Pyrrrolnitrin (3-chloro-4-(2’-nitro-3’-chlorophenyl)pyrrole) is a broad-spectrum antifungal compound isolated from *Pseudomonas pyrocina*. Four enzymes (PrnA, PrnB, PrnC, and PrnD) are required for pyrrrolnitrin biosynthesis from tryptophan. PrnB rearranges the indole ring of 7-Cl-L-tryptophan and eliminates the carboxylate group. PrnB shows robust activity *in vivo*, but *in vitro* activity for PrnB under defined conditions remains undetected. The structure of PrnB establishes that the enzyme belongs to the heme b-dependent indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) family. We report the cyanide complex of PrnB and two ternary complexes with both L-tryptophan substrates. The amide and carboxylate group of the substrate are orientated in a new conformation. Tyr321 and Ser332 play a key role in binding these groups. The structures suggest that catalysis requires an L-configured substrate. Isothermal titration calorimetry data suggest that tryptophan does not bind after cyanide (or oxygen) coordinates with the distal (or sixth) site of heme. This is the first ternary complex with a tryptophan substrate of a member of the tryptophan dioxygenase superfamily and has mechanistic implications.

Pyrrrolnitrin is a broad-spectrum potent antifungal compound (1) first isolated from *Pseudomonas pyrocinia* (2) and the active component of PYRO-ACE (treatment for fungal infections of skin). The gene cluster responsible for pyrrrolnitrin biosynthesis was identified in *Pseudomonas fluorescens* (BL915) (3, 4) and subsequently in *Pseudomonas pyrocina*, *Burkholderia cepacia* LT4-12-W, *Mycococcus fulvus* Mx f147, and other pyrrrolnitrin producing bacteria (5). Four conserved enzymes are involved in pyrrrolnitrin biosynthesis and named PrnA, PrnB, PrnC, and PrnD, reflecting their order in catalysis. Introduction of the entire cluster to *Escherichia coli* results in the production of pyrrrolnitrin, thereby demonstrating that the four genes are sufficient and essential for pyrrrolnitrin biosynthesis *in vivo* (3). Genetic manipulation in *P. fluorescens* BL915 has identified the intermediate products from each enzyme in the pyrrrolnitrin pathway, leading to the current model for the biosynthetic pathway (4). The first enzyme, PrnA (tryptophan 7-halogenase), incorporates the chlorine into the substrate tryptophan (6). Structural and biochemical analyses of both tryptophan 7 and 5-halogenase (7–10) have established a novel chemical mechanism of hypohalous acid formation at the flavin cofactor, followed by N-chlorolysine formation (9) and finally regioselective halogenation of tryptophan (controlled by orientation of substrate) (10, 11). PrnB converts 7-Cl-tryptophan into monodechloroamino-pyrrolnitrin, although the chirality of the PrnB substrate has remained unclear (4). The third enzyme, PrnC, like PrnA is a flavin-dependent halogenase and seems likely to operate by the same mechanism. The fourth enzyme, PrnD, contains a mononuclear nonheme Fe(II) and a [2Fe-2S] cluster and oxidizes the amino group to a nitro group, yielding pyrrrolnitrin (12).

The activity of PrnB has only been shown in crude extract and inferred from genetic studies (Fig. 1a). The structure of PrnB (13) established that it is a member of the heme-b dioxygenase superfamily and closely related to indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO).
The previous four PrnB binary complex structures with D/L-tryptophan and 7-Cl-L-tryptophan (13) all showed an atom of the substrate bound to the sixth position of the heme, suggesting that they were unlikely to be true models of the catalytic complex. In addition, the indole ring in the L-tryptophan complex was oriented very differently than in the other three complexes. We now report the binary complex of PrnB with a cyanide ion at the sixth position as a mimic of the iron peroxo complex, which makes it possible to obtain the ternary complexes with L-tryptophan and 7-Cl-L-tryptophan, respectively. These ternary complexes coupled to isothermal titration calorimetry analyses and site-directed mutagenesis have allowed us to explain a number of features of PrnB and to inform the general dioxygenase mechanism.

EXPERIMENTAL PROCEDURES

Structural Biology—His-tagged PrnB Cys mutant (C21S, C60S, or C185S) was overproduced in P. fluorescens BL915 ΔORF1-4 (deletion of the gene cluster of the pyrrolnitrin biosynthesis pathway), purified, and crystallized as previously described (13, 20) with two modifications; 7 mM sterile filtered hemin was supplemented to the growth medium to ensure full incorporation of heme (21), and 0.1 M Britis buffer (pH 6.2–6.4) was introduced to the crystallization condition and enhanced reproducibility of crystallization. The use of cyanide required appropriate safety considerations. We originally tried to crystallize PrnB preincubated with cyanide but produced only native crystals. Spectroscopic evaluation showed that the binding of cyanide is lost over time. We soaked native PrnB crystal in 6 mM KCN (buffered in 50 mM Tris-HCl, pH 7.2) for 10 min, flash-froze the crystals at 100 K, and collected data using the supplemented 20% polyethylene glycol 400 as cryoprotectant. Spectroscopy of protein immersed in the same solutions showed cyanide binding (supplemental Fig. S1). This produces the binary complex with clear density for CN at the sixth position. Ternary PrnB crystals were obtained by transferring the PrnB cyanide crystals into the same cyanide solution as above but supplemented with 5 mM L-tryptophan or with saturating 7-Cl-tryptophan racemate (~1.5 mM). Crystals were then frozen for data collection as above except that the cryoprotectant solution was supplemented with tryptophan ligands. All of the data sets were collected in house on a Rigaku Mac21127 HFM rotating anode with Osmic mirrors on a Rigaku Saturn 944 + CCD detector and processed using HKL2000 (22). Refinement started using Protein Data Bank entry 2v7j (with no ligands) with rigid body and moved to conventional positional and thermal refinement using REFMAC5 (23). Reflections for R-free calculation were copied from 2v7j (13). Manual adjustment employed Coot (24), and ligands were added only when density was clear with dictionaries from REFMAC library. The distance between the carbon of cyanide and iron of heme was restrained to 1.93 Å, but the angle of cyanide at the heme was not restrained. PrnB clearly and reproducibly bound 7-Cl-L-tryptophan from the racemate in the presence of cyanide but 7-Cl-D-tryptophan in the absence of cyanide. Using a racemate of a 5 mM concentration of each enantiomer of tryptophan, we only obtained L-tryptophan in the presence of cyanide; equally, with cyanide bound at the sixth position, we were unable to soak D-tryptophan into the ternary structure. The data processing and refinement statistics are summarized in supplemental Table S1. Unbiased $F_o - F_c$ electron density maps and $2F_o - F_c$ electron density maps for all ligands are shown in supplemental Figs. S2 and S3. All structures were validated by MOLPROBITY (25). All structure pictures were produced using PyMOL from DeLano Scientific (San Carlos, CA) unless indicated.

Site-directed Mutagenesis of PrnB—The gene of His-tagged PrnB Cys mutant was subcloned from the expression vector to
the pFastBac HTa vector (Invitrogen) by BamHI/HindIII digestion to give the plasmid pFastBac_PrnB_Cys, which was used as the template for site-directed mutagenesis. After the mutation was introduced by using the QuikChange site-directed mutagenesis kit (Stratagene), the insert was then transferred back into PrnB expression vector for mutant expression in *P. fluorescence* BL915 ΔORF1–4. The following primers were used to create the mutations: Y321F, 5′-GGGAGGGAACCCACTTCGGG-3′ and 5′-CCCCACTTCGGG-3′; S332A, 5′-GGGAGGGAACCCACTTCGGG-3′ and 5′-GGGGGACCCACTTCGGG-3′. The Y321F/S332A double site mutant was constructed sequentially by using both sets of primers. All of the mutants were purified as native enzymes, and heme occupancy was assessed by the ratio of the absorbances at 407 and 280 nm. The heme was then transferred into PrnB expression vector for mutant expression in *P. fluorescens* BL915 and 5′-CCCCGAAATCGGC-3′ and 5′-GCGGAGCGGGCGGGG-3′ were subjected to analytic HPLC analysis with methanol/H2O (65:35) as the eluent, using a Varian OmniSpher RP-18, 5-μm, 250 × 4.6-mm column. The flow rate was 0.5 ml/min, and compounds were detected at 220 and 303 nm.

**RESULTS**

**Complex Structure of PrnB with Cyanide**—As described previously, we used the triple Cys mutant of PrnB (C21S, C60S, and C185S) because it is fully functional but, unlike native protein, does not aggregate (13, 20). Crystals of native PrnB were soaked in 6 mM KCN to produce a binary complex structure of PrnB and cyanide. The protein structure in the binary complex is unchanged from earlier descriptions (Fig. 2a). Briefly, the monomer consists of 17 α-helices, two short β-strands, and a heme b molecule. His313 in α16 is the proximal ligand of the heme, and now cyanide (rather than water) functions as the distal/sixth ligand. Part of the loop (residues 324–

**In Vivo Activity Assay**—50-ml cultures of *P. fluorescens* BL915 ΔORF1–4 with a plasmid encoding PrnB (triple Cys mutant or the additional Y321F, S332A, Y321F/S332A mutants) were harvested by centrifugation at 3000 × g for 15 min at 4 °C after 48 h of growth. The cell pellets were suspended in 10 ml of phosphate-buffered saline solution. 0.4 mM 7-Cl-tryptophan (racemic) was added to each 1-ml cell suspension, followed by shaking at 500 rpm at 25 °C for 16 h. The cultures were extracted with one volume of ethyl acetate twice. The extracts were dried in a Savant vacuum concentrator (Stratagene), and residues were dispersed in 100 μl of methanol/H2O (65:35). The samples were clarified by centrifugation (13,000 rpm 10 min 25 °C) before 20-μl portions were subjected to analytic HPLC analysis with methanol/H2O (65:35) as the eluent, using a Varian OmniSpher RP-18, 5-μm, 250 × 4.6-mm column. The flow rate was 0.5 ml/min, and compounds were detected at 220 and 303 nm.

**Isothermal Titration Calorimetric Analysis on the Ligand Binding of PrnB and Its Mutants**—ITC experiments were carried out using a VP-ITC device (MicroCal, Northampton, MA) with fully degassed solutions. Protein samples were dia lyzed against 20 mM sodium phosphate buffer (pH 7.8), and all of the ligands were dissolved in the same buffer. The ligand solutions (0.55–0.97 mM) were injected at 25 °C into the sample cell containing ~1.4 ml of PrnB at 40–72 μM (triple Cys or additional mutants). Each titration consisted of a first 1-μl injection followed by up to 25 subsequent 10-μl injections of the ligand. Titration of L-tryptophan with PrnB preloaded with cyanide used 48 subsequent 5-μl injections. Because L-tryptophan did not bind to PrnB triple Cys preincubated with cyanide, it was not tested against the additional mutations. Calorimetric data were analyzed using MicroCal ORIGIN software using a single binding site model.

---

**Figure 2. Structures of PrnB.** a, the CN− complex of PrnB that mimics the oxyferrous complex. The nitrogen atom interacts with the oxyanion hole, highlighted in salmon. Much of the loop between Val323 and Leu340 is disordered, highlighted in orange. Blue, nitrogen atoms; red, oxygen atoms; brown, iron atoms; yellow, carbon of cyanide; green, carbon in heme. b, the ternary complex of PrnB, CN−, and the substrate 7-Cl-L-tryptophan. Much of the previously disordered loop, shown in orange, becomes ordered upon binding of substrate (7-Cl-L-tryptophan). Carbon atoms are colored white in tryptophan, chloride is colored purple, and other atoms are colored as in a. c, 7-Cl-L-tryptophan is recognized by extensive interactions. Hydrogen bonds are shown as dotted black lines, and the 3.6-Å contact between the nitrogen of cyanide and the C3 atom of 7-Cl-L-tryptophan is shown as a red dotted line. Atoms are colored as in b.
ternary complex. Arg206 is 3.5 Å from the carboxylate and has part of the loop structure that changes upon formation of the protein complexes (26, 27). There are small shifts on the main geometry of cyanide has been observed in a few cyanide-heme complexes (26, 27). There are small shifts on the main chain of the loops between α8 and α9 and between α13 and α14 compared with the ligand-free PrnB structure (13), which are distant from the active site and may reflect the inherent flexibility in the loops.

The binary complex crystals of PrnB and cyanide were soaked to produce the two ternary complex structures of PrnB, cyanide and L-tryptophan or 7-Cl-L-tryptophan. The protein structure and configuration of L-tryptophan and 7-Cl-L-tryptophan are identical to each other, save for the additional chlorine atom, so our discussion focuses on the 7-Cl-tryptophan. The main difference between the ternary cyanide complexes and the binary cyanide complex is that part of the previously disordered loop, Glu329–Tyr335, now becomes ordered in the experimental density (Fig. 2b). Ser332 makes a hydrogen bond with the amino group of the substrate. Concomitant with the ordering of the loop, the main chain from Pro337–Leu340 adopts a different orientation with a flip at Pro337 as the central point (Fig. 2, a and b). The flip of Pro337 leads to 1.6- and 0.9-Å shifts on the Cα of Ser338 and Met339. The active site heme and cyanide have undergone a very slight rigid body shift with respect to the protein backbone (<0.3 Å). The Fe-C-N angle is now 154°, and the polar contact with A224 is preserved. The hydrogen bond to Val225 has however lengthened to 3.3 Å (Fig. 2c).

The indole ring of the substrate is oriented in a perpendicular manner with respect to the heme plane in a hydrophobic pocket and sandwiched between Leu140 and Phe201 on one side and on the opposite side by the main chain of Gly223 (Fig. 2c). The indole ring nitrogen atom forms a hydrogen bond with the amide of Pro222. This arrangement is the same as seen in the binary complexes with 7-Cl-L-tryptophan, 7-Cl-D-tryptophan, and D-tryptophan but different from the binary complex of L-tryptophan (13). This supports our previous prediction that the binary complex of with L-tryptophan was an artifact (13). However, although the indole ring binding environment is conserved, the amine and carboxylate groups have moved in the ternary complexes, in effect by rotation around the CB–CG bond of 7-Cl-L-tryptophan by 68°. This breaks the coordination of tryptophan substrate to the iron atom seen in the binary complex (13). In the new ternary complexes, the amino group makes salt contact with the carboxylate of the heme, a hydrogen bond with the side chain of Ser332, and polar contact with nitrogen of the indole. These contacts complete a tetrahedral environment at the amino nitrogen atom. The carboxylate group of the substrate makes hydrogen bonds with the backbone amide of Ser332 and the hydroxyl of Tyr321 (Fig. 2c). These residues are part of the loop structure that changes upon formation of the ternary complex. Arg206 is 3.5 Å from the carboxylate and has adjusted its conformation upon binding the substrate to make the ion pair.

In the TDO L-tryptophan complex, the Ca, Cβ, amino group and carboxylate group are positioned in a similar position with respect to the heme (15). In TDO, Arg117 (equivalent to Arg206 in PrnB) makes a bidentate ion pair interaction with the carboxylate of L-tryptophan (15). The substrate tryptophan amino group also makes a tetrahedral arrangement with hydrogen bonding to Thr254 (equivalent to Ser332 in PrnB). There is no counterpart of Tyr321 in TDO. The indole ring is rotated relative to that seen in the PrnB structure and has a different set of interactions in TDO, although it is also perpendicular to the heme. As we noted previously, the most significant difference between PrnB and TDO (IDO) is the loop Gly223–Val225. In PrnB, this loop extends over the heme to a greater degree and has a closer contact with the indole ring (13).

Functional Analysis—The triple Cys mutant of PrnB was used as the template to create Tyr321F and Ser332 single mutants and the double mutant. Mutants Y321F and S332A (and the double mutant) demonstrate no activity in the in vivo assay (Fig. 3) despite being properly folded (supplemental material). Despite using both enantiomers of tryptophan and of 7-Cl-tryptophan, we were unable to obtain a ternary complex with a D-enantiomer. We used isothermal titration calorimetry to measure the binding of both L-tryptophan and D-tryptophan (we lacked sufficient quantities of the pure 7-Cl-tryptophan enantiomers). We note that the measured stoichiometry (which varies from 0.4–0.7) is less than 1, inconsistent with a solution of monomers. We speculate that this is due to experimental error or inactive protein due to either misincorporation of heme or partial misfolding loop of the flexible loop (residues 324–335) or partial loss of cyanide. In the absence of
cyanide, PrnB binds \(l\)-tryptophan at the active site heme (sixth position) in an endothermic manner (Fig. 4). The same enzyme binds \(d\)-tryptophan in fact more strongly than \(l\)-tryptophan and in an exothermic manner. This is consistent with our previous studies in which we observed that PrnB will preferentially form crystals with \(7\)-Cl \(d\)-tryptophan when a racemate is used. The endothermic binding of \(l\)-tryptophan to PrnB means that this is entropy-driven binding, suggesting that movement of the ordered water molecules around \(l\)-tryptophan and ordered water molecules in the active site to the bulk solvent is key to binding. As noted previously (13), \(l\)-tryptophan binds in a unique manner not seen for other PrnB ligands, where the amine nitrogen coordinates the central iron, and the indole ring is rotated. The \(pK_a\) of the amino nitrogen presumably makes a less enthalpically favorable interaction than the more negatively charged carboxylate seen in the other binary complexes (13). Further, the carboxylate of \(l\)-tryptophan in the binary complex interacts with the heme carboxylate groups in a presumably enthalpically unfavorable manner (13). In the \(l\)-tryptophan ternary complex (Figs. 2c and 4 and Table 1) and in the binary \(d\)-tryptophan (13) complex, the favorable interactions contribute favorably to the enthalpy of binding. In the absence of cyanide, both Y321F and S332A mutants show similar thermal traces upon \(l\)-Trp binding as the triple Cys mutant. However, for these mutants, no binding toward \(l\)-tryptophan is detected after cyanide titration, suggesting that they are incapable of forming a ternary complex. Cyanide titration also seems to abolish \(d\)-tryptophan binding, consistent with our inability to obtain a ternary co-complex of \(d\)-tryptophan. Although the activity of PrnB in vivo is reproducible and robust, we have continued to fail to demonstrate activity in vitro.

**DISCUSSION**

This is the first structural description of a ternary complex with substrate tryptophan of the TDO superfamily. PrnB is an unusual member of this family. TDO does not have a net requirement for electrons because it oxidizes its substrate by placing two oxygen atoms in it, whereas PrnB does not place oxygen in the final product but must reduce the oxygen to peroxide (2e) or water (4e) to regenerate the enzyme. Despite structurally similarity, the heme iron has a much more negative reduction potential in PrnB, a fact we had attributed to the loop
The ternary complex shows extensive recognition of the L-configured substrate, including protein structure rearrangement. Mutation of two important recognition residues, Tyr321 and Ser332, leads to loss of PrnB activity in vivo. The structure shows that a D-configured substrate’s amine group would clash with the cyanide, and attempting to relieve such a clash would result in clashes of the carboxylate group. Isothermal titration calorimetry demonstrates that it is only the ternary complex that displays this enantioselectivity.

The striking difference between the TDO tryptophan complex and the PrnB ternary complex is in the orientation of the indole ring relative to the heme. In the TDO complex, the indole nitrogen points toward the heme, and in PrnB, the indole nitrogen points away. The plane of the indole rings are offset by 27°. PrnB favors its indole binding orientation by making a hydrogen bond between Pro222 and the indole nitrogen. PrnB “holds” the indole ring perpendicular to the heme by “sandwiching” the indole ring within the protein structure. The orientation of the indole ring observed in TDO is not possible in PrnB because the ring would clash with protein side chains or the heme group, and the chlorine atom of 7-Cl-L-tryptophan would clash with Val44. Equally, the indole orientation in PrnB would clash with Tyr27 and Phe51 in TDO. The substrate orientations are mutually exclusive in TDO and PrnB.

Our ITC data suggest an ordered binding mechanism, oxygen (cyanide) first and 7-Cl-L-tryptophan second, in order to form the ternary complex. The ternary structure of PrnB strongly favors formation of oxygen carbon bond at C3 of the substrate (nitrogen of cyanide is 3.6 Å away) (Fig. 5a). This is of course consistent with indole chemistry, where electrophilic addition always occurs at the C3 position. The ternary complex shows that the amine group of the substrate is close to the nitrogen of cyanide, and we propose that oxyferrous complex deprotonates the amine of the substrate (Fig. 5a). This interaction occurs in PrnB with the cyanide nitrogen (equivalent to the heme-bound oxygen in dioxygenase). The interaction between the tryptophan amino group and the bound oxygen in TDO has recently been reported (29). Based on standard indole and peroxy chemistry, it is then possible to construct a mechanism for the indole rearrangement that proceeds by electrophilic addition of hydroxyl at C3 (Fig. 5a). The mechanism produces the ferryl intermediate (shown to exist in the TDO mechanism) (19) but does not require the iron alkylperoxo species previously proposed (13). We constructed a model of the hydroxylated three ring intermediate and ferryl intermediate (Fig. 5b). The organic compound was derived using the PRODRG server (30) with chirality at C2 and C3 of the indole based on the ternary complex (Fig. 2c). We manually positioned this in the active site overlapping the indole ring and the carboxylate group (Fig. 5b).

In vivo, the structure shows that a D-configured substrate’s amine group would clash with the cyanide, and attempting to relieve such a clash would result in clashes of the carboxylate group. Isothermal titration calorimetry demonstrates that it is only the ternary complex that displays this enantioselectivity.

The striking difference between the TDO tryptophan complex and the PrnB ternary complex is in the orientation of the indole ring relative to the heme. In the TDO complex, the indole nitrogen points toward the heme, and in PrnB, the indole nitrogen points away. The plane of the indole rings are offset by 27°. PrnB favors its indole binding orientation by making a hydrogen bond between Pro222 and the indole nitrogen. PrnB “holds” the indole ring perpendicular to the heme by “sandwiching” the indole ring within the protein structure. The orientation of the indole ring observed in TDO is not possible in PrnB because the ring would clash with protein side chains or the heme group, and the chlorine atom of 7-Cl-L-tryptophan would clash with Val44. Equally, the indole orientation in PrnB would clash with Tyr27 and Phe51 in TDO. The substrate orientations are mutually exclusive in TDO and PrnB.

Our ITC data suggest an ordered binding mechanism, oxygen (cyanide) first and 7-Cl-L-tryptophan second, in order to form the ternary complex. The ternary structure of PrnB strongly favors formation of oxygen carbon bond at C3 of the substrate (nitrogen of cyanide is 3.6 Å away) (Fig. 5a). This is of course consistent with indole chemistry, where electrophilic addition always occurs at the C3 position. The ternary complex shows that the amine group of the substrate is close to the nitrogen of cyanide, and we propose that oxyferrous complex deprotonates the amine of the substrate (Fig. 5a). This interaction occurs in PrnB with the cyanide nitrogen (equivalent to the heme-bound oxygen in dioxygenase). The interaction between the tryptophan amino group and the bound oxygen in TDO has recently been reported (29). Based on standard indole and peroxy chemistry, it is then possible to construct a mechanism for the indole rearrangement that proceeds by electrophilic addition of hydroxyl at C3 (Fig. 5a). The mechanism produces the ferryl intermediate (shown to exist in the TDO mechanism) (19) but does not require the iron alkylperoxo species previously proposed (13). We constructed a model of the hydroxylated three ring intermediate and ferryl intermediate (Fig. 5b). The organic compound was derived using the PRODRG server (30) with chirality at C2 and C3 of the indole based on the ternary complex (Fig. 2c). We manually positioned this in the active site overlapping the indole ring and the carboxylate group (Fig. 5b).

In this position, all of the interactions identified in the ternary complex are preserved. The closest approach between the protein and intermediate is 2.7 Å, and only a minor adjustment in protein structure would be required to alleviate unfavorable van der Waals interactions. The model argues against any deprotonation of the indole nitrogen (no base), consistent with recent results on TDO (17). The collapse to the final product would be driven by aromatization and elimination of carbon dioxide. Regeneration of the heme would require further reduction, presumably by the as yet unidentified protein or co-factor (Fig. 5a).
Insights into the Indolamine Dioxygenase Superfamily

The ternary complex helps to further the understanding of the TDO mechanism. PrnB shows no activity as a tryptophan dioxygenase, which had been previously attributed to unusual redox potential, because the previous TDO mechanism had favored attack at C3. However, recent data on the mechanism of TDO has favored formation of the iron peroxy intermediate at the indole C2 carbon by a radical mechanism (19) as the initial step. In PrnB, formation of such a species would require significant movement of substrate or heme to avoid steric clashes between them. This may explain the lack of tryptophan dioxygenase in PrnB. Reexamining the TDO tryptophan complex (15) and placing cyanide at a similar location as seen in PrnB reveals that formation of the iron alkylperoxy species at the C3 indole carbon is impossible in TDO unless there is significant movement of the heme or substrate to avoid a steric clash. Our data suggest that TDO would appear forced by structural considerations to form C2 peroxy species, consistent with recent calculations (19). This is the mirror opposite of PrnB, which is forced to attack the C3 position. The mutually exclusive nature of the site of attack is a consequence of the relative orientation of the C2–C3 indole bond, which occupies a similar location in the two complexes, but the vector from C2 to C3 is rotated by 180° between them. An alternative (non-radical mechanism) for TDO would have the same C3 hydroxyl species as predicted in PrnB (Fig. 5b) but the ferryl intermediate (not the amino group) would attack at C2 to give a dihydroxylated species. It is the different orientation of the C2 C3 vector that would enforce the choice of the C2 nucleophile.

CONCLUSION

The ternary complex of PrnB reveals that enantioselectivity is only established when heme is coordinated by an oxygen mimic, cautioning against overinterpreting binary complexes. The first ternary complex with tryptophan substrate of the TDO superfamily suggests that the chemical differences between dioxygenase and indole rearrangement chemistries are controlled by the relative orientation of a single indole bond with respect to the heme group. The data here fill an important gap in our understanding of this important chemistry and provide a sound basis for further theoretical approaches.

Acknowledgment—This project used the Scottish Universities Life Science Alliance structural biology facility.

REFERENCES

1. van Pee, K. H., and Ligon, J. M. (2000) Nat. Prod. Rep. 17, 157–164
2. Arima, K., Imanaka, H., Kousaka, M., Fukuda, A., and Tamura, G. (1964) Agric. Biol. Chem. 28, 575–576
3. Hammer, P. E., Hill, D. S., Lam, S. T., Van Pee, K. H., and Ligon, J. M. (1997) Appl. Environ. Microbiol. 63, 2147–2154
4. Körner, S., Hammer, P. E., Hill, D. S., Altmann, A., Fischer, I., Weislo, L. J., Lanahan, M., van Pee, K. H., and Ligon, J. M. (1998) J. Bacteriol. 180, 1939–1943
5. Hammer, P. E., Burd, W. H., Hill, D. S., Ligon, J. M., and van Pee, K. H. (1999) FEMS Microbiol. Lett. 180, 39–44
6. Hohaus, K., Altmann, A., Burd, W., Fischer, I., Hammer, P. E., Hill, D. S., Ligon, J. M., and van Pee, K. H. (1997) Angew Chem. Int. Ed. Engl. 36, 2012–2013
7. Dong, C., Flecks, S., Unversucht, S., Haupt, C., van Pee, K. H., and Naismith, J. H. (2005) Science 309, 2216–2219
8. Yeh, E., Cole, L. J., Barr, E. W., Bollinger, J. M., Jr., Ballou, D. P., and Walsh, C. T. (2006) Biochemistry 45, 7904–7912
9. Yeh, E., Blasiak, L. C., Koglin, A., Drennan, C. L., and Walsh, C. T. (2007) Biochemistry 46, 1284–1292
10. Zhu, X., De Laurentis, W., Leang, K., Herrmann, J., Ihlefeld, K., van Pee, K. H., and Naismith, J. H. (2009) J. Mol. Biol. 391, 74–85
11. Flecks, S., Patillo, E. P., Zhu, X., Enyedi, A. I., Seifert, G., Schneider, A., Dong, C., Naismith, J. H., and van Pee, K. H. (2008) Angew Chem. Int. Ed. Engl. 47, 9533–9536
12. Lee, J. K., Ang, E. L., and Zhao, H. M. (2006) J. Bacteriol. 188, 6179–6183
13. De Laurentis, W., Khim, L., Anderson, J. L., Adam, A., Johnson, K., Phillips, R. S., Chapman, S. K., van Pee, K. H., and Naismith, J. H. (2007) Biochemistry 46, 12393–12404
14. Sugimoto, H., Oda, S., Otsuki, T., Hino, T., Yoshida, T., and Shiro, Y. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 2611–2616
15. Forouhar, F., Anderson, J. L., Mowat, C. G., Vorobiev, S. M., Hussain, A., Abashidze, M., Bruckmann, C., Thackray, S. J., Seetharaman, J., Tucker, T., Xiao, R., Ma, L. C., Zhao, L., Acton, T. B., Montelione, G. T., Chapman, S. K., and Tong, L. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 473–478
16. Thackray, S. J., Mowat, C. G., and Chapman, S. K. (2008) Biochem. Soc. Trans. 36, 1120–1123
17. Chauhan, N., Thackray, S. J., Rafice, S. A., Eaton, G., Lee, M., Efimov, L.,
Basran, J., Jenkins, P. R., Mowat, C. G., Chapman, S. K., and Raven, E. L. (2009) *J. Am. Chem. Soc.* **131**, 4186–4187
18. Chung, L. W., Li, X., Sugimoto, H., Shiro, Y., and Morokuma, K. (2008) *J. Am. Chem. Soc.* **130**, 12299–12309
19. Lewis-Ballester, A., Batabyal, D., Egawa, T., Lu, C., Lin, Y., Marti, M. A., Capece, L., Estrin, D. A., and Yeh, S. R. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 17371–17376
20. De Laurentis, W., Leang, K., Hahn, K., Podemski, B., Adam, A., Korschwald, S., Carter, L. G., van Pee, K. H., and Naismith, J. H. (2006) *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **62**, 1134–1137
21. Littlejohn, T. K., Takikawa, O., Skylas, D., Jamie, J. F., Walker, M. J., and Truscott, R. J. (2000) *Protein Expr. Purif.* **19**, 22–29
22. Otwinowski, Z., and Minor, W. (1997) in *Methods in Enzymology* (Carter, C., Jr., and Sweet, R. M., eds) pp. 307–326, Academic Press, London
23. Murshudov, G. N., Vagin, A. A., Lebedev, A., Wilson, K. S., and Dodson, E. J. (1999) *Acta Crystallogr. D. Biol. Crystallogr.* **55**, 247–255
24. Emsley, P., and Cowtan, K. (2004) *Acta Crystallogr. D. Biol. Crystallogr.* **60**, 2126–2132
25. Davis, I. W., Leaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang, X., Murray, L. W., Arendall, W. B., 3rd, Snoeyink, J., Richardson, J. S., and Richardson, D. C. (2007) *Nucleic Acids Res.* **35**, W375–W383
26. Bolognesi, M., Rosano, C., Losso, R., Borassi, A., Rizzi, M., Wittenberg, J. B., Boffi, A., and Ascenzi, P. (1999) *Biophys. J.* **77**, 1093–1099
27. Sugishima, M., Sakamoto, H., Noguchi, M., and Fukuyama, K. (2003) *Biochemistry* **42**, 9898–9905
28. Chang, C. J., Floss, H. G., Hook, D. J., Mabe, J. A., Manni, P. E., Martin, L. L., Schröder, K., and Shieh, T. L. (1981) *J. Antibiot.* **34**, 555–566
29. Davydov, R. M., Chauhan, N., Thackray, S. J., Anderson, J. L., Papadopoulou, N. D., Mowat, C. G., Chapman, S. K., Raven, E. L., and Hoffman, B. M. (2010) *J. Am. Chem. Soc.* **132**, 5494–5500
30. Schütte-Kopf, A. W., and van Aalten, D. M. (2004) *Acta Crystallogr. D Biol. Crystallogr.* **60**, 1355–1363