Structure of Ddn, the Deazaflavin-Dependent Nitroreductase from *Mycobacterium tuberculosis*

Involved in Bioreductive Activation of PA-824

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**Figure S1. Characterization of WT and truncated Ddn**

(A) Preparative size exclusion chromatography illustrates the difference in behavior of full-length (red/top) and truncated (blue/middle) Ddn. While the full-length protein elutes near the void volume as a soluble aggregate, the NΔ30 truncation mutant elutes as expected for a monomeric protein. BioRad standards (black) are included for comparison. Both proteins were expressed from SpeedET. NΔ30 and standards were run in PBS; full-length was run in 25 mM citrate pH 6.5, 150 mM NaCl.

(B) NMR spectra of NΔ30 with and without coenzyme F$_{420}$. Dispersed peaks in these $^1$H-$^{15}$N-HSQC spectra suggest a well folded protein with a distinct three dimensional structure. Shifted peaks upon addition of coenzyme indicate tight binding. The apo spectrum (blue) includes 113 of an expected 114 peaks (including N-terminal Gly from TEV site). The oxidized (red) and reduced (green) coenzyme spectra contain 100 and 84 peaks respectively. Partial assignments for the apo- and oxidized F$_{420}$ spectra are presented in Supplementary Table 2.
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**Supplementary Figure S2, Related to Figure 2**

![Figure S2](image)

**Figure S2. Predicted N-terminal helix of Ddn**

Residues 13-28 are predicted to form a helix with a strongly hydrophobic face, as shown in this projection(Armstrong and Zidovetzki). This may be the cause of the aggregation of the full-length protein. It is unclear if Ddn is membrane-associated *in vivo*, but it has been identified in detergent extractions of *M. tuberculosis* as described in the main text(Sinha et al., 2002; Sinha et al., 2005). The N-termini of Ddn and similar predicted split barrel-like proteins vary greatly and may be important for their native functions.
**Figure S3. Comparison of NΔ30 and helix mutants**

Initially, mutations were made to the predicted N-terminal helix in order to remodel the hydrophobic face and prevent aggregation. Like the N-terminal truncations, these mutants behaved as monomeric proteins as determined by analytical size exclusion chromatography (see Table S1).

(A) The $^1$H-$^{15}$N-HSQC spectra of these mutants (F16A-F17D-W20D-I24A-W27D full-length Ddn shown here in blue) are very similar to that of NΔ30 (in red). This suggests that the N-termini of the mutants undergo conformational exchange.

(B) Constructs were made to introduce up to three salt bridges into the putative N-terminal helix in order to stabilize its structure. The $^1$H-$^{15}$N-HSQC spectra of these full-length Ddn mutants (D15E-F16A-F17D-W20D-I24A-W27D shown here) are very similar to that of NΔ30, except that there are approximately 18 additional NH peaks (out of 25 possible). This suggests a change in the kinetics of the conformational exchange process. However, the lack of shifts (beyond error in linewidths) among the shared peaks suggests that the N-terminus is not interacting with the core of the protein in the mutants.
Table S2. Partial NMR chemical shift assignments for NΔ30 with and without F$_{420}$

| Residue | Apo  | with F$_{420}$$^1$ | | Residue | Apo  | with F$_{420}$$^1$ |
|---------|------|------------------|--|---------|------|------------------|
| N32     | 120.541 | 120.622 | 8.518 | V73     | 122.177 | 8.745 |
| G33     | 120.883 | 120.795 | 8.302 | i74     | 131.897 | 8.29 |
| G34     | 108.815 | 108.807 | 8.302 | V75     | 117.375 | 9.056 |
| E35     | 120.424 | 120.469 | 8.151 | A76     | 121.683 | 8.069 |
| G36     | 109.654 | 109.592 | 8.376 | A77     | 119.301 | 8.016 |
| L37     | 121.455 | 121.406 | 8.103 | E83     | 118.509 | 7.981 |
| G38     | 109.342 | 109.389 | 8.45  | K84     | 121.756 | 8.046 |
| G39     | 108.52  | 108.558 | 8.185 | N85     | 117.474 | 8.563 |
| T40     | 113.247 | 113.358 | 7.954 | M87     | 122.169 | 8.699 |
| F41     | 121.997 | 122.035 | 8.176 | W88     | 115.119 | 7.603 |
| Q42     | 121.455 | 121.892 | 8.103 | Y89     | 122.769 | 5.667 |
| K43     | 122.457 | 122.565 | 8.242 | L90     | 117.212 | 7.318 |
| I44     | 123.723 | 124.119 | 8.043 | N91     | 117.542 | 8.607 |
| V46     | 116.069 | 115.187 | 8.003 | L92     | 119.53  | 7.719 |
| A47     | 125.545 | 126.718 | 9.109 | K93     | 120.47  | 7.552 |
| L48     | 116.067 | 117.917 | 8.479 | K94     | 120.391 | 6.951 |
| L49     | 130.664 | 129.968 | 8.838 | N95     | 114.945 | 7.808 |
| T50     | 127.455 | 126.572 | 9.742 | K97     | 119.204 | 7.487 |
| T51     | 116.14  | 115.949 | 8.868 | V98     | 119.101 | 8.645 |
| T52     | 115.916 | 117.721 | 8.907 | Q99     | 118.984 | 7.905 |
| G53     | 114.885 | 116.594 | 8.554 | V100    | 123.158 | 9.029 |
| R54     | 126.677 | 126.004 | 7.911 | Q101    | 127.278 | 8.859 |
| K55     | 118.293 | 119.584 | 8.57  | I102    | 128.32  | 8.462 |
| T56     | 114.935 | 111.517 | 8.549 | K103    | 126.059 | 9.124 |
| G57     | 110.231 | 110.698 | 7.912 | K104    | 121.281 | 8.256 |
| Q58     | 119.208 | 118.856 | 7.723 | E105    | 122.638 | 8.11 |
| R60     | 124.768 | 125.501 | 9.239 | V106    | 124.84  | 8.277 |
| V61     | 124.334 | 126.222 | 8.484 | L107    | 128.469 | 9.108 |
| N62     | 124.984 | 124.763 | 8.977 | D108    | 124.841 | 8.441 |
| L64     | 127.398 | 123.939 | 9.27  | L109    | 123.885 | 8.083 |
| Y65     | 121.496 | 116.77  | 7.663 | T110    | 116.92  | 9.203 |
| F66     | 113.639 | 132.978 | 7.908 | A111    | 132.901 | 9.82 |
| L67     | 116.244 | 117.447 | 8.871 | R112    | 117.976 | 8.941 |
| R68     | 121.148 | 121.884 | 8.764 | D113    | 120.002 | 8.8 |
| D69     | 123.367 | 123.4    | 7.981 | A114    | 123.756 | 8.558 |
| G70     | 116.14  | 116.203 | 8.986 | T115    | 117.226 | 9.805 |
| G71     | 116.377 | 116.24  | 9.023 | D116    | 122.009 | 9.174 |
| R72     | 116.153 | 116.139 | 7.792 | E117    | 119.693 | 8.6 |

$^1$Samples were titrated with F$_{420}$ to full occupancy before data acquisition for assignments.
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Supplementary Table S2, Related to Figure 3

Table S2 (cont.)

| Residue | Apo N (ppm) | Apo HN (ppm) | with F<sub>420</sub><sup>1</sup> N (ppm) | with F<sub>420</sub><sup>1</sup> HN (ppm) |
|---------|-------------|--------------|---------------------------------|------------------|
| Y122    | 115.978     | 8.595        |                                 |                  |
| W123    | 121.243     | 8.805        |                                 |                  |
| Q125    | 114.374     | 6.719        |                                 |                  |
| L126    | 122.24      | 7.953        |                                 |                  |
| Y136    | 119.599     | 7.76         |                                 |                  |
| Q137    | 119.419     | 8.276        |                                 |                  |
| S138    | 113.16      | 7.834        |                                 |                  |
| D141    | 120.699     | 7.342        |                                 |                  |
| R142    | 121.851     | 8.112        |                                 |                  |
| T143    | 111.755     | 8.202        |                                 |                  |
| I144    | 122.465     | 7.352        |                                 |                  |
| I146    | 121.225     | 7.973        |                                 |                  |
| V147    | 127.931     | 9.076        |                                 |                  |
| V148    | 126.807     | 9.144        |                                 |                  |
| C149    | 125.545     | 9.109        | 125.142                         | 9.084            |
| E150    | 123.352     | 8.981        | 123.03                          | 8.947            |
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Supplementary Figure S4, Related to Figure 5

**Figure S4. NMR characterization of nfa33440**

(A) The $^1$H-$^1$5N-HSQC spectrum of apo-nfa33440 includes 107 of 135 expected peaks (blue). Although no assignments have been made, the number of peaks is suggestive of a structured protein core with the N-terminus undergoing conformational exchange in the intermediate time regime. Robust shifts upon addition of $F_{420}$ confirm tight binding of the coenzyme. In the presence of $F_{420}$ (red), there are approximately 90 peaks observed. This is similar to the case for Ddn NAΔ30 in which parts of the active site are no longer visible upon binding to coenzyme.

(B) Addition of PA-824 does not change the number of peaks observed (red), but there are chemical shift perturbations of some of the weaker peaks. This further supports the hypothesis that it is the active site residues that are undergoing exchange and that binding of the substrates of the enzyme affects these dynamic processes.
Figure S5. NMR characterization of nfa33440 mutants
Proton spectra of the indicated mutants are shown. All samples were acquired in PBS using excitation sculpting for water suppression (Hwang and Shaka, 1995). The appearance of the spectra for all the mutants is very similar to wild-type protein and confirms they are well-folded with similar structures. The peaks for M59A have the largest chemical shift changes compared to wild-type and suggest this mutation may have had the most significant impact on the structure. The mutants W16A and T126A are missing peaks at 10.13 and 6.05 ppm respectively, probably corresponding to the amino acid amide protons.
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**Supplementary Table S3, Related to Table 2**

Table S3. Half-height linewidth\(^1\) of the 0.45 ppm methyl peak of nfa33440 mutants at different protein to \(F_{420}\) coenzyme ratios

| Sample | Apo | 1.4:1 \(F_{420}\) | 2:1 \(F_{420}\) | 3:1 \(F_{420}\) |
|--------|-----|----------------|---------------|---------------|
| I138A  | 23  | 37            | 20            |
| R136Q  | 24  | 37            | 24            |
| F136A  | 21  | 40            | 25            |
| E129A  | 16  | 37            | 19            |
| T126V  | 16  | 46            | 22            |
| Q73A   | 15  | 18            | 15            |
| S72V   | 18  | 26            |               |
| M59A   | 17  | 117           | 85            | 39            |
| F35A   | 18  | 50            | 39            |
| W16A   | 17  | 46            | 26            |
| WT     | 17  | 42            | 18            |

\(^1\)The linewidth is sensitive to \(F_{420}\) occupancy and increases when the protein is a mixture of free and bound - presumably due to exchange between apo and complex. Consequently, the concentration of coenzyme required to achieve a linewidth comparable to apo protein is a useful indication of the concentration required for 100% occupancy and thus the affinity. The results indicate that the only mutant with compromised coenzyme binding is M59A.
Table S4. PA-824 chemical shift difference and T1p intensity loss in the presence of nfa33440 mutants

| Sample | δ-δapo [Hz] | T1p intensity loss [%] |
|--------|-------------|------------------------|
|        | PA-824 Peak (ppm) | PA-824 Peak (ppm) |
|        | 7.775 | 7.459 | 7.444 | 7.339 | 7.325 | 7.775 | 7.459 | 7.444 | 7.339 | 7.325 |
| S72V   | -2.7 | -1.9 | -1.9 | -2.6 | -1.7 | no signals for PA-824 (DMSO peak only) |
| M59A   | -1.6 | -0.4 | -0.4 | -1.3 | -0.2 | 14 | 14 | 14 | 25 | 20 |
| F35A   | -2.2 | -2.5 | -2.5 | -2.5 | -2.3 | 13 | 20 | 18 | 18 | 25 |
| I138A  | -2.0 | -2.0 | -2.0 | -2.6 | -2.0 | 22 | 22 | 22 | 35 | 35 |
| R136Q  | -2.2 | -2.5 | -2.5 | -2.5 | -2.3 | 13 | 20 | 18 | 18 | 25 |
| Q73A   | -32.6 | -4.0 | -3.9 | -4.1 | -4.1 | 71 | 35 | 35 | 44 | 44 |
| W16A   | -37.3 | 2.3 | 2.2 | 3.8 | -4.1 | 72 | 32 | 32 | 58 | 58 |
| T126V  | -21.1 | -4.4 | -4.4 | -4.2 | -3.8 | 71 | 23 | 23 | 56 | 56 |
| E129A  | -30.8 | -3.1 | -3.1 | -2.7 | -3.1 | 82 | 34 | 34 | 55 | 55 |
| WT     | -17.3 | -3.6 | -3.5 | -3.0 | -3.4 | 78 | 25 | 25 | 57 | 57 |

1. Samples were prepared with the listed mutant at a protein-to-F420 ratio of 1:1.4 to 1:2 and a protein-to-PA-824 ratio of 1:0.6.
2. The chemical shift changes in Hz were measured for five peaks of PA-824 by subtracting the chemical shift in the presence of protein from those measured in buffer alone. For a two state system undergoing fast exchange (k>>2πΔδ), the chemical shift should reflect the average of the two populations (Wuthrich, 1986). Assuming that the chemical shift of the bound form is similar for all mutants, a larger chemical shift change reflects a greater bound fraction and provides an estimate of the relative affinity.
3. T1p data were also acquired to provide additional confirmation. In this experiment, spectra were acquired using a CPMG sequence with a short (10 ms) and long (200 ms) mix time, and the percent intensity loss was determined (Intensity loss = 1-(Intensity200ms/Intensity10ms)). The intensity loss is related to the fraction bound (Dalvit, 2009).
4. The grey shaded mutants appear to have altered PA-824 binding affinity based on T1p and chemical shift difference data.
5. For S72V there is no indication of free compound peaks despite the appearance of a DMSO peak (compound was added from a DMSO stock). These data suggest that compound binding is in the intermediate exchange regime (k≈2πΔδ) resulting in significant line broadening. This is unlike all the other mutants and wild-type where PA-824 is clearly in a fast-exchange regime and suggests that this mutant may have increased affinity for the prodrug. This may be due to a change in the binding pose or a stronger hydrophobic interaction with the substrate but does not represent a more efficient orientation for reduction based on the activity measurements.
Figure S6. PA-824 titration of nfa33440 S72V

(A) Overlaid proton spectra of 50 μM S72V with 30, 60, and 90 μM PA-824 in green, red, and blue respectively.

(B) Overlaid spectra for 50 μM wild-type protein alone (red) and upon the addition of 30 μM PA-824 (blue). In each panel the spectrum for PA-824 is offset in order to readily identify compound peaks. Sharp peaks corresponding to PA-824 were observed for wild-type nfa33440 at a protein-to-drug ratio of 1:0.6. Compound peaks were not observed for the S72V mutant until much higher concentrations (a protein-to-drug ratio of 1:1.8) and were very broad. These data suggest that the S72V mutant has higher PA-824 affinity.
Supplementary Figure S7, Related to Table 2

Figure S7. Cartoon of Ddn (Holo-1) and nfa33440 showing positions of residues mutated for functional studies. Residues tested that are also present in the structure are shown as sticks and colored in groups related to the presentation in Table 2.
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**Supplementary Figure S8, Related to Figure 7**

*Figure S8. Circular dichroism of the wild type Ddn (MPB-WT, black), maltose binding protein (MBP, red), F16A-F17A-W20A-Ddn (MBP-triple, green) and F16A-F17A-Ddn (MBP-double, blue) is represented as a plot of g-factor vs. wavelength*
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**Supplementary Table S5, Related to Figure 7**

Table S5. Relative percentage of secondary structure components as derived from circular dichroism measurements of the wild type Ddn (MPB-WT, black), maltose binding protein (MBP, red), F16A-F17A-W20A-Ddn (MBP-triple, green) and F16A-F17A-Ddn (MBP-double, blue)

|        | MBP-WT | MBP       | MBP-Triple | MBP-Double |
|--------|--------|-----------|------------|------------|
| helix  | 26+/-1.4% | 33+/-2.5% | 26+/-1.2%  | 28+/-1.4%  |
| sheet  | 30+/-1.5  | 28+/-2.4  | 31+/-1.2   | 30+/-1.3   |
| turn   | 11+/-1.1  | 12+/-1.9  | 11+/-1.0   | 12+/-1.1   |
| rem.   | 33+/-1.1  | 28+/-1.9  | 31+/-0.9   | 30+/-1.1   |

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