Protonation state of F420H2 in the prodrug-activating deazaflavin dependent nitroreductase (Ddn) from Mycobacterium tuberculosis†

A. Elaaf Mohamed, A. Elaaf Mohamed, Sundaram Arulmozhiraja, Ching Y. Lin, Matthew C. Taylor, Elmars R. Krausz, Colin J. Jackson* and Michelle L. Coote*

The protonation state of the deazaflavin dependent nitroreductase (Ddn) enzyme bound cofactor F420 was investigated using UV-visible spectroscopy and computational simulations. The reduced cofactor F420H2 was determined to be present in its deprotonated state in the holoenzyme form. The mechanistic implications of these findings are discussed.

The discovery of cofactor F420 (Fig. 1), and a new family of F420-dependent enzymes, in a wide range of Actinobacteria, including pathogenic mycobacteria,1–4 has led to increased interest in F420 dependent oxidoreductases (FDORs). The absence of F420 dependent biochemical reactions in humans, and their prevalence in Mycobacterium tuberculosis (TB), the causative agent of tuberculosis, has made the enzymes that depend on F420 (Fig. 2) a new family of anti-mycobacterial pro-drugs.1–4,6–11 While the deazaflavin F420 is structurally analogous to riboflavin based cofactors such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), it is functionally closer to nicotinamide (e.g. NAD) cofactors by being involved in redox reactions as a hydride carrier involved in two electron transfer mechanisms.12 Consistent with its involvement in redox reactions, F420 exists in both oxidized (F420) and reduced (F420H2) forms, as shown in Fig. 2. The oxidized cofactor exhibits strong and distinct UV-visible spectroscopic properties, with a characteristic absorbance peak at 420 nm that is different to the spectroscopic properties of the reduced species.5,11 This allows for the study of F420 dependent reactions using UV/vis spectroscopy to follow changes in the amounts of the oxidized cofactor being formed or consumed.1,4,6,7,11 These spectroscopic properties are also sensitive to pH changes, with the structural changes due to deprotonation of the 8-OH and 1-NH groups in the oxidized and reduced species (Fig. 2), respectively, influencing the absorbance bands.

Although the role of both Ddn and F420 in the activity of pretomanid is known,1,6,8,11 the precise catalytic mechanism is not fully understood. Thus, a better understanding of this reaction and the configuration and protonation state of the reactants within the enzyme’s active site is essential to further improving this class of anti-mycobacterial pro-drugs.

Fig. 1 Structures of F420 (left) and pretomanid (right).

The protonation state of F420H2 in the prodrug-activating deazaflavin dependent nitroreductase (Ddn) from Mycobacterium tuberculosis†

A. Elaaf Mohamed, F. Hafna Ahmed, Sundaram Arulmozhiraja, Ching Y. Lin, Matthew C. Taylor, Elmars R. Krausz, Colin J. Jackson* and Michelle L. Coote*

The protonation state of the deazaflavin dependent nitroreductase (Ddn) enzyme bound cofactor F420 was investigated using UV-visible spectroscopy and computational simulations. The reduced cofactor F420H2 was determined to be present in its deprotonated state in the holoenzyme form. The mechanistic implications of these findings are discussed.

The discovery of cofactor F420 (Fig. 1), and a new family of F420-dependent enzymes, in a wide range of Actinobacteria, including pathogenic mycobacteria,1–4 has led to increased interest in F420 dependent oxidoreductases (FDORs). The absence of F420 dependent biochemical reactions in humans, and their prevalence in Mycobacterium tuberculosis (TB), the causative agent of tuberculosis, has made the enzymes that depend on F420 (Fig. 2) a new family of anti-mycobacterial pro-drugs.1–4,6–11 While the deazaflavin F420 is structurally analogous to riboflavin based cofactors such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), it is functionally closer to nicotinamide (e.g. NAD) cofactors by being involved in redox reactions as a hydride carrier involved in two electron transfer mechanisms.12 Consistent with its involvement in redox reactions, F420 exists in both oxidized (F420) and reduced (F420H2) forms, as shown in Fig. 2. The oxidized cofactor exhibits strong and distinct UV-visible spectroscopic properties, with a characteristic absorbance peak at 420 nm that is different to the spectroscopic properties of the reduced species.5,11 This allows for the study of F420 dependent reactions using UV/vis spectroscopy to follow changes in the amounts of the oxidized cofactor being formed or consumed.1,4,6,7,11 These spectroscopic properties are also sensitive to pH changes, with the structural changes due to deprotonation of the 8-OH and 1-NH groups in the oxidized and reduced species (Fig. 2), respectively, influencing the absorbance bands.

Although the role of both Ddn and F420 in the activity of pretomanid is known,1,6,8,11 the precise catalytic mechanism is not fully understood. Thus, a better understanding of this reaction and the configuration and protonation state of the reactants within the enzyme’s active site is essential to further improving this class of anti-mycobacterial pro-drugs.

While the deazaflavin F420 is structurally analogous to riboflavin based cofactors such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), it is functionally closer to nicotinamide (e.g. NAD) cofactors by being involved in redox reactions as a hydride carrier involved in two electron transfer mechanisms.12 Consistent with its involvement in redox reactions, F420 exists in both oxidized (F420) and reduced (F420H2) forms, as shown in Fig. 2. The oxidized cofactor exhibits strong and distinct UV-visible spectroscopic properties, with a characteristic absorbance peak at 420 nm that is different to the spectroscopic properties of the reduced species.5,11 This allows for the study of F420 dependent reactions using UV/vis spectroscopy to follow changes in the amounts of the oxidized cofactor being formed or consumed.1,4,6,7,11 These spectroscopic properties are also sensitive to pH changes, with the structural changes due to deprotonation of the 8-OH and 1-NH groups in the oxidized and reduced species (Fig. 2), respectively, influencing the absorbance bands.

The protonation state of the cofactor will significantly influence the catalytic mechanism owing to the availability or unavailability of labile hydrogens. However, in the current literature this has not been addressed and F420H2 is often presented as its neutral species.6 In this study we have determined

* Research School of Chemistry, The Australian National University, Canberra, ACT 2601, Australia. E-mail: colin.jackson@anu.edu.au, michelle.coote@anu.edu.au
b CSIRO Land and Water, Black Mountain Laboratories, Canberra, ACT 2601, Australia
† ARC Centre of Excellence for Electromaterials Science
‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c6mb00033a
The protonation state of F₄₂₀ and F₄₂₀H₂ when bound to Ddn, and a homolog of Ddn from *Mycobacterium smegmatis*, MSMEG_2027, using a combination of UV-visible spectroscopy and time-dependent density functional theory (TD-DFT) calculations. Initially, absorbance spectra of both F₄₂₀ and F₄₂₀H₂ were measured in an aqueous environment as a function of pH so as to assign the neutral and deprotonated spectra (Fig. 3; Fig. S1 in ESI†). However, in order to ensure that these assignments do not change in the lower polarity environment within the enzyme active site, we also used TD-DFT to calculate the neutral and deprotonated spectra under both aqueous and low polarity solvent environments (Fig. 4).

For F₄₂₀, the characteristically intense absorbance peak at 420 nm gives way to a weaker peak at 400 nm at lower pH values with the observed pKₐ of 6.5 (Fig. 3a) agreeing with previously reported values of 6.3,14,15 The spectroscopic red-shift from 400 to 420 nm is also indicative of stabilization of the π-π* transitions stemming from the deprotonation of the 8-OH group. For F₄₂₀H₂, its characteristic peak at 320 nm did not exhibit the strong red-shift upon deprotonation observed in F₄₂₀ instead it was the neutral species that was slightly red-shifted to 322 nm with the deprotonated species at 319 nm and consistent with literature for both F₄₂₀ as well as the structural analogue 1,5-dihydrodeazariboflavin.15,16 Rather, a more noticeable spectral change is seen at 260 nm with a peak present in the deprotonated species. Both the 320 nm and 260 nm peaks exhibit a pKₐ near neutrality at 7.1 (Fig. 3b). These results agree with previously published literature values for F₄₂₀H₂ of 6.9 and 7.2 for F₄₂₀H₂ and 1,5-dihydrodeazariboflavin.15,16

Although the computationally derived electronic transitions for both species deviated from the experimental values, their relative values, and the trends that are observed, agree closely with the experimental spectrum (Fig. 4). For both species it was also noted that the electronic transitions responsible for the absorbance peaks remained in agreement for both the aqueous and low polarity environment with ε = 80.1 and the low polarity environment with ε = 12. This uniform behaviour enables us to directly compare experimental UV-visible spectra of the cofactor in aqueous conditions with that of when the cofactor is bound within the active site to complete the holoenzyme. The full comparisons of the simulated electronic transitions with experiment are in Fig. S2 in ESI.†

To determine the protonation state in the enzyme, the bound absorbance spectrum was measured with F₄₂₀H₂ bound to the Ddn and 2027 enzymes (Fig. 5; Fig. S4 in ESI†). The results
cofactor F$_{420}$H$_2$ is bound and stabilized within the active site in aqueous conditions. On this basis we can conclude that the protonation state of F$_{420}$/F$_{420}$H$_2$ is essential for further mechanistic studies involving the cofactor F$_{420}$H$_2$ and Ddn. This result suggests that the proton transfer step of the reaction, which completes the 2H$^+$/2e$^-$ reduction, has to be facilitated via a source other than from the cofactor. Additionally, the deprotonation of the reduced cofactor enables it to readily form the more stable neutral molecule F$_{420}$ following the hydride transfer instead of a less stable carbocation that would result if the protonated species were involved. There are several ionisable sidechains in the vicinity of the cofactor that could act as general acids in this reaction; their potential involvement is currently under investigation. Conversely, it has also been suggested that the protonation of pretomanid is completed by water molecules from the surrounding environment. A proposed mechanism utilizing water molecules from the surrounding environment is shown in Fig. 7, where initial hydride transfer is followed by a proton transfer event. These steps result in the formation of oxidized F$_{420}$ and reduced pretomanid-H$_2$, which then undergoes heterolytic bond cleavage and bond formation to produce the anti-mycobacterial nitric oxide species.

Fig. 6 Crystal structure of F$_{420}$ bound within Ddn (PDB ID: 3R5R), with the substrate pretomanid docked via simulation. The electrostatics of the surrounding residues reveals the positively charged surroundings which help bind and stabilize the negatively charged deprotonated cofactor. The residues that contribute to the positive environment are shown and labelled. Scale is in kT/e.

substrate within the active site, which shows how the deprotonated species might be stabilized by the surrounding positively-charged electrostatic environment.

Correctly assigning the protonation state of F$_{420}$/F$_{420}$H$_2$ is essential for further mechanistic studies involving the cofactor F$_{420}$H$_2$ and Ddn. This result suggests that the proton transfer step of the reaction, which completes the 2H$^+$/2e$^-$ reduction, has to be facilitated via a source other than from the cofactor. Additionally, the deprotonation of the reduced cofactor enables it to readily form the more stable neutral molecule F$_{420}$ following the hydride transfer instead of a less stable carbocation that would result if the protonated species were involved. There are several ionisable sidechains in the vicinity of the cofactor that could act as general acids in this reaction; their potential involvement is currently under investigation. Conversely, it has also been suggested that the protonation of pretomanid is completed by water molecules from the surrounding environment. A proposed mechanism utilizing water molecules from the surrounding environment is shown in Fig. 7, where initial hydride transfer is followed by a proton transfer event. These steps result in the formation of oxidized F$_{420}$ and reduced pretomanid-H$_2$, which then undergoes heterolytic bond cleavage and bond formation to produce the anti-mycobacterial nitric oxide species.

The holoenzyme structure solved by Cellitti et al. was obtained with the oxidized species F$_{420}$ at pH 6.5. Therefore the cofactor would be deprotonated, as would be true for F$_{420}$H$_2$ at physiological pH: both molecules would lack the N1 hydrogen, although the hydroxyl group at C8 would most likely be deprotonated in F$_{420}$H$_2$. Since the C8–OH group extends into the solvent, this difference is unlikely to significantly affect substrate binding, making the crystal structure a good model for the holoenzyme structure. Our results provide some insight into the substrate specificity of Ddn since the substrate range would be relatively broad if the proton transferred to C2 could be donated from a side-chain or protein-stabilized water
molecule, there are significant constraints on the substrate range, given that the substrates must be in sufficiently close contact with the general acid for proton transfer to occur. This model is consistent with previous work that showed there is little enantioselectivity with smaller pretomanid analogs, such as CGI-17341 and phenyl oxazole, since the C2 atom will be in the same approximate position in either enantiomer.11

In summary, we determined the protonation state of the cofactor F$_{420}$H$_2$ when bound to the Ddn active site. This has a significant impact on our understanding of the F$_{420}$H$_2$ mediated activation mechanism as it implies that maintaining the deprotonated state of F$_{420}$H$_2$ contributes to the catalytic efficiency of the enzyme and that the proton source to complete the reaction is not F$_{420}$H$_2$ itself, but a separate residue or nearby molecule involved within the active site.

MLC and CJJ gratefully acknowledge funding from the Australian Research Council in the form of Discovery Project funding (DP130102144) and ARC Future Fellowships. MLC also acknowledges generous allocations of supercomputing time on the National Facility of the Australian National Computational Infrastructure.

Notes and references


