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Tryptophan 359 regulates flavin thermodynamics and coenzyme selectivity in *Mycobacterium tuberculosis* FprA

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Running title: Regulation of thermodynamics and catalysis in FprA

SYNOPSIS

Mycobacterium tuberculosis (*Mtb*) flavoprotein reductase A (FprA) is a NAD(P)H-dependent FAD-binding reductase that is structurally related to mammalian adrenodoxin reductase, and which supports catalytic function of *Mtb* cytochrome P450s. Tryptophan 359, proximal to the FAD, was investigated in light of its potential role in controlling coenzyme interactions, as observed for similarly located aromatic residues in diflavin reductases. Phylogenetic analysis indicated that a tryptophan residue corresponding to Trp359 is conserved across FprA-type enzymes and in adrenodoxin reductases. W359A/H mutants of *Mtb* FprA were generated, expressed and the proteins characterized to define Trp359's role. W359A/H mutants exhibited perturbed UV-visible absorption/fluorescence properties. The FAD semiquinone formed in wild-type NADPH-reduced FprA was destabilized in the W359A/H mutants, which also had more positive FAD midpoint reduction potentials (-168/-181 mV, respectively, *versus* the standard hydrogen electrode, compared to -230 mV for wild-type FprA). The W359A/H mutants had lower ferricyanide reductase k_{cat} and NAD(P)H K_{m} values, but this led to improvements in catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) with NADH as reducing coenzyme (9.6/18.8 $\mu\text{M}^{-1} \text{min}^{-1}$, respectively, compared to 5.7 $\mu\text{M}^{-1} \text{min}^{-1}$ for wild-type FprA). Stopped-flow spectroscopy revealed NAD(P)H-dependent FAD reduction as rate-limiting in steady-state catalysis, and to be retarded in mutants (e.g. limiting rate constants for NADH-dependent FAD reduction were 25.4 s^{-1} for wild-type FprA and 4.8 s^{-1} /13.4 s^{-1} for W359A/H mutants). Diminished mutant FAD content (particularly in W359H FprA) highlighted the importance of Trp359 for flavin stability. The results demonstrate that the conserved Trp359 is critical in regulating FprA FAD binding, thermodynamic properties, catalytic efficiency and coenzyme selectivity.

Keywords: *Mycobacterium tuberculosis*, flavoprotein reductase A, adrenodoxin reductase, coenzyme selectivity, electron transfer.

Abbreviations: AdR – adrenodoxin reductase; FprA – flavoprotein reductase A from *Mycobacterium tuberculosis*; e.t. – electron transfer; HQ – hydroquinone; PDA – photodiode array; SQ – semiquinone.

INTRODUCTION

The *Mycobacterium tuberculosis* H37Rv (*Mtb*) FprA protein (product of gene *Rv3106*) [1] is a FAD-binding, NAD(P)H-dependent reductase enzyme and a homologue of the eukaryotic adrenodoxin reductase (AdR) proteins [2]. AdR is a mitochondrial enzyme whose major role is as a redox partner for cytochrome P450 (P450) systems involved in steroid metabolism. AdR's roles include electron transfer to the P450_{sc} enzyme, leading to cholesterol side-chain cleavage to pregnenolone, the committed step in steroid hormone synthesis [3]. The FprA atomic structure confirmed its structural similarity to bovine AdR [4-6]. A slightly more distant relative of FprA is the yeast AdR homologue Arh1p, a mitochondrial protein with essential roles in control of mitochondrial and cellular iron levels [7] (Figure 1). The characterization of catalytic, thermodynamic and biophysical properties of FprA revealed further similarities to eukaryotic AdR, including the formation of a long-wavelength absorption species on reduction by NADPH, but not NADH [e.g. 8,9]. Structural relationships suggested that FprA might be a physiologically relevant redox partner for the *Mtb* P450 enzymes, participating in a similar type of P450 redox system as that seen in the eukaryotic mitochondrion [10].

The *Mtb* genome sequence identified 20 different genes encoding P450 enzymes. A number of these genes have been expressed and two of the P450s (CYP51B1 and CYP121) structurally characterized [11-14]. Bacterial P450s are typically the terminal oxidases of redox chains involving a NAD(P)H-binding ferredoxin/ferredoxin reductase, a ferredoxin (or flavodoxin), and the P450 [10,15]. The *Mtb* genome is devoid of flavodoxin genes, but contains several putative ferredoxin genes, including ones directly adjacent to P450 genes. One of these ferredoxins (Fdx, the product of gene *Rv0763c*) is adjacent to the CYP51B1 sterol demethylase P450 [16]. Functional P450 redox chains were reconstituted for both *Mtb* CYP51B1 and CYP121 P450s using NAD(P)H, FprA, Fdx and the relevant P450, and the sterol demethylase activity of *Mtb* CYP51B1 was demonstrated using this type of system. Thus, FprA was shown to be a functional redox partner for a typical *Mtb* class I P450 system [16, 17]. An unusual property of FprA is its ability to stabilize a neutral blue FAD semiquinone (SQ) on reduction by NADPH. Using NADH or dithionite, FprA's FAD is instead converted fully to its 2-electron reduced hydroquinone (HQ) form [8]. It was hypothesised that electronic comproportionation between a NADPH-bound molecule of oxidized FprA (in charge-transfer complex with the coenzyme) and a NADPH-bound molecule of reduced (HQ) FprA results in formation of two molecules of NADPH-bound flavosemiquinone FprA [8,18]. From previous steady-state kinetic studies, NADPH is FprA's preferred coenzyme [8,18]. However, the factors underlying coenzyme selectivity and the unusual differences in the reductive reactions of FprA with NADH/NADPH have not been examined in detail.

A common feature in NAD[P]H-binding diflavin reductases and ferredoxin reductases is the presence of an aromatic residue "shielding" part of the isoalloxazine ring from solvent and blocking access of coenzyme nicotinamide to the flavin [e.g. 19,20]. Studies of mutants at this position in diflavin reductases (e.g. nitric oxide synthase [NOS], the fatty acid oxidase flavocytochrome P450 BM3 [BM3] and human cytochrome P450 reductase [CPR]) showed that replacement of the aromatic residue can result in a considerable coenzyme preference "switch" from NADPH towards NADH, and often also result in substantial improvements for NADH-driven activities [e.g. 20-22]. For human CPR, the specificity switch (i.e. the ratio of the specificity constant [k_{cat}/K_m] with NADH as coenzyme divided by that with NADPH as coenzyme) was ~1000-fold in a W676A mutant for cytochrome *c* reductase activity [20]. With the BM3 CPR domain, a huge improvement

of ~6000-fold in NADH preference was determined from ferricyanide reductase activity measurements with a W1064A mutant [21]. In studies of the related pea chloroplast ferredoxin NADP⁺ reductase (FNR) enzyme, Karplus *et al.* observed that replacement of the FAD-shielding Tyr308 residue resulted in much tighter NADP⁺ binding to Y308G/S mutants, and in a switch of coenzyme preference towards NADH of 300-400 fold [23,24]. These data suggested Y308 had an important role in coenzyme dissociation. X-ray crystallography of the Y308S mutant revealed a productive FNR-coenzyme complex in which the nicotinamide ring of NADP(H) was located close to the flavin isoalloxazine ring, with a distance of ~3 Å between the flavin N5 and nicotinamide C4 atoms [23].

In view of the distinctive reductive reactions of FprA with NADH and NADPH, and in light of previous data indicating importance of aromatic residues in controlling coenzyme reactivity in other flavoprotein reductases, we examined the FprA structure to identify an aromatic residue in the vicinity of the FAD that might exert control over coenzyme binding and reactivity. To date, there has been no study of the function of any aromatic FAD-shielding residues in AdR-like enzymes. In FprA, the residue Trp359 is located close to the FAD isoalloxazine ring and could play a role similar to that of the conserved aromatic residues in FNR and the diflavin reductases (Figure 2). The tryptophan is strongly conserved in related AdR's and FprA homologues (Figure 1). To explore Trp359's role in FprA redox and catalytic properties, we generated the W359A/H mutants and investigated their spectroscopic features and interactions with NADPH/NADH coenzymes. The data reveal substantial changes in W359A/H mutant enzyme (i) affinity for NAD(P)H, (ii) microscopic rate constants for FAD reduction by coenzymes, (iii) flavin-binding properties, and (iv) redox potentials of the bound FAD. Our data are consistent with an important role for Trp359 in the control of FprA flavin binding and coenzyme reactivity.

EXPERIMENTAL

Construction of FprA mutants: Oligonucleotide-directed mutagenesis of the *fprA* gene was done with the Stratagene QuikChange kit, using the plasmid pKM3b [8]. Oligonucleotide primers were from MWG Biotech, UK. The following primers were used to create the W359A and W359H FprA variants: 5'- CGA ATA CGT CGT CGG GCA TAT CAA GCG CGG C - 3' (W359H forward primer); 5'- GCC GCG CTT GAT ATG CCC GAC GAC GTA TTC G - 3' (W359H reverse primer); 5'- CGA ATA CGT CGT CGG GGC GAT CAA GCG CGG - 3' (W359A forward primer); 5'- CCG CGC TTG ATC GCC CCG ACG ACG TAT TCG - 3' (W359A reverse primer). PCR reactions were done using *Pfu* DNA polymerase (Stratagene). The conditions were: 50 ng template DNA, 150 ng primers, 100 μM dNTPs, 2.5 units of *Pfu* polymerase in a 50 μl reaction volume. The PCR reactions were performed using a Techne Genius Thermocycler for 18 cycles of 95 °C (30 s), 63 °C (1 min), and 68 °C (8 min) for denaturation, annealing, and extension steps, respectively.

Plasmids (pET11a containing prospective *fprA* mutants) were transformed into *E. coli* XL1-Blue cells and transformant colonies were grown on LB agar plates containing ampicillin at 50 μg ml⁻¹. Small scale plasmid preparations were done using the Sigma GenElute purification kit. Purified plasmids were sequenced to verify mutations. Sequencing was done at MWG Biotech. Entire genes were sequenced using primers described previously for the wild-type (WT) *fprA* [8] using MWG's

T7 and T7 term oligonucleotide primers. Desired mutations, and absence of secondary mutations, were confirmed in several plasmid isolates for both the W359A and W359H variants.

Purification of WT and W359A/H FprA mutants: WT FprA and W359A/H mutant genes were expressed in the T7 RNA polymerase lysogen strain HMS174 (DE3) (Novagen). HMS174 (DE3)/pFprAW359A/H transformants (10–30 litres) were grown at 37 °C with vigorous agitation (220 rpm) until mid-logarithmic growth phase ($OD_{600} \sim 0.8$ – 1.0) in 2xYT media [25]. Culture temperature was then decreased to 25 °C, and expression of the WT and mutant *fprA* genes was induced by addition of IPTG (1 mM). The culture growth was continued for 16–20 h post-induction. Cells (30–50 g wet weight) were harvested and disrupted, and FprA W359A/H mutant proteins were purified to homogeneity using methods established previously for the WT FprA enzyme [8]. The purity of the FprA proteins was assessed by spectral analysis (using the ratio of flavin-specific absorption [A_{452}] to protein-specific absorption [A_{274}]) and by SDS/PAGE analysis of protein samples at different stages in the purification process. The FprA concentration was determined using the oxidized enzyme coefficient: $\epsilon_{452} = 10.6 \text{ mM}^{-1} \text{ cm}^{-1}$ [8].

Attempts were made to reconstitute FAD cofactor into flavin-depleted W359H/A FprA protein by both (a) extensive washing of mutant FprA proteins immobilized on Q-Sepharose resin with FAD (1 mM) incorporated into the binding buffer (50 mM Tris/HCl, 1 mM EDTA, pH 7.2), followed by washing with FAD-free binding buffer and elution in binding buffer plus 250 mM KCl, and (b) mixing and incubating FprA proteins with FAD (1 mM in binding buffer), followed by protein separation using a PD10 gel filtration column (Pharmacia).

Steady-state and stopped-flow kinetic assays: The WT and W359A/H FprA mutant-catalysed reduction of potassium ferricyanide was analysed by steady-state kinetic measurements using both NADH and NADPH as electron donors. The kinetic parameters (K_m , k_{cat}) for ferricyanide reduction were determined in buffer A (100 mM potassium phosphate, pH 7.5) at 30 °C. The enzyme concentration was 30 nM for WT and W359A FprA's, and ~ 100 nM for W359H FprA. Ferricyanide concentration was maintained at 1 mM. This concentration was previously determined as saturating, and confirmed in this work [8]. Ferricyanide reduction was determined at 420 nm ($\Delta\epsilon_{420} = 1020 \text{ M}^{-1} \text{ cm}^{-1}$). All measurements were made in triplicate at each substrate concentration and averages reported varied by $<5\%$ from the mean.

Stopped-flow studies were performed using an Applied Photophysics SX.18 MVR stopped-flow spectrophotometer contained within a glove box (Belle Technology, Portesham, UK) to maintain anaerobic conditions (<5 ppm oxygen). Flavin reduction by NADH/NADPH (up to 1 mM) was monitored in single wavelength mode at 452 nm (at the FprA flavin absorption peak). Flavin reduction rates for mutant proteins were measured at 30 °C. All measurements were carried out in buffer A. All buffers were deoxygenated by evacuation and extensive bubbling with oxygen-free argon. Prior to stopped-flow studies, protein samples were fully oxidized by treatment with potassium ferricyanide. The excess ferricyanide was removed by rapid gel filtration in the glove box (Pharmacia PD-10 column).

Appropriate exponential functions were used to fit absorption transients from stopped-flow experiments, using Spectrakinetics software (Applied Photophysics). The observed rate constants for FprA W359A/H flavin reduction by NADH (k_{obs}) showed a hyperbolic dependence on the coenzyme concentration. Equation 1 was used to fit the data [26] in order to determine the apparent

enzyme-coenzyme dissociation constant, K_d , and the limiting rate constant for hydride transfer to the FAD (k_{lim}). The values reported are averages of at least three separate determinations at each coenzyme concentration.

$$k_{obs} = \frac{k_{lim}[S]}{K_d + S} \quad \text{Equation 1}$$

Stopped-flow, multiple-wavelength scanning absorption studies were also done using a photodiode array (PDA) detector and X-SCAN software (Applied Photophysics). Reactions were typically performed over timescales between 0.3 to 4 seconds after the initial mixing event, with the first spectrum recorded 1.3 milliseconds after mixing. NADH and NADPH (up to 200 μ M) were mixed with oxidized enzymes (typically 10 μ M).

Spectroscopic analysis: All UV-visible absorption spectra and steady-state assays for FprA enzymes were recorded using a Cary UV-50 Bio UV-visible scanning spectrophotometer (Varian UK). Samples were suspended in buffer A for spectral analysis. Samples (typically ~25 μ M) were analysed using a 1 cm pathlength quartz cuvette. Fluorescence measurements were made on a Cary Eclipse fluorimeter (Varian UK). Flavin excitation was at 430 nm, and emission data were collected from 450-700 nm. Slit widths were set at 10 nm and 5 nm for excitation and emission, respectively. FprA samples for fluorimetry were in buffer A and at a final concentration of 5-25 μ M.

Analysis of flavin redox potentials: Potentiometric redox titrations to determine the midpoint reduction potentials of the FAD cofactor in the W359A/H FprA mutants were performed in a Belle Technology glove-box under a nitrogen atmosphere, as described previously and for WT FprA [8,27]. All solutions were degassed under vacuum with argon. Oxygen levels were always maintained at less than 2 ppm. 100 mM potassium phosphate, pH 7.0, containing 10 % v/v glycerol (titration buffer) was used in all potentiometric titrations. FprA proteins (typically 30–50 μ M in ~5 ml titration buffer) were titrated electrochemically according to the method of Dutton [28]. Mediators (2 μ M phenazine methosulfate, 5 μ M 2-hydroxy-1, 4-naphthoquinone, 0.5 μ M methyl viologen, and 1 μ M benzyl viologen) were added to facilitate electrical communication between the enzyme and the electrode, prior to titration [28,29]. The electrochemical potential of the solution was measured using a Hanna pH 211 meter coupled to a Pt/Calomel electrode (ThermoRussell Ltd.) at 25 ± 2 °C. The electrode was calibrated using the Fe^{3+}/Fe^{2+} EDTA couple as a standard (+108 mV). A factor of +244 mV was used to correct relative to the standard hydrogen electrode. Data manipulation and analysis were performed using Origin 7.5 software (OriginLab, Northampton MA). For the W359A/H mutant titrations, absorbance data at 452 nm were plotted against applied potential to determine the relevant FAD oxidized/HQ redox couples. A 2-electron Nernst equation was used to fit the data in order to define the midpoint reduction potential for the 2-electron transition, as described previously [29,30].

Materials: Restriction enzymes and DNA ligase were purchased from NEB or Stratagene. The QuikChange mutagenesis kit was from Stratagene. All other reagents were from Sigma Aldrich (Poole, UK) and were of highest grade available.

RESULTS AND DISCUSSION

Generation and purification of FprA W359A/H mutants: The W359A/H FprA mutants were created by site-directed mutagenesis as described in the *Experimental* section. The W359A mutant was expressed at levels comparable to WT FprA and the encoded protein was readily purified using methods successfully applied for WT FprA [8]. However, expression levels of the W359H mutant were typically < 25 % of those for the WT FprA, and the overall yield of pure enzyme was also much lower (typically 0.5-1 mg *per* litre of transformant culture compared with ~4 mg *per* litre for WT FprA). Purification of the W359A/H FprA mutants is shown in the online supplemental material, Figure 1.

The W359A mutant was consistently found to have lower FAD content than WT FprA, typically with 80 ± 5 % FAD bound. However, the pure W359H mutant invariably had much lower FAD content than either W359A or WT FprA (stoichiometric FAD incorporation occurs in WT) [8]. In addition to the lower production and recovery of W359H FprA, its FAD content decreased somewhat during the purification process. In contrast, negligible FAD loss occurred for the W359A FprA protein during purification. Leaching of FAD was particularly noticeable during ultrafiltration steps for concentration of the W359H protein. To minimise the FAD loss, purification steps were done more rapidly and ultrafiltration time kept to a minimum. Even with these precautions, pure W359H FprA invariably contained ≤ 40 % bound FAD. For enzyme assays, the W359H FprA protein was quantified by bound flavin content ($\epsilon_{451} = 10.6 \text{ mM}^{-1} \text{ cm}^{-1}$), to ensure that rate constants accurately reflected only the holoenzyme activity. Attempts were made to reconstitute FAD into flavin-depleted W359H enzyme, as described in the *Experimental* section. Modest increases in FAD content of recovered protein (~5 % from A_{451} to A_{280} ratio) were achieved, but the process was not considered profitable since only small improvements in flavin incorporation were achieved and further protein loss occurred due to the additional step in the purification regime. Thus, it appears that the W359H mutant leads to considerable instability of the FAD. The W359A mutant is much less severely affected and is isolated predominantly as holoenzyme. It should also be noted that the apoprotein form of WT FprA can be at least partially reconstituted with FAD, again pointing to reduced affinity for the FAD cofactor in the W359H mutant.

UV-visible absorption and fluorescence spectroscopic features: UV-visible absorption spectra are shown for WT FprA and for the W359A and W359H mutants in Figure 3. The spectra detail the absorption features of the oxidized enzymes, and the effects of reducing the enzymes with a 10-fold molar excess of NADH/NADPH, and with sodium dithionite. Previous studies of WT FprA revealed that reduction of FprA using either dithionite or the coenzyme NADH led to essentially complete conversion of FprA flavin from its oxidized (quinone) form to its 2-electron reduced (HQ) form; while reduction by NADPH produced a partially reduced FprA with a substantial amount of 1-electron reduced (neutral, blue SQ) FAD [8]. Figure 3A demonstrates these phenomena. The formation of blue SQ in WT FprA was assigned to electronic comproportionation between NADPH-bound forms of the oxidized and 2-electron reduced species [8]. The UV-visible spectroscopic properties of W359A (Figure 3B) and W359H (Figure 3C) FprA proteins reveal interesting differences to WT FprA. For W359A, there is little evidence for SQ flavin formation in the reactions with dithionite or NAD(P)H. NADPH effects a slightly more complete reduction of the FAD than does NADH (at the same molar excess), and low intensity of absorption in the 530-650 nm region for the NAD(P)H-reduced enzymes suggests that flavosemiquinone represents only a very minor proportion of species present, and that similar amounts of SQ are present for both the

NADH- and NADPH-reduced enzymes. For W359H, the situation is similar to the WT and W359A mutants for the reductions with dithionite and NADH. However, NADPH-reduced enzyme shows a lower level of reduction to the HQ form than is observed with NADH (and dithionite), and a slightly larger absorption feature in the 530–650 nm region suggests that some blue SQ is present at equilibrium, albeit $\leq 10\%$ of that observed for NADPH-reduced WT FprA. Thus, both W359A/H mutations considerably destabilize the FAD blue SQ in NADPH-reduced enzymes by comparison with the WT FprA.

In the oxidized forms of the W359A/H FprA mutants, some changes in UV-visible absorption properties are also observed. The major absorption band at 452 nm (WT) is shifted to 451 nm (W359A/H). However, the strong absorption shoulder located at ~ 473 nm for WT FprA is also much less prominent in the W359A/H mutant proteins, suggesting a change in the FAD isoalloxazine ring environment induced by the mutations. In addition, while low flavin content of W359H FprA prevents accurate determination of the position of the shorter wavelength FAD absorption band, the relevant band in the W359A mutant is at 375 nm compared to 380 nm for WT FprA. Thus, it appears from spectral analysis of both oxidized and reduced W359A/H FprA proteins that the mutations result in (i) changes in flavin content and incorporation (particularly for W359H), (ii) perturbations to the flavin environment in the oxidized enzymes, and (iii) altered thermodynamic properties and destabilization of the FAD SQ in NADPH-reduced mutants. The absence of any obvious charge-transfer bands in the optical spectra suggests that NAD(P)H-reduced W359A/H mutants do not remain tightly bound to NAD(P)⁺ product, and diminished affinity for NADP⁺ is also inferred from the fact that the IC_{50} value for NADP⁺ in inhibition of steady-state ferricyanide reduction (with NADPH as reducing coenzyme) is $1.5 \pm 0.3 \mu\text{M}$ for WT FprA, whereas the values are ~ 3 -fold greater for the W359A/H FprA mutants (data not shown).

A further interesting observation made for the WT FprA protein was that, although its FAD fluorescence is very low (quantum yield of 0.01 compared to 0.03 for free FAD) [8,31], reduction of FprA with NADH/NADPH did not substantially affect the FAD fluorescence intensity or emission maximum (despite the effects of reductants on the absorption spectrum – see Figure 3A). Previous data for WT FprA were confirmed here, and reduction of the W359A/H mutant proteins with sodium dithionite (as with WT FprA) resulted in a near-complete bleaching of the FAD fluorescence. However, addition of a 10-fold molar excess (250 μM) of NADH/NADPH resulted in $\sim 70\%$ decrease in FAD fluorescence in W359A FprA. For W359H FprA, dithionite again bleached FAD fluorescence. NADH effected a $\sim 75\%$ decrease in W359H flavin fluorescence, whereas NADPH produced only $\sim 25\%$ fluorescence decrease (data not shown). With 430 nm excitation, the emission maximum was also shifted from 525 nm (WT) to ~ 520 nm in both the W359A/H mutant proteins. While reasons underlying unusual fluorescence properties of the FprA FAD remain uncertain, W359A/H mutations clearly result in alterations in levels of flavin reduction by NAD(P)H coenzymes (Figure 3), and cause perturbations in flavin environment that influence fluorescence properties.

Steady-state kinetic properties: Previous studies on WT FprA have indicated it was an efficient electron transferase with ferricyanide as electron acceptor, and that the NADPH K_m value is considerably lower than that for NADH [8, 18]. We investigated steady-state ferricyanide reductase activity of WT and W359A/H mutants at a near-saturating ferricyanide concentration (1 mM). Results are detailed in Table 1. Data demonstrate some loss of NADPH-dependent ferricyanide reductase activity in the W359A/H mutants (decreased k_{cat} values), but also lower W359A/H mutant

K_m values for NADPH (Table 1) This leads to negligible changes in the catalytic efficiency of the mutants with NADPH as electron donor, with the k_{cat}/K_m ratios being indistinguishable within error between WT FprA and the W359A/H mutants. With NADH as electron donor, the WT k_{cat} value was of similar magnitude to that obtained with NADPH as donor, but the k_{cat} values for the W359A/H FprA mutants were substantially decreased (Table 1). However, the W359H FprA K_m for NADH was >13-fold lower than that for WT FprA, and the value for W359A FprA was also considerably decreased (~8-fold). Overall, the data indicate that catalytic efficiency (k_{cat}/K_m ratio) of W359A FprA activity with NADH is 1.7-fold greater than for WT FprA, while that for W359H FprA is 3.3-fold greater. Ratios of catalytic efficiencies with NADH and NADPH as electron donors are detailed in Table 1. These data indicate a 1.7-fold switch in coenzyme selectivity towards NADH for W359A FprA, and a 4.1-fold switch for W359H FprA. Thus, W359A/H mutations result in decreased k_{cat} (lower activity) and K_m (increased coenzyme affinity) for both NADPH/NADH coenzymes, giving rise to improved catalytic efficiency with NADH as the reducing coenzyme.

The W359A/H mutations thus produce modest switches in coenzyme selectivity from NADPH towards NADH, but these are not of the same magnitude as those observed for human CPR and P450 BM3 enzymes [20,21]. The 4.1/1.7-fold coenzyme selectivity switches towards NADH in the FprA W359H/A mutants have their origins mainly in considerably improved K_m values for NADH (and NADPH), which offset the lower k_{cat} values observed for both mutants with both coenzymes (Table 1).

Stopped-flow kinetic analysis: Preceding studies of stopped-flow kinetics of FprA FAD reduction demonstrated that the limiting FAD reduction rate constants (k_{lim} values) at high (>500 μ M) NADPH/NADH concentration were broadly similar at 20–25 s^{-1} [8]. For ferricyanide reduction, these rate constants (40–50 electrons s^{-1} , since oxidation of each NAD(P)H molecule results in the transfer of 2 electrons to the FprA FAD) are compatible with observed k_{cat} values for ferricyanide reduction (35 electrons s^{-1} transferred to ferricyanide with NADPH as coenzyme, 42 s^{-1} with NADH, Table 1) and demonstrate that coenzyme-dependent flavin reduction is the major rate-limiting step in steady-state FprA-dependent ferricyanide reduction with both NADH/NADPH as the reducing coenzymes. The fact that both NADPH- and NADH-dependent ferricyanide reduction (k_{cat} values) are decreased for the W359A/H mutants thus suggests that the electron transfer rate from NAD(P)H to FAD should be similarly decreased in these mutants. To investigate further, we determined rates of NAD(P)H-dependent FAD reduction for W359A/H FprA enzymes using stopped-flow absorption spectroscopy, and by following the A_{452} decrease on flavin reduction. Studies were done over a range of coenzyme concentrations (up to 1 mM).

For both the W359A/H mutants (as for WT FprA), flavin reduction progress curves with both NADPH/NADH coenzymes were monophasic and data were fitted well using a single exponential expression [8]. There was no apparent dependence of flavin reduction rate on NADPH concentration for either W359H/A, with the apparent k_{lim} values being $16.5 \pm 2 s^{-1}$ and $15.0 \pm 2.0 s^{-1}$, respectively. There was no evidence of increased flavin reduction rate at lower NADPH concentrations, as was observed for the WT FprA enzyme [8]. Thus, the NADPH-dependent W359A/H FAD reduction rate constant is decreased by comparison to that for WT FprA, as was predicted from the lower steady-state k_{cat} values. With NADH as the reducing coenzyme, stopped-flow reduction transients remained monophasic and there was a hyperbolic dependence of the observed FprA FAD reduction rate (k_{obs}) on NADH concentration, as was also observed for the WT

FprA. A hyperbolic function was used to fit the data and produced parameters of $k_{\text{lim}} = 13.4 \pm 0.4 \text{ s}^{-1}$ and apparent affinity for NADH (K_d) = $119.2 \pm 12.0 \text{ } \mu\text{M}$ for W359H; and $k_{\text{lim}} = 4.8 \pm 0.1 \text{ s}^{-1}$, $K_d = 26.8 \pm 1.7 \text{ } \mu\text{M}$ for W359A. In both cases, the k_{lim} values are lower than that for WT FprA ($25.4 \pm 0.7 \text{ s}^{-1}$), and are again consistent with the decreased W359A/H k_{cat} values for ferricyanide reduction in the steady-state studies. The slower NAD(P)H-dependent FAD reduction in these mutants possibly results from non-optimal geometry of interaction with the reducing coenzymes. Figure 4A shows the hyperbolic dependence of FAD reduction rate on NADH concentration for W359H FprA, with a typical stopped-flow transient (a single exponential function is used to fit the data) shown as the inset. Entire W359A/H FprA spectra were also collected during reductive transient reactions with NAD(P)H using a photodiode array (PDA) stopped-flow attachment. There was no evidence of any significant accumulation of blue SQ during W359A/H FprA reductive reactions. Figure 4B shows exemplary PDA spectral data for the reaction of W359A FprA with NADH (100 μM).

Thermodynamic properties: The midpoint reduction potential of the WT FprA FAD is $-230 \pm 12 \text{ mV}$, and reduction potentials of the W359A/H variants were determined to probe for variations in thermodynamic properties resulting from changes in FAD environment, and to evaluate whether SQ species were stabilized in equilibrium potentiometric titrations using single electron reductant (dithionite) and oxidant (ferricyanide) molecules. Both the W359A/H enzymes were found to be reduced cleanly from their oxidized to HQ forms with no evidence for significant formation of SQ intermediates during either the reductive or oxidative phases of the redox titration. Figure 5 shows selected spectra from the W359A FprA redox titration, with data analysis (a 2-electron Nernst function was used to fit the A_{452} versus applied potential data) as inset. The midpoint reduction potential for the 2-electron reduction of the W359A FprA is $-168 \pm 4 \text{ mV}$, and that for W359H FprA is $-181 \pm 8 \text{ mV}$ versus the standard hydrogen electrode (SHE). Both values are substantially more positive than that for ligand-free WT FprA (-230 mV) [8]. Evidently, removal of the Trp359 aromatic side chain impacts on flavin environment and causes a significant increase ($\sim 50\text{--}60 \text{ mV}$) in midpoint reduction potential for the 2-electron reduction of the FAD in the W359A/H FprA mutants. The more positive FprA W359A/H FAD reduction potentials likely result from alterations in flavin solvent exposure, and indicate that Trp359 is important in controlling the FAD environment and its thermodynamic properties. A similar conclusion can be made from observations indicating altered UV-visible absorption/fluorescence properties of the W359A/H mutants, and since both W359A/H FprA mutants become more fully reduced by NADPH than does the WT FprA (Figure 3). The absence of stabilization of FprA blue SQ in the NADPH-reduced W359A/H mutants could be related to diminished affinity of the reduced enzymes for NADP(H). However, it might also have its origins in the further separation of the redox couples for the oxidized/SQ (E_1) and SQ/HQ (E_2) FAD transitions by comparison to those for WT FprA, and specifically if E_2 has become substantially more positive than E_1 in the W359A/H FprA mutants.

The greater driving force for FAD reduction (i.e. the more positive redox potentials of the W359A/H FAD cofactors) does not compensate for structural alterations, and the W359A/H hydride transfer rates from NAD(P)H coenzymes are lower than those for the WT FprA enzyme (Figure 4). Thus Trp359 likely optimises geometry of interaction of NAD(P)H nicotinamide to enable efficient electron transfer to the FAD, and in so doing overcomes any unfavourable change in FAD redox potential.

In conclusion, our kinetic and mechanistic characterization of the W359A/H mutants of *Mtb* FprA represents the first evaluation of the role of this conserved tryptophan residue in the bacterial FprA

and eukaryotic AdR enzyme classes. The results show that this aromatic residue is important in controlling FAD environment and redox properties, and also NAD(P)H coenzyme interactions. The W359A/H mutations destabilize FAD binding and diminish NAD(P)H-dependent FAD reduction rate and steady-state k_{cat} values. However, the apparent affinity (K_m) for both NADPH/NADH is improved and there are resultant switches in catalytic efficiency from NADPH towards NADH in the mutants. While Trp359 in FprA may not be an aromatic “lid” over the FAD cofactor that must be displaced to enable productive redox reactions with NAD(P)H (as is the case in the diflavin reductases), it is evidently an important residue that controls structure of the FAD binding site, interactions with NAD(P)H coenzymes, FAD thermodynamic properties and the efficiency of FprA catalysis.

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TABLES

Table 1. Steady-state kinetic parameters for WT FprA and W359A/H mutants. Steady-state parameters were determined as described in the *Experimental* section. Rate constants were determined using a coefficient of $\Delta\epsilon_{420} = 1020 \text{ M}^{-1} \text{ cm}^{-1}$ for ferricyanide reduction. A hyperbolic function was used to fit the observed rate *versus* NAD(P)H concentration data to derive k_{cat} and K_{m} parameters.

FprA enzyme	NADPH			NADH			
	k_{cat} (min^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\mu\text{M}^{-1} \text{ min}^{-1}$)	k_{cat} (min^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\mu\text{M}^{-1} \text{ min}^{-1}$)	$k_{\text{cat}}/K_{\text{m}}(\text{NADH})/$ $k_{\text{cat}}/K_{\text{m}}(\text{NADPH})$
WT	2091 ± 97	6.5 ± 1.4	322 ± 84	2488 ± 120	437 ± 49	5.7 ± 0.9	0.018 ± 0.007
W359A	1539 ± 24	5.0 ± 0.4	308 ± 30	517 ± 27	54 ± 7	9.6 ± 1.3	0.031 ± 0.007
W359H	410 ± 33	1.6 ± 0.3	256 ± 69	627 ± 28	34 ± 3	18.8 ± 2.6	0.073 ± 0.029

Table 1

FIGURES

Figure 1. Relationships between FprA and diverse adrenodoxin reductase (AdR) and AdR-like enzymes. Figure 1A shows a phylogram demonstrating evolutionary relationships between *Mtb* FprA and various AdR-like enzymes. Data were generated from a multiple protein alignment using the ClustalW program via the EBI server. Figure 1B shows a segment of a multiple amino acid sequence alignment of *Mtb* FprA with the other AdR-like enzymes. Trp359 in FprA is completely conserved (shown in bold). *Mtb* FprA is most highly related to other mycobacterial AdR-like enzymes (99.5 % identity with *M. bovis* FprA), but exhibits high levels of amino acid sequence identity with e.g. certain insect AdR's (39 % identity with *D. melanogaster* AdR) and with mammalian AdR's (39 % identity with bovine AdR; 43 % identity with human AdR). Enzymes shown are human AdR (humanAdR); horse (*Equus caballus*) AdR (horseAdR); rat (*Rattus norvegicus*) AdR (RatAdR); bovine (*Bos taurus*) AdR (BostaurusAdR); *Drosophila melanogaster* AdR (DrosophAdR); *M. tuberculosis* FprA (Mtb); *M. bovis* FprA (Mbov); *M. avium* FprA (Mavium); *M. ulcerans* FprA (Mulc); *Saccharomyces cerevisiae* Yrh1p (Yrh1p); and *Plasmodium falciparum* AdR (PfalcAdR).

Figure 2. Structural organization of *Mtb* FprA. Figure 2A shows the atomic structure of *Mtb* FprA with secondary structural elements indicated in cyan (α -helices) and magenta (β -sheets). NADP⁺ coenzyme (green) and FAD cofactor (yellow) are shown in spacefill [6]. The proximity of the FAD (isoalloxazine ring) and NADP⁺ is evident. Figure 2B focuses on the immediate environment of the FAD-binding site. The FAD (lower) and NADP⁺ (upper) are in stick representation with carbon atoms in yellow and other atoms in conventional colours. Trp359 is indicated in magenta/blue, and is located on a loop region projecting towards the flavin ring system.

Figure 3. UV-visible absorption properties of WT FprA and W359A/H mutants. UV-visible absorption spectra are shown for WT FprA (panel A, 28 μ M), W359A mutant (panel B, 26.5 μ M) and W359H mutant (panel C, 25.5 μ M). Spectra are shown for oxidized enzymes (thick solid line); for enzymes reduced by NADPH (300 μ M, thin solid line); NADH (300 μ M, dashed line) and sodium dithionite (dotted line). Substantial amounts of blue SQ are stabilized only for NADPH-reduced WT FprA.

Figure 4. Transient kinetic analysis of the reduction of W359A/H mutants. Panel A (main figure) shows the hyperbolic dependence of FAD reduction rate (from FAD A₄₅₂ bleaching) for the reaction of the W359H FprA mutant with various concentrations of NADH. Equation 1 was used to fit the data to produce parameters of $k_{\text{lim}} = 13.4 \pm 0.4 \text{ s}^{-1}$ and $K_d = 119.2 \pm 12.0 \text{ }\mu\text{M}$. The inset shows a typical stopped-flow transient for reaction of W359H FprA with NADH (100 μ M), with the dotted line indicating the fit obtained using a single exponential function. Panel B shows data from PDA analysis of the reaction of FprA W359A (10 μ M) with NADH (100 μ M). Selected spectra are shown, collected at regular intervals following the mixing event from 0.0038 s to 0.9715 s. Negligible absorption change was observed after this time. The arrow indicates direction of flavin absorption change. Absence of absorption change in the 500-700 nm region indicates no significant formation of the neutral blue FAD SQ during the reaction.

Figure 5. Potentiometric analysis of the FprA W359A mutant. The main figure shows spectral changes on redox titration of W359A FprA (45 μ M). The inset shows a plot of absorption change (A₄₅₂) versus applied potential. A 2-electron Nernst function was used to fit the data and to define

the midpoint reduction potential of the W359A FAD oxidized/HQ transition as -168 ± 4 mV. The midpoint FAD potential for the W359H FprA mutant was similarly determined as -181 ± 8 mV.

Stage 2(a) POST-PRINT

Figure 1A

HumanAdR	-KLGVIPNVEGRVMD-----VPGLYCSGWVKRGPTGVIATTMTDSFLTQMLLQDLK-	432
horseAdR	-KLGVIPNMEGRVVD-----VPGLYCSGWVKRGPTGVIATTMTDSFLTQMLLQDLK-	398
ratAdR	-KLGIIIPNTEGRVFN-----APGLYCSGWVKRGPTGVITTTMTDSFLTSQVLLKDLK-	429
BostaurusAdR	-KLGVPNMEGRVVD-----VPGLYCSGWVKRGPTGVITTTMTDSFLTQIILLQDLK-	427
DrosophAdR	-RRGRVHNINGRILKDDATGEVDPGLYVAGWLGTPGTGVIIVTTMNGAFVAKTICDDIN-	407
Mtb	-QSGTIPNVGGRING-----SPNEYVVGWIKRGPTGVIGTNKKDAQDVTDTLIKDLGN	388
Mbov	-QSGTIPNVGGRING-----SPNEYVVGWIKRGPTGVIGTNKKDAQDVTDTLIKDLGN	388
Mavium	-KTATIPNTGGRVEG-----SRNEYVVGWIKRGPTGVIGTNKKDSQDVTDTLLADLAN	388
Mulc	-NSATIPHSGRVAG-----SSNEYVVGWIKRGPTGVIGTNKKDSQDVTDTLLLEDLAH	389
Yrh1p	FDKDHIANKQGRVLTSSGEIFPHLYASGWIRKGSQGVIASTMQDAFEVGDRIQDLVV	436
PfalcAdR	-YNKSVDKYKDDILN-----KKFAIFKAGWFQTGAKGNIASHIINTKQNTTEILNFLN-	441
	: : . : : ** . * . * * : . : : :	

Figure 1B

Figure 2A

Figure 2B

Figure 3

Figure 4

Figure 5