Unusual Spectroscopic and Ligand Binding Properties of the Cytochrome P450-Flavodoxin Fusion Enzyme XplA

Soi H. Bui 1, Kirsty J. McLean 1, Myles R. Cheesman 9, Justin M. Bradley 1, Stephen E. J. Rigby 1, Colin W. Levy 1, David Leys 1, and Andrew W. Munro 1,2

From the 1 Manchester Interdisciplinary Biocentre, Faculty of Life Sciences, University of Manchester, 131 Princess Street, Manchester M1 7DN, United Kingdom and the 2 School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom

Background: XplA is a P450-flavodoxin fusion catalyzing reduction of the explosive RDX.

Results: XplA has unusual properties of high P450 affinity for imidazole and weak flavodoxin FMN binding.

Conclusion: Detailed structural and spectroscopic analysis of XplA explain non-standard P450/flavodoxin properties reported previously.

Significance: Specialization of XplA as an RDX reductase involves perturbations to its cofactors’ thermodynamic properties and evolution of a constrained P450 active site.

The Rhodococcus rhodochrous strain 11Y XplA enzyme is an unusual cytochrome P450-flavodoxin fusion enzyme that catalyzes reductive denitration of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazene (RDX). We show by light scattering that XplA is a monomeric enzyme. XplA has high affinity for imidazole (Kd = 1.6 μM), explaining previous reports of a red-shifted XplA Soret band in pure enzyme. The true Soret maximum of XplA is at 417 nm. Similarly, unusually weak XplA flavodoxin FMN binding (Kd = 1.09 μM) necessitates its purification in the presence of the cofactor to produce hallmark flavin contributions absent in previously reported spectra. Structural and ligand-binding data reveal a constricted active site able to accommodate RDX and small inhibitory ligands (e.g. 4-phenylimidazole and morpholine) while discriminating against larger azole drugs. The crystal structure also identifies a high affinity imidazole binding site, consistent with its low Kd, and shows active site penetration by PEG, perhaps indicative of an evolutionary lipid-metabolizing function for XplA. EPR studies indicate heterogeneity in binding mode for RDX and other ligands. The substrate analog trinitrobenzene does not induce a substrate-like type I optical shift but creates a unique low spin EPR spectrum due to influence on structure around the distal water heme ligand. The substrate-free heme iron potential (−268 mV versus NHE) is positive for a low spin P450, and the elevated potential of the FMN semiquinone/hydroquinone couple (−172 mV) is also an adaptation that may reflect (along with the absence of a key Thr/Ser residue conserved in oxygen-activating P450s) the evolution of XplA as a specialized RDX reductase catalyst.

The capacity of bacteria to transform and metabolize synthetic organic compounds is now a well recognized hallmark of the metabolic flexibility and rapid evolutionary capacity of such organisms (1). Examples include evolution of aerobic and anaerobic pathways to catabolism of polychlorinated biphenyls, major pollutants of the environment (2). Aerobic pathways are widespread in Gram-negative bacteria, often involving a biphenyl 2,3-dioxygenase to initiate the degradation pathway (3). Reductive dechlorination is observed in anaerobic bacteria occupying polychlorinated biphenyl-contaminant sediments, and dehalogenases from Dehalococcoides and Clostridium spp. are likely to contribute toward anaerobic polychlorinated biphenyl degradation (4, 5). There are several other examples of microbial degradation of unusual compounds for energy generation and/or detoxification. For example, bacterial pathways for the breakdown of molecules, such as camphor (in Pseudomonas putida) and thiocarbamate herbicides (in Rhodococcus spp.), have also been well studied (6–8).

Recent decades have seen increasing concern regarding the environmental accumulation of explosives and the ramifications for their toxicity in biological systems (9). Among the most widely used explosives are the nitro-substituted molecules 2,4,6-trinitrotoluene and hexahydro-1,3,5-trinitro-1,3,5-triazene (RDX). These are listed as “priority pollutants” and as “possible human carcinogens” by the United States Environmental Protection Agency (10–12). Strategies for clean-up of 2,4,6-trinitrotoluene- and RDX-contaminated soils include...
bioremediation using plants or microbes, and published studies have shown the feasibility of the phytoremediation approach for 2,4,6-trinitrotoluene using, for example, tobacco expressing Enterobacter cloacae pentaerythritol tetranitrate reductase (which also degrades the explosive glycerol trinitrate) and Arabidopsis thaliana expressing an Escherichia coli nitroreductase (13, 14). In addition, A. thaliana expressing a cytochrome P450 enzyme system (XplA) from Rhodococcus rhodochrous (strain 11Y) was also shown to detoxify RDX (15).

The RDX-degrading P450 enzyme XplA is an unusual member of the P450 enzyme superfamily. The P450s are monooxygenase enzymes that catalyze reductive scission of dioxygen bound to their heme b cofactor and generally source electrons required for NAD(P)H with delivery via one or more redox partner systems (16). In bacterial P450 systems, these are often soluble NAD(P)H-dependent ferredoxin reductase and iron-sulfur ferredoxin proteins. This is a so-called class I P450 redox system, analogous to the adrenodoxin reductase/adrenodoxin system responsible for driving steroidalogenic P450s in eukaryotic mitochondria (17, 18). However, the majority of mammalian and other eukaryotic P450s receive electrons from the diflavin enzyme NADPH-cytochrome P450 reductase in a membrane-associated class II redox system (16, 17). Recent years have seen recognition of a wider diversity of redox systems driving P450 enzymes, including the well characterized Bacillus megaterium P450 BM3 (CYP102A1) fatty acid hydroxylase, a soluble P450-NADPH-cytochrome P450 reductase fusion enzyme (18), and a sterol demethylase (CYP51) class P450 fused to its cognate ferredoxin, MccY5P51FX from Methylcoccus capsulatus (19, 20). XplA falls into a different class of P450 redox system, with a flavodoxin-like module (N-terminal) fused to the P450 (1, 21, 22). XplA receives electrons from a NADPH-dependent flavoprotein reductase (XplB) encoded by the xplB gene immediately upstream of xplA on the R. rhodochrous strain 11Y genome (23).

In previous studies of XplA (formally CYP177A1), Bruce and co-workers (24) have expressed and purified the enzyme and have solved the crystal structure for its P450 (heme) domain (residue 154 to the end of the 552-amino acid flavocytochrome). Reductive denitration of RDX nitro groups was observed in turnover studies performed using the XplA/XplB combination. Similar outcomes were observed whether the reaction was done anaerobically or aerobically, with nitrite, formaldehyde, and methylenedinitramine detected under anaerobic conditions and 4-nitro-2,4-diazabutanal (not methylenedinitramine) formed aerobically. P450-mediated oxidation was not considered to be responsible for differences observed (21). This was consistent with previous studies of RDX biotransformation in whole cells of Rhodococcus sp. strain DN22 cells and by mammalian CYP2B4 and NADPH-cytochrome P450 reductase in vitro. The authors of this study concluded that monoxygenation of RDX was unlikely and that nitro group reduction by P450s occurs (25). Bhushan et al. (25) noted that the presence of oxygen retarded the rate of CYP2B4-mediated RDX breakdown to 4-nitro-2,4-diazabutanal, nitrite, formaldehyde, and ammonium and ascribed this to competition between dioxygen and RDX for the ferrous P450 heme iron. Studies using 18O2 and 2H2O also demonstrated that a labeled oxygen was incorporated into 4-nitro-2,4-diazabutanal product only from H218O, apparently ruling out redox partner-dependent P450 heme iron-mediated oxygen insertion chemistry from either CYP2B4 or the Rhodococcus (XplA) P450 (25). This conclusion is also consistent with the observation that a phylogenetically conserved cytochrome P450 Ser/Thr residue that participates in oxygen activation is absent in XplA (1, 26).

Our focus was drawn to XplA in light of its being an unusual P450-redox partner fusion (27) and also as a consequence of unusual spectral features reported for this enzyme, including a red-shifted Soret maximum and absence of a defined absorbance shoulder in the 450–460 nm region that would be typical for a flavin-binding protein (15, 21). We provide here a detailed study of the spectroscopic and thermodynamic properties of XplA. These reveal several peculiar features, including unusually weak binding of FMN to the XplA flavodoxin module, high affinity of the P450 heme for imidazole (and Tris) that bind weakly to most P450s, and a very positive heme iron reduction potential. Our data reveal several non-standard properties for a P450 system adapted as a reductive (rather than an oxidative) catalyst.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of CYP177A1**—The xplA gene construct was codon-optimized for E. coli and synthesized (Entelechon, GmbH) and cloned into the expression vector pET15b (Novagen) using NdeI and BamHI sites placed at the 5’- and 3’-ends of the gene, respectively. The gene also encodes a His10 tag at the N-terminal end of XplA (supplemental Fig. S1). The expression plasmid pET15b(xplA) was transformed into HMS174 (DE3) (Novagen) cells. Conditions used for cell growth and protein expression were 37 °C in 1-liter cultures of LB medium (ForMedium, Hunstanton, UK), until A600 reached ~0.6–0.8. At this point, the temperature was lowered to 20 °C, and cells were allowed to equilibrate prior to induction of xplA expression by the addition of 100 μM isopropyl β-D-thiogalactoside and cell growth for a further 24 h. Cells were harvested by centrifugation (6000 rpm, 15 min, 4 °C) using a Beckman JLA8.1 rotor. The cell pellet was washed in ice-cold 50 mM Tris (pH 7.2) and 1 mM EDTA buffer and stored frozen at −20 °C.

The frozen pellet was thawed in 50 mM potassium phosphate, 250 mM KCl, 10% glycerol (buffer A) with 10 mM imidazole (pH 8.0) supplemented with protease inhibitor mixture (Roche Applied Science), lysozyme (10 μg/ml), and DNase (10 μg/ml). Cells were lysed by sonication with 15-s bursts, at 50-s intervals and 40% amplitude for 30 min using a Bandelin SONOPULS sonicator. Temperature was kept at <10 °C. Cell debris was removed by centrifugation at 22,000 rpm for 30 min at 4 °C. The supernatant was loaded directly onto a Ni-NTA column (Qiagen) equilibrated with buffer A. The column was washed with five column volumes of buffer A, followed by five column volumes of buffer B with 20 mM imidazole. The protein was eluted using a one-step gradient with 100 mM imidazole in buffer A. Alternatively, the column was washed in 5 mM histidine in buffer B prior to elution using 100 mM histidine. The partially purified XplA protein was concentrated in an Amicon ultrafil-
tration device with a 30,000 molecular weight cut-off and subsequently diluted with 50 mM Tris (pH 7.2) and 1 mM EDTA. It was then dialyzed against 2 liters of the same buffer with four changes of buffer. Dialyzed protein was filtered using a 0.22-μM filter and concentrated using Vivaspin 20-ml concentrators (30,000 molecular weight cut-off; Generon) to a ~500-μl final volume. Concentrated protein was loaded onto a Superdex 200 10/300 GL (GE Healthcare) gel filtration column (24 ml, flow rate 0.5 ml/min) equilibrated with 10 mM HEPES, 150 mM NaCl (pH 7.5) (buffer B), collecting 0.5-ml fractions. Fractions containing XplA were analyzed for purity with SDS-PAGE and by their $A_{198}/A_{280}$ ratio. The purest fractions were pooled and concentrated using Vivaspin 20-ml concentrators to ~1 ml and dialyzed against buffer B plus 50% glycerol prior to storage at ~80°C.

Expression and Purification of CYP177A1 Heme Domain—To generate a plasmid construct expressing the XplA heme domain (XplA-HD), residues 159–552 of XplA, an NdeI restriction site was engineered in the linker region separating the FMN and heme domains. To achieve this, XplA-HD was generated by first using oligonucleotide primers XplA-HDF (5’-GTCGTTGGTGCTACATGACGGCCTC-3’) and XplA-HDR (5’-GAGGCCGCAATGACGGCCACACG-3’), where non-complementary nucleotides introduced are in boldface type and the new site generated for restriction enzyme NdeI is underlined. The mutagenesis PCR experiment was done using the QuickChange II site-directed mutagenesis kit (Stratagene), following the manufacturer’s protocol. Plasmids from resulting transformants were sequenced to confirm the desired mutation and digested with NdeI to excise the FMN domain. The digestion products were run on a 0.8% agarose gel to separate plasmid and excised fragment. The plasmid was extracted and purified from the gel using a QIAquick gel extraction kit (Qiagen), according to the manufacturer’s instructions, and religated to make the pET15b(xplA-HD) construct, with gene sequencing as before to confirm correct clones. pET15b(xplA-HD) was subsequently transformed into HMS174 (DE3) cells, and the XplA-HD was expressed and purified as done for intact XplA.

UV-visible Spectroscopy—UV-visible absorption spectra were recorded on a Cary UV-50 Bio UV-visible scanning spectrophotometer (Varian) using 1-cm path length quartz cells. Unless otherwise stated, spectra were recorded using 10–50 μM enzyme in 50 mM HEPES, 150 mM NaCl (pH 7.2) (buffer C). The concentration of the XplA was initially determined using the method of Omura and Sato (28), using an extinction coefficient of ε$_{450-490}$ = 91 mm$^{-1}$ cm$^{-1}$ in the reduced CO-bound minus reduced absorption difference spectrum. CO binding to XplA was performed by reduction with a few grains of solid sodium dithionite (DT), followed by slow bubbling of CO into the reaction mixture. Spectral data for the XplA complex with β-mercaptoethanol (β-ME) were collected in 50 mM potassium phosphate (pH 7) at pH values from 6 to 8.

Optical titrations to determine $K_d$ values for XplA ligands were carried out at 20°C with ligands solubilized in appropriate solvents. Spectra were recorded (250–800 nm) after each addition of the ligand. RDX substrate, trinitrobenzene (TNB) and polycyclic azole drugs were solubilized in DMSO. Dithiothreitol (DTT) and β-ME were solubilized in buffer C. 4-Phenyldiazole (4-PIM) solutions were in 50% ethanol, buffer C mixtures. Unless otherwise stated, other ligands used were prepared in buffer C, and binding titrations were done in the same buffer.

Following completion of titrations, difference spectra were generated by subtraction of the ligand-free spectrum from those generated at each point in the titration. The $K_d$ values were determined by fitting the data for the ligand-induced absorption change (normally peak minus trough data, using the same wavelength pair for each difference spectrum in a particular titration) versus ligand concentration using either a standard (Michaelis-Menten) hyperbolic function or a quadratic function (Equation 1) for tight binding ligands, using Origin software (OriginLab, Northampton, MA).

$$A_{obs} = \frac{A_{max}}{2E_r} \times (S + E_r + K_d) - \frac{((S + E_r + K_d)^2)}{(4 \times S \times E_r)}$$ (Eq. 1)

In Equation 1, $A_{obs}$ is the observed absorbance change at ligand concentration $S$, $A_{max}$ is the absorbance change at ligand saturation, $E_r$ is the XplA concentration, and $K_d$ is the dissociation constant for the XplA-ligand complex.

In studies to determine the $K_d$ value for TNB, the apparent $K_d$ value for the competitive binding of RDX (which induces a large optical shift upon binding XplA) was determined at three different concentrations of TNB (250 μM, 500 μM, and 1 mM), and data were fitted using Equation 1. The $K_d$ for TNB was then determined using Equation 2.

$$K_r = \frac{K_d \times [TNB]}{[E_{app}]} - K_d$$ (Eq. 2)

In Equation 2, $K_r$ is the dissociation constant for RDX determined in the absence of TNB, [TNB] is the concentration of TNB used in the relevant titration with RDX, $[E_{app}]$ is the apparent $K_d$ for RDX at the given [TNB], and $K_r$ is the $K_d$ value for TNB. The $K_d$ reported for TNB is then the statistical average of the $K_r$ values determined at the three different TNB concentrations.

Multicolumn Laser Light Scattering (MALLS)—Intact XplA and XplA-HD protein samples for MALLS (5 μM) were in 50 mM KP$_r$ 300 mM KCl (pH 7.5). Buffer was filtered and degassed immediately before experiments. A 500-μl sample was run on a size exclusion chromatography column (S-200) using a flow rate of 0.71 ml/min. The eluent passed through a DAWN-EOS MALS spectrometer (Wyatt Technology Corp., Santa Barbara, CA) detector with an Optilab rEX refractometer (Wyatt Technologies) and a quasielastic light scattering detector (Wyatt) to measure the refractive index and the hydrodynamic radius ($R_h$) values, respectively. The average molecular mass was calculated using ASTRA version 5.21 (Wyatt) and was derived using a Zimm fitting procedure (29).

Redox Potentiometry—To determine the XplA heme iron Fe$^{3+}$/Fe$^{2+}$, FMN oxidized/semiquinone (OX/SQ), and FMN semiquinone/hydroquinone (SQ/HQ) midpoint reduction potentials, redox titrations were performed in an anaerobic glove box (Belle Technology, Weymouth, UK) under a nitrogen atmosphere.
MCD spectra were recorded using JASCO J/810 and J/730 dichrographs in the near UV-visible and near-IR regions, respectively, using an Oxford Instruments superconducting solenoid with a 25-mm ambient bore to generate a magnetic field of 6 tesla. A 0.1-cm path length quartz cuvette was used to record near-IR spectra with sample concentrations the same as those used for EPR data collection. UV-visible spectra were recorded for XplA with 50 mM HEPES in 2H2O (pH*, 7.0) as those used for EPR data collection. MCD spectra were recorded using JASCO J/810 and J/730 dichrographs in the near UV-visible and near-IR regions, respectively, using an Oxford Instruments superconducting solenoid with a 25-mm ambient bore to generate a magnetic field of 6 tesla. A 0.1-cm path length quartz cuvette was used to record near-IR spectra with sample concentrations the same as those used for EPR data collection.

FMN Binding and Quantification by Fluorescence Spectroscopy—The flavin content of XplA was quantified using the methods of Aliverti et al. (33). Following gel filtration, a sample of pure XplA was diluted to 1 ml with 10 mM HEPES (pH 7.5). The concentration of this protein solution was determined by absorbance spectrum, as described elsewhere under “Experimental Procedures.” The protein was recovered, transferred to a 1.5-ml Eppendorf microcentrifuge, and wrapped in foil. The flavin was subsequently removed by heat denaturation at 100 °C for 10 min. The solution was then cooled on ice, and precipitated protein was removed by centrifugation at 13,000 rpm for 10 min at 4 °C. The fluorescence emission spectrum of the supernatant between 480 and 600 nm was recorded using a Varian Cary Eclipse fluorescence spectrophotometer with an excitation wavelength of 450 nm and excitation/emission slits openings at 20 nm. The emission intensity at 524 nm following each titration addition was corrected for buffer contribution and subtracted from the intensity of the free FMN and then plotted against the concentration of apoenzyme.

Stopped-flow Spectroscopy—Stopped-flow absorption measurements were made using an Applied Photophysics SX18 MVR stopped-flow spectrophotometer in an anaerobic glove box (Belle Technology) to maintain oxygen levels at <2 ppm. Multiple wavelength data were collected using a photodiode array detector and XSCAN software. XplA-CO binding rates were measured at 25 °C by monitoring formation of the ferrous-CO complex at 448 nm in buffer C. All experiments were carried out with anaerobic buffer saturated by extensive bubbling with CO gas. Reactions were initiated by mixing a solution of DT-reduced enzyme (10 μM) with a solution containing various concentrations of CO (23–139 μM from a 975 μM saturated stock solution) (35).

To analyze the formation of the XplA oxyferrous complex, the XplA heme domain (XplA-HD) was used in order to ensure that further reduction of the oxyferrous heme complex did not occur due to electron transfer from the flavodoxin module. XplA-HD (~500 μM) was first mixed with excess DT under anaerobic conditions. A sample was taken, sealed, and analyzed by UV-visible spectroscopy to confirm full reduction. The reduced sample was then passed down a PD10 column in the glove box to remove excess DT. The eluted sample was diluted to ~10 μM and checked as before to confirm that the heme was still fully reduced. The reduced sample was then mixed in the stopped-flow instrument with buffer C containing varying concentrations of O2 from 20 to 100 μM. Entire spectral accumulation allowed identification of the spectral changes involved in formation of the oxy complex of XplA-HD, and absorbance changes at 435 nm (where there is a large increase in absorption for the oxyferrous complex) were recorded against time in single wavelength mode. Data were fitted using a single exponential function using Spectrakinetcs software (Applied Photophysics), and the observed reaction rates (kobs) were plotted.
against the [O₂] using Origin software and fitted to a linear function to obtain the second order O₂ binding rate constant.

**Pyridine Hemochromogen Assay**—The pyridine hemochromogen method was used to quantify XplA heme and to determine an extinction coefficient at the XplA Soret maximum. Analysis was done according to the method of Berry and Trumpower (36).

**XplA Crystallization and Structural Analysis**—The XplA-HD was crystallized using pure protein concentrated to 25 mg ml⁻¹ in 10 mM HEPES, pH 7.5. Diffraction quality crystals were obtained using the sitting drop method by mixing equal volumes of protein solution (plus 1 mM RDX) with mother liquor from a number of conditions using a Molecular Dimensions Morpheus Screen 1 screening kit (400-nl total volume) and by incubating at 4 °C and at room temperature. Crystals appeared within 2–3 days when incubated at 4 °C and within 1 day with incubation at room temperature. A single crystal obtained using 50 mM MgCl₂, 0.1 M imidazole/MES, pH 6.5, 30% PEG550MME-PEG20.000 was flash-cooled in liquid nitrogen, and diffraction data were collected at beamline IO4 of the Diamond synchrotron (Didcot, UK). Data were reduced and scaled with the X-ray Detector Software suite (37). Diffraction data were observed to 2.3 Å, and the crystal belonged to the I4₁ space group with cell parameters a = b = 136.12 Å, c = 75.13 Å. The structure was solved using molecular replacement with the available XplA heme domain structure (Protein Data Bank code 2W1Y). Positional and B-factor refinement was carried out using Refmac5 from the CCP4 suite (38), and data collection and final refinement statistics are given in supplemental Table S1.

**Materials**—All chemicals and reagents used for experimental procedures were purchased from Sigma-Aldrich or Fisher. RDX was purchased from Vitas-M Laboratory, Ltd. (Moscow, Russia).

**RESULTS**

**Expression and Purification of XplA**—An *E. coli* codon optimized version of the *xplA* gene was synthesized and cloned into a T7 promoter plasmid (pET15b) to enable high level expression in *E. coli* (plasmid pET15b(xplA)). The codon-optimized gene sequence is given in supplemental Fig. S1. Expression trials of a variety of *E. coli* T7 RNA polymerase lysogen strains indicated that HMS174 (DE3) produced the highest amount of soluble XplA protein, and this strain/plasmid combination was used for production of the flavohemoprotein XplA. Purification from cell extract was achieved in two steps, involving affinity chromatography (Ni-NTA) and gel filtration chromatography (Superdex 200) using an AKTA purifier. An SDS-polyacrylamide gel demonstrating isolation of pure XplA is given as supplemental Fig. S2. The XplA expression cell cultures typically attained a dark green-blue color, suggestive of the formation of indigo through XplA-mediated oxidation of indole in the cells, as has been observed in previous studies of other P450s (e.g. see Refs. 39 and 40) (supplemental Fig. S3).

**Spectrophotometric Analysis of Purified XplA**—The purified XplA was analyzed first by UV-visible spectroscopy to establish the properties of its heme center. XplA purified using imidazole elution from a Ni-NTA column as a first step in the isolation procedure had its Soret band at ~421 nm, with the smaller α- and β-bands at ~570 and 542 nm (Fig. 1A). The α-band is weaker, and its peak is less defined, appearing more as a shoulder on the β-band. The Soret peak position was consistent with that reported previously by Jackson et al. (21) but is red-shifted by comparison with other predominantly low spin ferric heme P450s (e.g. the P450 BM3 heme domain at 418 nm and the *P. putida* camphor hydroxylase P450cam at 417 nm) (41, 42). In addition, the absence of an absorbance feature in the ~450–480 nm region (where the longer wavelength band for an oxidized flavin would be observed) suggested that the protein may be deficient in FMN. Again, a similar lack of absorbance shoulder in this region is seen in spectra reported in previous studies of XplA (15, 21). The affinity of XplA for FMN is discussed further below and under “Quantification of FMN Binding”.

In view of the unusually long wavelength of the XplA Soret band (421 nm), we considered whether imidazole (used to elute XplA from the Ni-NTA) might have particularly high affinity for the P450 heme and remain as a ligand to the heme iron in at least a proportion of the enzyme. The polar imidazole is usually a weak inhibitory ligand for P450s, although substituted imidazoles can be much tighter binding as a consequence of their more apolar nature and favorable interactions of their substituent groups with active site amino acid side chains. For instance, the P450 BM3 Kᵢₜ for imidazole is several mM, whereas the Kᵢₜ values for 4-phenylimidazole and the fatty acid derivative 12-(imidazolyl)dodecanoic acid are 0.85 and 8 μM, respectively (32, 43). In order to explore further whether imidazole might be retained by XplA upon protein purification, we performed more extensive dialysis with several buffer changes following the Ni-NTA chromatography step. The XplA purified in this way exhibited a distinctive spectrum with Soret maximum at 417 nm. The Soret peak intensity at 417 nm was slightly greater (relative to A₃₈₅) than that at ~421 nm in the less extensively dialyzed form. The α/β bands were also of more equal intensity and shifted to ~569 and 540 nm, respectively (Fig. 1A). In an alternative Ni-NTA purification regime, histidine (100 mM) was used instead of imidazole to elute XplA, and the spectrum of the purified enzyme was again shifted to a 417 nm Soret species. Thus, we conclude that XplA purified using imidazole (in the absence of extended dialysis) is partially imidazole-bound, explaining the altered spectrum reported previously (15). Consistent with this conclusion, the spectrum for the fully imidazole-bound XplA enzyme shows a markedly lower intensity of the α-band (576 nm) compared with the β-band (543 nm), and the Soret feature is further red-shifted to 424.5 nm. This species is readily reduced by dithionite to give a ferrous-imidazole adduct with absorption maxima at 439, 538, and 566 nm, and with a broad low intensity band centered at ~620 nm (Fig. 1B). Also of note in comparing different forms of XplA is the change in intensity of the absorbance band at ~362 nm, which is increased considerably in imidazole-bound XplA. It should also be noted that early purification trials using 50 and 10 mM Tris at the post-Ni-NTA stages (ultrafiltration and gel filtration steps, respectively) revealed a Soret band shift to ~421–422, suggesting that Tris itself was also able to bind to the XplA heme iron. Optical binding assays using Tris (performed using XplA isolated using HEPES buffer) confirmed this.
to be the case, although affinity for Tris was relatively weak ($K_d = 4.57 \pm 0.42 \text{ mM}$, Soret maximum of adduct at 423 nm).

Fig. 1C shows the UV-visible absorption spectra for the oxidized, reduced, and reduced/CO-bound forms of (imidazole-free) XplA. The heme is very readily reduced by dithionite (see "Redox Potentiometry of XplA"), giving a ferrous heme species with absorption maximum at 408 nm and a single merged peak in the Q-band region at $\sim 542$ nm. The Fe(II)CO form appears to be nearly completely in the cysteine thiolate-coordinated form, with a peak at 446.5 nm. Only a small shoulder at $\sim 420$ nm (indicative of the thiol-coordinated P420 form) is observed. The Soret intensity of the Fe(II)CO P450 species is comparable with that for the oxidized enzyme, whereas previous studies indicated it to have a lower intensity (albeit also featuring a much more substantial P420 content) (15).

Quantification of FMN Binding—The XplA protein has an N-terminal domain whose protein sequence is related to those of bacterial flavodoxins. Protein sequence alignment with several bacterial flavodoxins (Flds) reveals modest amino acid sequence identity with XplA (e.g. 30% identity with $E.\ coli$ FldA and 24% with $Rhodococcus jostii$ (strain RHA1) Fld). Among the most similar Fld sequences identified in the NCBI database (discounting XplA homologues) were those for an uncharacterized $Erwinia carotovora$ flavodoxin-like protein (51%) and for cindoxin from $Citrobacter braakii$ (39%). Cindoxin supports catalytic function of the cineole oxidizing P450cin (supplemental Fig. S4) (44). Flavodoxins typically have high affinity for their FMN cofactor (e.g. 18 nM for the $Clostridium beijerinkii$ Fld and 14.6 nM/25.2 nM for the $Bacillus subtilis$ YkuN/YkuP Flds) (45, 46). An XplA $K_d$ for FMN in this range should thus have resulted in a purified enzyme with nearly complete FMN incorporation. However, the spectra for the oxidized XplA enzyme presented in Fig. 1A do not show any strong signal in the $\sim 450 - 480$ nm region that could be assigned to the presence of the longer of the two major absorption bands for an oxidized FMN cofactor. The spectrum shown has similar characteristics in this region to those previously reported for XplA (15, 21). Jackson et al. (21) suggested previously that the majority of XplA was FMN-bound and that the heme Soret obscured spectral contributions from the oxidized FMN. A stoichiometry of $\sim 1.5$ mol of DT reductant to XplA enzyme (8–10 nmol of DT to 6 nmol of XplA) was reported to fully reduce the XplA sample by Jackson et al. (21). This could only be true if each molecule of DT breaks down completely to form two molecules of sulfite as the relevant reductant and with the assumption that all of the relevant cofactor redox couples (heme Fe$^{3+}$/Fe$^{2+}$, FMN oxidized/semiquinone, and FMN semiquinone/hydroquinone) are rather more positive than that for DT itself ($\sim 420$ mV versus NHE at pH 7). However, Jackson et al. (21) quantified their XplA sample based on protein rather than...
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An experiment was then done to determine XplA affinity for FMN. Fig. 2A shows results from a fluorimetric titration of FMN-free XplA enzyme (prepared as described under “Experimental Procedures”) against FMN cofactor. The FMN fluorescence is progressively quenched through binding to XplA, and the fitting of fluorescence change ($\Delta F_{324}$ versus [XplA]) was done using Equation 1 to determine a $K_d$ value of $1.09 \pm 0.14 \mu M$ for the cofactor, an affinity much weaker than for most other flavodoxins studied to date. The weak FMN $K_d$ is consistent with the lack of clear flavin absorption features seen in XplA in this study (e.g. see Fig. 1A) and previous work (15, 21), indicating that FMN is probably lost from XplA upon its purification from the expression host.

Reconstitution of XplA with FMN Cofactor—Consistent with the weak affinity reported above for the XplA FMN cofactor, it was observed that during XplA purification steps (including gel filtration and ultrafiltration stages), yellow-colored FMN cofactor separated for the hemoprotein. Studies were done with varying buffer and ionic strength conditions in attempts to stabilize the binding of FMN, and these revealed that elevation of salt concentration (i.e. ~150 mM NaCl) led to the retention of much higher amounts of FMN in the purified XplA. With the high salt regime employed from the start of the purification scheme, FMN content (relative to heme) reached ~50–60%. Further improvements in FMN content were achieved by incubation of XplA (at ~100–200 $\mu M$) with FMN (~1 $\mu M$) in buffer B at 4 °C overnight, prior to separation of protein from free FMN by gel filtration using a PD10 column. Final XplA FMN content then reached ~90%. The difference in FMN content following XplA reconstitution is shown in Fig. 2B, where the enzyme before FMN reconstitution shows residual imidazole binding (Soret maximum at ~423 nm) and low absorption in the region of 450–500 nm, where oxidized flavin absorbs maximally. Upon extensive incubation with FMN and gel filtration as described above, the Soret is blue-shifted due to imidazole dissociation, and a prominent shoulder appears between 450 and 500 nm due to FMN binding. In order to retain FMN in XplA samples, subsequent experiments (titrations etc.) were done using 150 mM NaCl (in buffer C).

Determination of Absorption Coefficient for XplA Heme—As shown in Fig. 1C, XplA binds CO to form a complex with absorption maximum at 446.5 (the cysteine thiolate-coordinated Fe(II)CO species) and a minor feature at ~422 nm that probably reflects the cysteine thiol-bound P420 Fe(II) CO species. Using the extinction coefficient described by Omura and Sato (28), the P450 concentration of the sample used in Fig. 1C was estimated at ~4.4 $\mu M$, once account was taken of the small proportion of P420 formed. Independent estimation of the XplA heme coefficient was also done using the pyridine hemochromagen method described by Berry and Trumpower (36). This indicated an extinction coefficient of $\epsilon_{417} = 93 \text{ mm}^{-1} \text{cm}^{-1}$ for the oxidized XplA heme at its Soret maximum. Following reconstitution of XplA with FMN, a coefficient of $\epsilon_{417} = 100 \text{ mm}^{-1} \text{cm}^{-1}$ was used to account for the absorption contribution of the bound flavin. This coefficient was then used to enable estimation of concentration of the oxidized form of XplA once reconstituted with FMN.

MALLS Characterization of XplA and XplA-HD—MALLS data for intact XplA and XplA-HD are shown in supplemental Fig. S5. For intact XplA, there was a single, monodisperse species of calculated apparent molecular mass 60.6 kDa, compared

cofactor content. Using an estimate of the extinction coefficient of $93 \text{ mm}^{-1} \text{cm}^{-1}$ at the oxidized XplA Soret peak (see “Determination of Absorption Coefficient for XplA Heme”), a different stoichiometry of ~4 $\mu M$ XplA reduced by ~10 $\mu M$ DT is suggested from the previous study, which clearly points to substoichiometric cofactor content. In order to resolve this issue (and particularly in light of the absence of a clear spectral contribution from the FMN), we quantified the amount of FMN bound in our purified XplA samples, which exhibited spectral properties in the flavin region similar to those reported previously (15, 21). The FMN content in these samples was typically ~20%.
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The main figure shows spectra from a titration of FMN-bound XplA (3.6 μM; solid line spectrum) with RDX, with a type I P450 spectral shift from a predominantly LS ferric heme iron (Soret maximum at 417 nm) to a mainly HS form at 396 nm at the end of the titration (dashed line) and with a CT band at ~650 nm. Intermediate spectra are shown as dotted lines, and converge on a Soret isosbestic point at ~407 nm. Inset, plot of change in Soret absorption induced on RDX binding (specifically the change in peak and trough absorption computed from individual difference spectra generated by the subtraction of the spectrum for RDX-free XplA from each successive RDX-bound spectrum in the titration) with data fitted using Equation 1 to produce a $K_d$ value of 7.5 ± 0.2 μM.

with the predicted mass of 62.0 kDa based on the amino acid sequence. For XplA-HD, a single monodisperse species of calculated mass 46.7 kDa was found, compared with the predicted mass of 45.7 kDa. These data are consistent with both intact XplA and XplA-HD being monomeric proteins.

Optical Analysis of XplA Binding to RDX Substrate—Previous studies demonstrated the reductive denitration of RDX by XplA (15). Fig. 3 shows an optical titration of XplA with RDX, clearly demonstrating the extensive conversion of the low spin (LS) XplA heme iron to the high spin (HS) state on binding RDX. The RDX-bound HS form of XplA absorbs maximally at 396 nm and has an isosbestic point with LS XplA at ~407 nm. A small absorbance feature at ~650 nm in the RDX-bound enzyme is a charge transfer (CT) species typical of HS substrate-bound P450s. The dissociation constant for RDX was determined by fitting induced absorption change versus RDX concentration using Equation 1, yielding a $K_d$ of 7.5 ± 0.2 μM. This value is rather lower than that reported by Jackson et al. (57.9 ± 2.8 μM) (21), and there are also differences in the spectral features observed between the earlier RDX-XplA titration and that reported here. Most notable are the much less prominent feature at ~360 nm in our titration (the heme δ band) and the lack of a strong 650 nm CT species and other unusual spectral features associated with HS heme accumulation seen in the previous study (21). The RDX titration of Jackson et al. (21) shows a developing spectral trough at ~390 nm that undergoes an inflection partway through the titration to form the expected HS peak at ~395 nm. In contrast, our RDX titration shown in Fig. 3 reveals a more typical accumulation of the HS ferric heme feature on RDX binding, comparable with those of other well characterized microbial P450s upon binding their substrates (e.g. see Refs. 47 and 48).

Interactions of XplA with Trinitrobenzene and Other Ligands—

The binding of the structurally related trinitrobenzene was investigated by spectrophotometry, but there was no evidence of a HS heme shift. However, TNB binding was demonstrated by determination of XplA $K_d$ values for RDX in the presence of three different concentrations of TNB (250, 500, and 1000 μM). RDX binding produced HS changes, and using Equation 2 for competitive binding and the aforementioned $K_d$ value for RDX (7.5 μM), the $K_d$ for TNB was established as 198 ± 41 μM (supplemental Fig. S6). The data indicate a distinct active site binding mode for TNB (compared with RDX) that does not involve heme water ligand displacement but does overlap with that for RDX, such that TNB can be displaced by the stronger binding RDX substrate.

XplA titrations were also performed with imidazole and various substituted and polycyclic azole compounds, which typically ligate the heme iron of P450s via a nitrogen atom and induce a red shift (type II shift) in the Soret maximum. Imidazole produced the expected shift to 424.5 nm (supplemental Fig. S7) with a $K_d$ of 1.6 ± 0.1 μM, a substantially tighter value than for other bacterial P450s, such as the Mycobacterium tuberculosis CYP121 and CYP51B1 enzymes (50 and 11.7 mM, respectively) (47, 49). The unusually high affinity for imidazole is clearly consistent with its retention as a heme ligand in XplA purification schemes involving nickel affinity chromatography. As a consequence of this finding, XplA purification was subsequently done using histidine (rather than imidazole) to elute the protein from Ni-NTA resin (see “Experimental Procedures”). A titration with l-histidine showed no evidence of interaction of the ligand with the XplA heme iron.

Spectral titrations with several azole antifungal drugs (ecnazol, fluconazol, ketoconazol, miconazol, clotrimazol, and voriconazol) failed to produce any significant XplA Soret shifts indicative of heme iron coordination. This suggested that the binding of bulkier, hydrophobic azoles was disfavored in the constrained environment of the XplA active site. The smaller 1-phenylimidazol also failed to produce a type II binding spectrum, although 4-phenylimidazol did bind to shift the Soret band to 423 nm ($K_d = 0.25 ± 0.05$ μM). The azole binding properties of XplA are thus unusual in the P450 superfamily, with extremely high affinity for the polar imidazole and capacity to bind only smaller substituted imidazoles.

In early studies, we observed spectral changes induced on addition of DTT to XplA during enzyme purification. The binding of DTT, β-ME, and dimethyl sulfide was thus investigated by spectral titration, and each of these compounds exhibited heme binding. In the cases of DTT and β-ME (in buffer C at pH 7.2), the single XplA Soret feature is split into three components with absorption maxima at 374, 423.5, and 453.5 nm and 372, 423.5, and 453.5 nm, respectively. Based on previous studies by Sono et al. (50) and Ullrich et al. (51), the outer bands result from a hyperporphyrin spectrum due to bis-thiolate coordination of the XplA ferric heme iron, whereas the 424 nm feature results from distal coordination of the iron by a thiol ligand. A titration of XplA with DTT and the respective data fit are shown in Fig. 4A, with the $K_d$ value for DTT being 70.5 ± 1.1 μM. Fig. 4B shows selected spectra for the β-ME-bound XplA ($K_d = 58.5 ± 3.2$ μM) collected at pH values between 6 and 8. At pH 6, the dominant Soret feature is at 419.5, but as pH is ele-
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In view of the tight binding of imidazole, we also investigated the interactions of XplA with other nitrogen-containing monooxygenases: morpholine, pyrrolidine, and piperazine. Each of these molecules bound the heme iron, producing type II spectral shifts to 422, 423.5, and 422 nm, respectively. Morpholine bound tightest with a $K_d$ of 31.9 ± 0.5 μM, compared with pyrrolidine ($K_d$ = 474 ± 51 μM) and piperazine ($K_d$ = 971 ± 45 μM). The polar molecule cyanide also bound XplA comparatively tightly for a P450 ($K_d$ = 294 ± 17 μM) with $K_d$ values of 1.68 ± 0.06 mM for P450 BM3 and 4.6 ± 0.2 mM for P450cam, with a Soret band of diminished intensity at 436.5 nm and development of δ- and Q-band features at 366 and 559 nm (52, 53). Nitric oxide (NO) bound XplA to produce a spectrum with Soret shift to 431 nm, and prominent bands at 543 and 573 nm. Supplemental Table S2 collates spectral shift and $K_d$ data for the various XplA ligands. Supplemental Fig. S8 shows selected XplA ligand-bound spectra.

EPR and MCD Spectroscopic Analyses of XplA—Further spectroscopic analysis of the interactions of XplA with RDX substrate and various heme-binding ligands was done using EPR and MCD. Fig. 5A shows EPR spectra for the ligand-free XplA enzyme and for its complexes with RDX, DTT, 4-PIM, and TNB. The native XplA exhibits two sets of g values at $g_z = 2.56$, $g_y = 2.26$, and $g_x = 1.84$ and at 2.49, 2.26, and 1.86, respectively. Despite the high $g_z$ values (e.g. the comparable g values for ligand-free flavocytochrome P450 BM3 are at 2.42, 2.26, and 1.92) (54), the XplA sample used for EPR was free of imidazole as judged by the position of its Soret band. Moreover, the dominant set of g values for the 4-PIM complex were at 2.60, 2.25, and 1.84 (with a minor species at 2.49, 2.25, and 1.87), suggesting that the $g_z = 2.60$ form is characteristic of hexacoordinate XplA heme iron with an imidazole nitrogen sixth ligand. The imidazole complex of XplA gave identical sets of g values to the 4-PIM complex. Thus, we assign the two species in the ligand-free XplA enzyme with 0.5 mM DTT, 4-PIM, and TNB.
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FIGURE 5. EPR and near UV-visible MCD spectroscopy of XplA. A, X-band EPR spectra for XplA in the as-purified ligand-free state, and for XplA in complex with 4-PIM, TNB, DTT, and RDX. Ligand-free XplA has two low spin heme species that probably arise from different H2O ligand environments. The addition of TNB induces a single aqua ligand-bound state. The 4-PIM-bound XplA shows a residual component for H2O-bound XplA and a major species for 4-PIM N-coordinated heme (g_z = 2.60) distinct from those in the ligand-free and TNB-bound enzyme. The DTT-bound XplA reveals several species (including a proportion of H2O-bound enzyme). The broad g_y (= 2.38 – 2.42) and g_z = 1.91 encompass DTT binding in thiol (higher end g_y) and thiolate (lower end g_z) forms, whereas the g_x = 2.01 signal probably results from FMN reduced to a semiquinone state. The RDX-bound enzyme shows a complex set of low spin g values that probably arise from different water ligand orientations and interactions with the RDX as well as ferric high spin g values at 7.95, 3.95, and 1.77.

B, near UV-visible MCD spectra for XplA in ligand-free (dotted line), imidazole-bound (boldface solid line), β-ME-bound (dashed line), and RDX-bound (thin solid line) forms. The spectrum for ligand-free enzyme is consistent with a low spin Cys thiolate/H2O-coordinated P450, whereas RDX binding leads to development of a high spin population, as evident from development of the 650 nm band. Imidazole and DTT binding give signals consistent with formation of low spin distal N- and S-coordinated XplA (respectively), as discussed under "EPR and MCD Spectroscopic Analyses of XplA."

Fig. 5B shows results from MCD analysis of XplA in the near UV-visible region. The MCD spectrum is consistent with the expected Cys^-/H2O axial ligation of the ferric heme. The pattern of bands between 300 and 700 nm indicates the existence of solely low spin conformers (57), whereas the position of the CT_{LS} band (1190 nm; see supplemental Fig. S9) in conjunction with the decreased peak-to-trough intensity at 418 nm (58) defines thiolate as one of the ligands to the heme iron. The red shift of the Soret band to 425 nm in the imidazole complex is indicative of complete displacement of water by a nitrogenous ligand, the 425 nm peak-to-trough intensity and position of the CT_{LS} band being consistent with thiolate (Cys^-) remaining as the proximal ligand. Binding of β-ME gives the expected hyperporphyrin spectrum of the ferric complex with a negative Gaussian feature centered at 374 nm and a derivative at 454 nm arising from bis-thiolate coordination, with the 425 nm intensity arising from thiolate/thiol coordination (50). Dissociation of water to give a high spin pentacoordinate heme upon binding of RDX is evidenced by the appearance of negative Gaussian intensity at 650 nm. However, persistence of the derivative band at 419 nm indicates the presence of both high and low spin conformers presumably due to incomplete dissociation of the distal water in the sample. In the near IR MCD spectrum, a porphyrin-to-ferric heme iron CT band is located at ~1190 nm (supplemental Fig. S9), a slightly longer wavelength than has been observed for other P450s analyzed to date (e.g. ~1080 nm for P450 BM3) (32). This suggests that the t_g heme iron d-orbitals are closer in energy to the porphyrin orbitals in XplA than is typical in the Cys thiolate-coordinated P450s, possibly due to the electrostatic environment of the thiolate ligand.

Redox Potentiometry of XplA—Having successfully reconstituted XplA with its FMN cofactor, we used spectroelectrochemical methods to determine the midpoint reduction of the heme and FMN cofactors. Fig. 6A shows selected spectra from the redox titration, illustrating the progression from the fully oxidized enzyme to the reduced form that has heme in its ferrous state and the FMN cofactor reduced to its hydroquinone form. The oxidized XplA has its ferric heme iron Soret maximum at 417 nm, with the reduced enzyme heme maximum at 407 nm. Data fitting at 417 nm gives a midpoint potential (E) of ~268 ± 5 mV (versus NHE) for the heme iron Fe^{3+}/Fe^{2+} couple (Fig. 6A, inset). Elsewhere in the spectra, there is an initial decrease in absorption in the region around 470 nm during the early stages of the reductive phase, followed by an increase in absorption at more negative potentials. In the region around 550 nm, there is an initial increase and then decrease in absorption upon titration with DT, followed by a larger increase in absorption as XplA becomes fully reduced (Fig. 6A). The origins of these changes lie in the initial single electron reduction of the FMN cofactor to its blue SQ form (producing an absorption decrease in the 470 nm region and an increase at ~550 nm), followed by its further reduction to the HQ state (producing decreases in absorption in both regions). The reduction of the heme iron then occurs in the potential range overlapping partially with the SQ/HQ transition of the flavin. The heme reduction is then associated with increases in absorption at both the 470 and 550 nm regions of the spectrum (Fig. 6A). Consistent with this conclusion, there is an isosbestic point at

to its environment and ligation geometry give rise to the different species. A further g_z feature is seen at 1.93. Its corresponding g_x is less defined but, according to the Bohan formula (56), should be located at 2.33 (see the asterisk in Fig. 5A). This final component of the spectrum (2.33/2.26/1.93) is again assigned to a discrete form of water-ligated XplA interacting with RDX in the active site.

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Breakdown of XplA Fe(II)CO Complex by RDX and CO Binding Kinetics—As shown in Fig. 1C, XplA readily forms a Fe(II)-CO adduct upon the addition of CO to the DT-reduced enzyme, with the Soret peak at 446.5 nm, indicative of a predominantly cysteine thiolate-coordinated CO complex. The relatively positive P450 heme iron (and flavin FMN) potentials underlie the efficient reduction of XplA by DT. Under anaerobic conditions in CO-saturated solution, we found that the addition of RDX resulted in the efficient conversion of the Fe(II)-CO complex to an HS Fe(II) RDX-bound form (supplemental Fig. S10). This indicated that RDX (K_d = 7.5 mM, added at 1 mM) probably displaces the CO and reoxidizes the heme iron by XplA-catalyzed nitro group reduction. To investigate CO binding, we measured the kinetics of Fe(II)-CO complex formation (at 446 nm) using stopped-flow mixing of DT-reduced XplA heme domain with CO at various concentrations. ΔA_{446} reaction transients were fitted accurately using a monophasic exponential function, and a linear dependence of k_{obs} values on [CO] was established, giving a second order rate constant for CO association (k_{on}) of 17.82 ± 6.00 mM⁻¹ s⁻¹ and an apparent rate constant (k_{off}) for CO dissociation of 7.48 ± 0.35 s⁻¹, leading to a K_d value of 0.42 ± 0.12 mM (supplemental Fig. S11). Thus, although affinity of XplA for CO may be greater than that for RDX, at high concentrations of both molecules, the rapid dissociation of CO enables access of RDX to the ferrous heme iron to oxidize the iron and prevent further CO ligation. Thus, although CO is often considered as a powerful inhibitor of P450 function, in the case of XplA, the nature of the reductive reaction catalyzed with RDX and the tight binding of RDX immediately adjacent to the heme iron in the constrained XplA active site result in an effective breakdown of the Fe(II)CO complex.

Oxy Complex of XplA—Previous studies of XplA showed differences in product formation from RDX under aerobic and anaerobic conditions, with nitrite and formaldehyde accompanied by predominantly methylenedinitramine anaerobically or by 4-nitro-2,4-diazabutanol in aerobic reactions (21). Although it was considered unlikely that XplA would directly hydroxylate RDX (21, 54), we examined the ability of the P450 to form an oxyferrous complex using a stopped-flow approach. Oxygenated buffer (100 mM O_2) was mixed with deoxygenated ferrous XplA heme domain in a glove box environment, and entire spectra were collected using a photodiode array attachment.

Crystal Structure of XplA—The XplA-HD was crystallized in a different space group from previously published structures.

FIGURE 6. Determination of redox potentials for the heme and FMN centers in XplA. A, selected spectral data from a spectroelectrochemical redox titration of FMN-bound XplA (~8 μM). The oxidized enzyme is shown with a thin solid line. Spectra collected in the early phase of the reductive titration (from ~75 to ~120 mV) are shown as dotted lines and reflect absorbance changes predominantly due to reduction of the FMN. Spectra collected at more negative potentials are shown as dashed lines and report mainly on heme iron reduction. The spectrum for the fully reduced XplA enzyme is shown as a thick black line. Arrows, directions of absorption change during the reductive phase of the titration. At ~470 nm (near the absorption maximum for oxidized FMN), there is a decrease in absorbance in the early phase, reflecting conversion of FMN from oxidized through SQ to HQ. At more negative potentials, there is an increase in absorbance in this region due to heme iron reduction. In the region at ~550 nm, there is an initial increase in absorbance due to FMN SQ formation, followed by a decrease as the SQ is reduced to FMN HQ and then a final increase as heme is reduced and the Q-band absorbance becomes more prominent. Inset, plot of A_{446} versus potential fitted using the Nernst equation to give a heme iron Fe^{3+}/Fe^{2+} midpoint potential (versus NHE) of E = -268 ± 5 mV. B, fit of A_{630} data (following FMN SQ formation and its further reduction to HQ) versus potential fitted using a 2-electron Nernst equation (30), giving midpoint potentials for the FMN_{OX/SQ} (E_F) and FMN_{SQ/HQ} (E_S) couples of ~80 ± 5 mV and ~172 ± 8 mV, respectively.

~504 nm in spectra collected at potentials between approximately +50 and ~125 mV, consistent with the XplA FMN OX/SQ transition occurring prior to any significant reduction of the heme iron. Once the FMN is fully reduced to the HQ state, a heme Fe^{3+}/Fe^{2+} isosbestic point is also apparent at ~412 nm. Fig. 6B shows a fit of absorbance versus potential data at 630 nm, where the increase and decrease in FMN SQ absorbance is minimally affected by heme contributions. FMN potentials of E_{1(OX/SQ)} = -80 ± 5 mV and E_{2(SQ/HQ)} = -172 ± 8 mV were determined.
(21). A comparison with the previously solved 2WIY structure reveals little overall change in the structure (root mean square deviation of 0.518 Å over 392 Ca atoms), with the exception of the residues comprising the BC-loop region (residues 220–241) (Fig. 7A). This loop has undergone a minor rearrangement as a consequence of the binding of a PEG molecule from the mother liquor at the active site region. The PEG extends into the heme binding pocket and is in van der Waals contact with a range of hydrophobic residues from the BC-loop (Trp224, Trp230, and Leu238). In addition, hydrogen bonding contacts are observed with Gln325 and Thr236, the latter mediated via water molecule Wat-4. The bound PEG is in van der Waals contact with the sixth ligand to the heme (W4 in Fig. 7B).

Although imidazole was completely removed from XplA prior to crystallization, and despite the fact that 1 mM RDX substrate was used during crystallization, the only conditions in which XplA crystallized were those containing imidazole as a component of the crystallization buffer. Electron density from the crystals clearly indicates that an imidazole is bound to the XplA heme iron (Fig. 7C). In addition to van der Waals contacts

FIGURE 7. Structure of the imidazole-coordinated XplA heme domain. A, overlay of the XplA heme domain-PEG-imidazole ternary complex (in green, Protein Data Bank code 4EP6) with the previously determined XplA-imidazole complex structure (Protein Data Bank code 2WIY; in gray). The region most affected by PEG binding, the BC-loop region, is colored in blue for the ternary complex. PEG and imidazole ligands are shown in atom colored sticks. B, stereo view of the ligand-protein interactions observed in the PEG-XplA complex. Selected residues contacting PEG are shown as sticks with a schematic representation for the BC-loop region (residues 220–241). The \( 2F_o - F_c \) electron density corresponding to the bound PEG is shown contoured at 1.5 \( \sigma \). C, stereo view of the ligand-protein interactions observed in the imidazole-XplA complex. Selected residues contacting imidazole are shown as sticks with a schematic representation for the I-helix region (residues 388–398). The \( 2F_o - F_c \) electron density corresponding to the bound imidazole is shown contoured at 1.5 \( \sigma \).
with several I-helix residues (Val\textsuperscript{391}, Met\textsuperscript{394}, and Ala\textsuperscript{395}), a water-mediated hydrogen bonding network is made between imidazole, Wat-1, Wat-2, and Pro\textsuperscript{437}–Gin\textsuperscript{438}.

The unusually high affinity of XplA for imidazole (as discussed above) can be understood from the strong complementarity between the XplA distal heme binding pocket and the ligand. It was suggested previously that the I-helix conformation relative to the heme iron is key to determining the affinity of azole-type ligands to the heme iron (59). In the case of XplA, the I-helix is oriented such that imidazole coordinates the heme iron with ideal geometry. This is due not only to the severe kink in the I-helix as it passes over the heme plane, but also to the fact that residue 395 is an Ala as opposed to the usual Thr/Ser residues observed in the majority of P450 oxygenases at this position. This avoids a significant clash between the imidazole and the hydroxyl side chain that is usually present at this position. The imidazole ligand is furthermore bound within a hydrophobic pocket with high shape complementarity made by residues Val\textsuperscript{391}, Met\textsuperscript{394}, and Ala\textsuperscript{395}. In addition to the iron-imidazole ligation, an extended water-mediated hydrogen bonding network is established with the other imidazole nitrogen atom. The unusually tight binding of imidazole to XplA can thus be explained by the geometry of the XplA active site.

In contrast, other bulkier azole ligands bind much less tightly or not at all to XplA (e.g. histidine, 1-phenylimidazole, and fluconazole). This can similarly be understood from the strong complementarity between ligand and active site observed in the imidazole-XplA complex. Any substituents on the imidazole ring would cause considerable van der Waals clashes with Val\textsuperscript{391}, Met\textsuperscript{394}, and Ala\textsuperscript{394}.

**DISCUSSION**

XplA is an unusual member of the P450 superfamily, which has become specialized to catalyze reductive denitration as an initial step in the degradation and detoxification of the military explosive RDX (21). It is an example of a growing class of P450 enzymes fused to redox partners or other protein modules, with a flavodoxin domain attached at the N-terminal of the XplA P450 (15, 22). The xplA gene was identified in several types of bacteria able to oxidatively exploit RDX as a source of nitrogen (61, 62). XplA and its FAD-binding reductase partner XplB appear to be plasmid-encoded, along with several other genes involved in transport and degradation of RDX (63).

Previous reports indicated that XplA had a Soret band slightly red-shifted (to ~420 nm) by comparison with the low spin ferric Soret features in other P450s and was also devoid of any obvious absorbance feature that could be associated with the binding of FMN (15, 21). However, our data indicate that the true XplA Soret is located at 417 nm (Fig. 1C). This difference (and other minor alterations in the Q-band and 8-band spectral regions) originates from a nitrogenous ligand (imidazole or possibly Tris) that remains bound to a proportion of XplA enzyme used in these previous studies. The improved behavior of the imidazole-free (417 nm) XplA is also evident in the spectrum for the ferrous form (with a prominent fused peak in the Q-band region) and with the nearly complete formation of the thiolate-coordinated P450 Fe\textsuperscript{2+}–CO species at 446.5 nm (Fig. 1C). In contrast, previous studies of the 420 nm form showed split Q-band features in the reduced spectrum and a substantial proportion of the P420 (thiol-coordinated) form in the Fe\textsuperscript{2+}–CO state (15, 21). In previous studies, Jackson et al. (21) reported a Soret shift to 389 nm for ferrous XplA. However, inspection of their data indicates that their peak is in fact at >400 nm, and we show here that the imidazole-free form Soret shifts to ~408 nm on heme reduction. Our data indicate an unusually high imidazole affinity (K\textsubscript{d} = 1.6 μM) for XplA, where this polar ligand typically has millimolar affinity for the hydrophobic active sites of most P450s (e.g. K\textsubscript{d} = 11.7 mM for the M. tuberculosis CYP51B1 sterol demethylase P450) (47). Titration of the imidazole-free XplA with RDX results in a typical type I spectral shift and a much tighter K\textsubscript{d} (7.5 μM) than previously reported for the 420 nm Soret form of XplA (57.9 μM) (21). The tight binding of RDX at the heme distal face and the reductive reaction catalyzed by XplA is highlighted by the ability of RDX to oxidize the Fe\textsuperscript{2+}–CO complex of XplA under anaerobic conditions, presumably due to displacement of the CO ligand and efficient electron transfer from the ferrous iron to denitrate RDX. The XplA heme can also clearly form an oxyferrous complex that is sufficiently stable to characterize spectrophotometrically (supplemental Fig. S12A), which might suggest that XplA retains the capacity to perform oxidative reactions on other substrates. Formation of indigo in XplA expression cells hints at this capacity, although we could not detect indole binding to XplA spectrophotometrically or its transformation to indigo by XplA in vitro (data not shown). However, the reduction potential for the XplA heme iron Fe\textsuperscript{2+/3+} couple in the substrate-free (low spin) state is quite positive for a P450 (~268 ± 5 mV) (Fig. 6A). Previous studies of Phe\textsuperscript{393} mutants in the P450 BM3 enzyme indicated that positive shifts in the heme iron potential were correlated with faster heme reduction by the redox partner but also with greater oxyferrous complex stability, diminished driving force for electron transfer to bound dioxygen from ferrous heme iron, and much less efficient oxidative catalysis (64, 65). Thus, the positive potential of the XplA heme iron may be an important clue regarding its evolution into a predominantly reductive catalyst. Other factors pointing to a reductive role for XplA are (i) the absence of the Ser/Thr residue found in virtually all P450 oxidases and involved in O\textsubscript{2} binding/activation and (ii) the constrained XplA active site that may disfavor oxygen binding to ferrous iron once RDX binds very close to the heme iron. The extensive HS heme iron shift induced on RDX binding probably produces a large increase in heme potential to enable electron delivery from the XplA FMN hydroquinone.

A further unusual feature of XplA is the weak affinity for FMN in its flavodoxin domain (K\textsubscript{d} = 1.09 μM). The affinity of other flavodoxins for FMN is almost invariably ~10\textsuperscript{2}–10\textsuperscript{4}-fold greater (e.g. K\textsubscript{d} = 0.24 nm for the Desulfovibrio vulgaris flavodoxin) (66). To avoid purification of mainly FMN-depleted XplA, the use of buffer containing ~150 mM NaCl proved effective in stabilizing the FMN-bound form, and incubation of FMN with XplA purified in this way (prior to gel filtration to separate non-bound cofactor) led to a predominantly holoprotein form (~90% FMN-bound). The reason for the weak binding of FMN to XplA is not immediately obvious from its protein sequence. For instance, aromatic residues that typically sand-
wich the FMN isoalloxazine system are retained as Tyr \(^{59}\) (at the re-face) and Phe \(^{99}\) (si-face). However, our attempts to express the FMN domain independently were unsuccessful, pointing to a lack of stability of this module in absence of its fused P450 partner. Spectroelectrochemical titrations revealed that the XplA FMN (like its heme) had positive redox potentials for its FMN\(_{\text{OX/SQ}}\) (~80 mV) and FMN\(_{\text{SQ/HQ}}\) (~172 mV) couples. Although the FMN\(_{\text{OX/SQ}}\) value is in a similar range to those of many other flavodoxins (e.g. ~105 mV for the YkuN and YkuP flavodoxins from \(B.\ subtilis\)), the FMN\(_{\text{SQ/HQ}}\) couple is substantially more positive than for typical flavodoxins (e.g. ~382 mV/377 mV for YkuN/YkuP) (46), resulting in less stabilization of the 1-electron reduced neutral blue semiquinone form of FMN in XplA (Fig. 6B). The FMN\(_{\text{SQ/HQ}}\) couple is the likely electron donor to the XplA heme, and natural mutations to the FMN binding site that have resulted in the relatively positive potential of this couple may have enabled efficient electron transport between XplB, the XplA FMN, and the heme while resulting in diminished FMN affinity for the protein.

MALLS studies of XplA and the XplA heme domain (XplA-HD) demonstrate that both are monomeric proteins (supplementary Fig. S5). XplA is thus different from the P450 BM3 (CYP102A1) P450-NADPH-cytochrome P450 reductase fusion enzyme, which is functional as a dimer with intermonomer electron transfer (67). However, the more recently characterized CYP116B1 phthalate dioxygenase reductase-P450 fusion enzyme was also shown to be monomeric (68). Thus, the XplA monomer probably interacts with its NAD(P)H-dependent XplB partner flavoprotein reductase in a 1:1 complex. The crystal structure for the XplA heme domain reported here is consistent with the earlier reported structure in terms of overall fold and active site organization (24). Close inspection of the distal pocket enables rationalization of the reason for the extraordinarily high affinity for imidazole. Aside from the iron-imidazole nitrogen ligation, an extended hydrogen bonding network to the other imidazole nitrogen and steric complementary of residues around the imidazole enable its nearly linear geometry of coordination to the iron. Indeed, an adaptation consistent with the evolution of XplA toward reductive P450 catalysis also contributes to enhancing its affinity for imidazole. The presence of the relatively small Ala\(^{395}\) in the XplA I-helix avoids steric clash with imidazole, whereas the vast majority of P450 oxidizes have Ser/Thr at this position in order to facilitate activation of oxygen bound to the heme iron, probably through protonation of a ferric-hydroperoxo intermediate in the catalytic cycle (69). XplA may thus be incapacitated as an oxygenase by this substitution, unless it can oxidize a substrate possessing a hydroxyl group as a surrogate for the absent Ser/Thr (70). Some clues to an evolutionary origin for XplA may come, however, from the binding of PEG from the crystallization buffer in the XplA active site channel, possibly pointing to a fatty acid binding role for a progenitor of this RDX-reducing enzyme.

The unusually constricted nature of the XplA distal pocket is also evident in the inability of P450 to bind bulky azole antifungal drugs, whereas molecules such as fluconazole and econazole have been shown to ligate strongly to various other P450s (e.g. see Refs. 47 and 71). 4-PIM binds very tightly (\(K_a = 0.25 \mu M\)), whereas 1-phenylimidazole does not ligate the XplA heme iron. Inspection of the imidazole-bound XplA structure does not provide a clear rationale for the difference in binding of the phenylimidazoles and may point to structural plasticity of XplA. Indeed, EPR analysis of, for example, the ligand-free, RDX- and 4-PIM-bound forms indicates heterogeneity in binding modes of the water and the substrate/inhibitor molecules. Only when bound to the substrate analog TNB is a single EPR species observed, suggesting that the binding mode of TNB favors a unique structural configuration around the sixth ligand on the heme iron (Fig. 5A). The inability of XplA to bind histidine was exploited to enable purification of the P450 using Ni-NTA affinity chromatography (avoiding using the tightly binding imidazole heme ligand). XplA is also able to bind the small sulfur-containing ligands DTT, β-ME, and dimethyl sulfide, with the first two molecules giving split (hyperporphyrin) Soret spectra and showing a capacity to coordinate XplA heme iron in both their thiol and thiolate forms (Fig. 4). Similar spectral characteristics were reported recently for the DGCR8 hemoprotein enzyme involved in microRNA processing. DGCR8 is considered to be the first natural example of a protein-bound heme that is axially coordinated by two cysteine residues (60).

In conclusion, we have demonstrated non-standard properties of the P450-flavodoxin fusion enzyme XplA, which reconcile unusual spectral features of the enzyme reported previously (15, 21). The XplA P450 has evolved as a reductase (rather than oxidase) of RDX, and structural alterations to its heme- and FMN-binding domains have led to reduction potentials for low spin heme iron Fe\(^{3+}/Fe^{2+}\) and FMN\(_{\text{SQ/HQ}}\) couples being much more positive than those seen in typical P450s and flavodoxins, but consistent with non-oxidative P450 catalysis. These evolutionary steps have also led to a constricted P450 active site with high affinity for RDX (but also for the small heterocyclic inhibitor imidazole) and also to substantially diminished affinity for FMN in the flavodoxin domain. These properties necessitate careful control of purification conditions to ensure production of an inhibitor-free holoenzyme form. The production of homogeneous FMN-replete and imidazole-free XplA may enable crystallization of the holoenzyme and determination of the structure for this P450-flavodoxin fusion.

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