manchester M1 7DN, U.K.

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 Arg_{199} and Arg_{200} 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 lowerNADHK_m values. Despite a lowered k_{cat}, the R199A/R200A mutant exhibited a 200-fold coenzyme specificity switch towards NADH, although substrate inhibitionwas observed at highNADH concentrations (K_i **=**250 μM). 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 $FADH_2$-$NADP^+$ charge-transfer intermediate) for R200A FprA, albeit more slowly (k_{lim} **=**58.1 s^{-1} compared with >300 s^{-1}) than in WT. All mutations caused positive shifts in FAD potential (**~**40–65 mV). Binding of an NADPH analogue (tetrahydro-NADP) induced negative shifts in potential (**~**30–40 mV) only for variants with the R200A mutation, indicating distinctive effects of Arg_{199}/Arg_{200} 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*.