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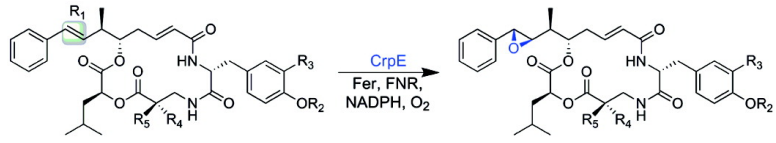

Desepoxy Cryptophycin Analogues

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Analysis of the Cryptophycin P450 Epoxidase Reveals Substrate Tolerance and Cooperativity

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Abstract: Cryptophycins are potent anticancer agents isolated from *Nostoc* sp. ATCC 53789 and *Nostoc* sp. GSV 224. The most potent natural cryptophycin analogues retain a β-epoxide at the C2′−C3′ position of the molecule. A P450 epoxidase encoded by c*rpE* recently identified from the cryptophycin gene cluster was shown to install this key functional group into cryptophycin-4 (Cr-4) to produce cryptophycin-2 (Cr-2) in a regio- and stereospecific manner. Here we report a detailed characterization of the CrpE epoxidase using an engineered maltose binding protein (MBP)−CrpE fusion. The substrate tolerance of the CrpE polypeptide was investigated with a series of structurally related cryptophycin analogues generated by chemoenzymatic synthesis. The enzyme specifically installed a β-epoxide between C2′ and C3′ of cyclic cryptophycin analogues. The k_{cat}/K_m values of the enzyme were determined to provide further insights into the P450 epoxidase catalytic efficiency affected by substrate structural variation. Finally, binding analysis revealed cooperativity of MBP−CrpE toward natural and unnatural desepoxy cryptophycin substrates.

Introduction

Over two-thirds of pharmaceutical agents introduced within the past two decades are natural products or natural product derivatives.[1] Secondary metabolites from cyanobacteria have been of increasing interest due to their structural complexity and diverse biological activities. Over 600 peptides or peptide derivatives have been isolated and identified from terrestrial, aquatic, and marine-derived cyanobacteria, mainly produced by secondary metabolic processes.[1,2] Although it has become evident recently that some cyclic peptides (e.g., patellamides) are derived via ribosomal processes that include post-translational modification, cyclization and other modification events,[3] most are assembled by nonribosomal peptide synthetases (NRPSs).

Cryptophycins are potent anticancer agents isolated from the cyanobacterial symbiont *Nostoc* sp. ATCC 53789 and its close genetic relative *Nostoc* sp. GSV 224.[4,5] Biological activity stems from their ability to stimulate cellular microtubule instability, inhibit microtubule assembly, and induce tubulin self-association, resulting in the G2/M phase transition block in the cell cycle at picomolar concentrations.[6,7] The cryptophycin mode of action and cellular target resembles both the vinca alkaloids and taxol,[8,9] two widely used clinical anticancer drugs. Furthermore, cryptophycins are not active substrates of P-glycoprotein (P-gp) and/or multiple drug resistance-associated protein (MRP), two transporters significantly overexpressed in both vinca alkaloid- and taxol-resistant cancer cells that contribute to recalcitrant tumors.[10,11]

There are more than 25 cryptophycin analogues produced by the *Nostoc* sp. ATCC 53789.[12] The major isolate is cryptophycin 1 (Cr-1) (Figure 1) that is composed of four subunits linked in clockwise order of δ-hydroxyoctenoic acid (Unit A), 3-chloro-*O*-methyl-D-tyrosine (Unit B), methyl-β-alanine (Unit C), and L-leucic acid (Unit D).[13] Variations found among cryptophycin analogues mainly occur on Units A and B.[12] Approximately half of the cryptophycin analogues include a β-epoxide at the C2′−C3′ of subunit A, as opposed to a *trans*-double bond that is introduced during assembly of this region of the molecule. All β-epoxy cryptophycins exhibit >100 times increased potency compared to their styrene counterparts, indicating that this functional group is crucial for cryptophycin biological activity.[14]

The clinical potential, validated mode of action, and synthetically challenging structure of cryptophycins, as well as lack of

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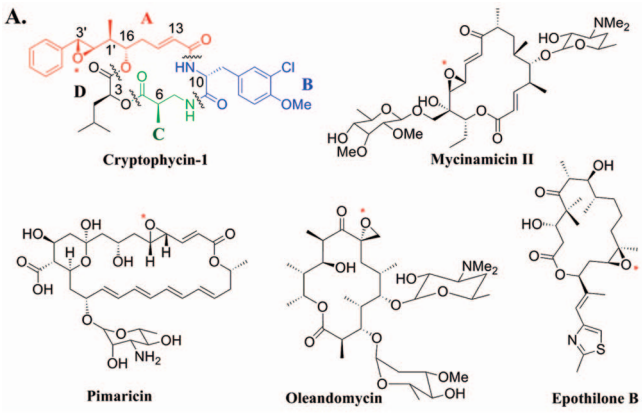

Name	R_1	R_2	R_3	R_4	R_5
Cr-438	*trans*-Styrene	Me	Cl	H	Me
Cr-424	*trans*-Styrene	Me	H	H	Me
Cr-441	*trans*-Styrene	H	Cl	H	Me
Cr-430	*trans*-Styrene	H	H	H	Me
Cr-537	*trans*-Styrene	Me	Cl	H	H

Name	R_1	R_2	R_3	R_4	R_5
Cr-1	β-Epoxide	Me	Cl	H	Me
Cr-3	*trans*-Styrene	Me	Cl	H	Me
Cr-2	β-Epoxide	Me	H	H	Me
Cr-4	*trans*-Styrene	Me	H	H	Me
Cr-16	β-Epoxide	H	Cl	H	Me
Cr-17	*trans*-Styrene	H	Cl	H	Me
Cr-41	β-Epoxide	H	H	H	Me
Cr-43	*trans*-Styrene	H	H	H	Me
Cr-BE	β-Epoxide	Me	H	Me	Me
Cr-B	*trans*-Styrene	Me	H	Me	Me
Cr-539	β-Epoxide	Me	Cl	H	H
Cr-538	*trans*-Styrene	Me	Cl	H	H

Figure 1. Chemical structure of several epoxy natural products and MBP−CrpE substrates. (A) Cryptophycin-1 (Cr-1) and other examples of natural products containing an epoxide moiety installed by P450s. (B) Six *seco* and six cyclic cryptophycin substrates used in MBP−CrpE studies. The six observed reaction products are also shown.

large-scale fermentation methods to produce cryptophycins have stimulated development of synthetic methods to provide suitable amounts of material for clinical studies. Installation of the β-epoxide at the C2′−C3′ position of Unit A has been the most daunting step in the synthesis of these natural products.[14] Initial efforts utilized *m*CPBA or dimethyl dioxirane (DMD) for the oxidation of the *trans*-styrene to form the epoxide.[15,16] In this approach, about one-third of key cryptophycin precursors were converted into unfavorable α-epoxy cryptophycin, whose potency is at least 100 times lower than the β-epoxy product.[15] Although several other stereospecific epoxidation methods have been developed, there remains significant need for improvement in yield and selectivity.[17,18]

Recently, the respective cryptophycin biosynthetic gene clusters were identified and characterized in *Nostoc* sp. ATCC 53789 and *Nostoc* sp. GSV 224.[12] Each is composed of two polyketide synthase (PKS) genes, *crpA* and *crpB*, two NRPS genes, *crpC* and *crpD*, and four tailoring enzyme genes including a P450 (*crpE*), putative 2-ketoglutarate-dependent enzyme (*crpF*), decarboxylase (*crpG*), and flavin-dependent halogenase (*crpH*). Units A, B, C, and D of cryptophycin are assembled and cyclized to the macrocyclic depsipeptide by Crp A−D, whereas individual tailoring enzymes are responsible for modification of specific subunits and postassembly modification. Characterization of the cryptophycin gene cluster has enabled the development of useful tools for preparation of new cryptophycin analogues by chemoenzymatic strategies. For example, CrpD thioesterase (TE) has provided efficient access to desoxy-deschloro-cryptophycin-1 from the corresponding *seco*-cryptophycin precursor.[19] Recently, CrpE was shown to be a P450 epoxidase and subsequently employed for tandem assembly of cryptophycins with CrpD TE in a single reaction vessel.[12,19]

P450s are a superfamily of heme-thiolate enzymes composed of more than 6000 members (http://drnelson.utmem.edu/CytochromeP450.html). Their catalytic function depends on the consumption of NADPH/NADH and O_2. In bacteria, fungi, and plants, P450s are widely found in the biosynthesis of antibiotics and other natural products with biological activities by catalyzing a variety of reactions such as hydroxylation and epoxidation. Several bacterial P450 epoxidases have been demonstrated to participate in secondary metabolite pathways (Figure 1).[20–24] For example, epothilones C and D are converted into epothilones A and B by a P450 epoxidase (EpoK) in the myxobacterium *Sorangium cellulosum*,[25,26] resulting in the most active forms of this important class of antitumor natural products. However, only a few biochemical studies have been conducted to investigate these bacterial P450 epoxidases functioning in secondary metabolic pathways.

We report here a full biochemical characterization of CrpE P450 epoxidase. Purified CrpE was reconstituted with redox partners and tested for function against a series of cryptophycin substrate analogues to assess selectivity. CrpE tolerated all naturally occurring variations on Unit B and Unit C of cryptophycin structural analogues. Binding studies and kinetic analysis of CrpE, using both natural and unnatural substrates, were performed to provide information on structural factors that influence enzyme efficiency. Finally, spectral titration analysis of CrpE revealed that substrate cooperativity likely occurs in this unusual bacterial P450.

Experimental Methods

Chemoenzymatic Preparation of MBP−CrpE Substrates. Detailed information about the chemical synthesis and characterization of the CrpD TE substrates can be found in the Supporting Information. Unit A (**7**) (Figure S4) was synthesized according to the procedure by Georg et al.[27] CrpD TE was overexpressed and purified following the previously described method.[19] Macrocyclic substrates were generated from *seco*-cryptophycin intermediates in

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a 50 mL reaction consisting of 50 mM Tris-Cl, pH 8.0, and 10 μM CrpD TE. The reactions were allowed to incubate overnight at room temperature. Following incubation, the reaction mixtures were extracted twice with equal volumes of ethyl acetate. The combined ethyl acetate extracts were dried with Na_2SO_4 and concentrated in vacuo. The resulting residue was dissolved in DMSO and water (1:1), and the products were separated by a XBridge Prep C18 5 μM column. Product analysis was achieved by MS.

In Vitro Assay of MBP−CrpE. MBP−CrpE reactions contained 100 μg/mL of ferredoxin from spinach, 0.2 unit/mL ferredoxin $NADP^+$ reductase from spinach, 1.4 mM NADPH, 10 mM glucose-6-phosphate, 8 units/mL glucose-6-phosphate dehydrogenase in 100 μL storage buffer (50 mM sodium phosphate solution, pH 6.4). The alternative redox partners tested were *Escherichia coli* NADPH flavodoxin reductase and flavodoxin or rat NADPH cytochrome P450 reductase. A DMSO stock solution of Cr-3 (2 mM) was added to the reaction mixture to reach a final concentration of 5 μM, such that the final concentration of DMSO in the reaction was 5%. The reaction mixture was prewarmed at room temperature for 2 min. The reactions were initiated by addition of MBP−CrpE to a final concentration of 0.2 μM. Reaction mixtures were incubated at room temperature for 2–4 h. In order to investigate optimal conditions for MBP−CrpE reaction, reactions were performed at 4, 15, 25, 30, 37, and 42 °C. In addition, optimal pH of the reaction was determined by monitoring product formation as a function of reaction pH (pH of 5, 6, 6.4, 7, 8, and 9). From these analyses, optimal reaction conditions were determined to be 50 mM sodium phosphate buffer, pH 6.4, at 25 °C. Cr-4, Cr-17, Cr-43, Cr-B, Cr-538, *SNAC*-Unit A, and *seco*-cryptophycin Cr-438, Cr-424, Cr-442, Cr-430, and Cr-537 (each at a final concentration of 5 μM) were used investigate MBP−CrpE substrate selectivity. Following incubation at 25 °C for 2–4 h, reaction mixtures were extracted twice with equal volume of ethyl acetate. The combined ethyl acetate extracts were dried and subsequently concentrated in vacuo. The concentrated compounds were dissolved into DMSO/water (1:1) and separated by analytical reverse-phase HPLC (XBridge C18 column, 1 mL/min, 30–100% acetonitrile/water + 0.1% TFA, 40 min, 218 nm).

Kinetic Analysis of MBP−CrpE Reactions. MBP−CrpE reactions contained 100 μg/mL of ferredoxin, 0.2 unit/mL of ferredoxin $NADP^+$ reductase, and 1.4 mM of NADPH in 100 μL storage buffer. Substrate concentrations ranged from 2 to 100 μM for Cr-3, Cr-4, Cr-17, Cr-43, and Cr-B and 2–60 μM for Cr-538. The reactions contained 5% DMSO (v/v). The reaction mixture was prewarmed at room temperature for 2 min, after which MBP−CrpE was added to a final concentration of 0.2 μM to initiate each reaction. The reactions were incubated at room temperature for 10 min and quenched with addition of two volumes of methanol. After centrifugation at 13 200 rpm for 5 min, the 300 μL solutions were subjected to HPLC analysis. The areas of the product peaks were calculated and converted to moles by fitting into the Cr-4 standard curve. The kinetic data were analyzed with KaleidaGraph software (Adelbeck Software) and fit to the linear function. All experiments were performed in duplicate.

Binding Analysis of MBP−CrpE. Binding affinities of ligands to MBP−CrpE were determined using 0.3 μM of enzyme in a total volume of 1.0 mL of 50 mM sodium phosphate solution, pH 6.4. A Carey 300 Bio UV–visible spectrophotometer was used to record the spectrum from 350 to 450 nm. Ligands used in the assay were dissolved in DMSO. Organic solvent final concentration was less than 1.5% (v/v). An equal volume of DMSO was added to the reference cuvette. The change in absorbance (ΔA) was determined by subtracting the absorbance at 422 nm from the absorbance at 388 nm. Nonlinear regression (KaleidaGraph) was used to fit data to the equation $\Delta A = \Delta A_{max} S^n/(K_d^n + S^n)$, where n is a measure of cooperativity. One micromolar GroEL solution was used in the control experiment with Cr-538 as the ligand.

Results

Overexpression of *crpE* in *E. coli.* The 1.4 kb *crpE* gene was identified at the 3′-end of cryptophycin biosynthetic gene cluster[12] and cloned into the pSJ8 expression vector to produce CrpE tagged with maltose-binding protein (MBP) at its amino terminus. The MBP−CrpE fusion protein, when coexpressed with select chaperones (Supporting Information), resulted in significant levels of soluble protein that displayed the expected red-brown color.[28] A protein of molecular weight ~57 kDa (presumed to be GroEL) was copurified with MBP−CrpE (Figure S1, Supporting Information), and all attempts to separate this contaminant from the fusion protein by addition of ATP and urea[29,30] were unsuccessful (Figure S1). Thus, the studies described in this report were performed on the MBP−CrpE fusion protein in the presence of this bound polypeptide.

Reconstruction of P450 MBP−CrpE Reaction System. CrpE was hypothesized to be an epoxidase based on bioinformatic analysis. Comparison of its sequence to four bacterial P450 epoxidases from secondary metabolic pathways (EpoK, PimD, MycG, and OleP) revealed that all share two conserved motifs (Figure S2).[20,21,23,24,31,32] A Ser or Thr residue in motif I is likely responsible for O_2 binding and cleavage, and an invariant Cys residue in motif II presumably serves as the fifth ligand for the heme iron. The presence of these conserved sequence motifs suggested that CrpE is a P450 enzyme. Subsequently, UV–visible CO difference spectrum analysis of MBP−CrpE gave direct evidence for this conclusion (Figure S1). Although TEV protease digestion provided native CrpE, this protein lacked suitable stability (estimated half-time of 2 h) for biochemical studies, and therefore the studies reported in this paper utilized the MBP fusion protein.

The native *Nostoc* sp. electron donor partners for CrpE are unknown. In our studies, spinach ferredoxin (Fer) and ferredoxin $NADP^+$ reductase (FNR) were used to provide electrons for MBP−CrpE catalytic turnover (Figure 2). An NADPH regenerating system (e.g., glucose-6-phosphate and glucose-6-phosphate dehydrogenase[20]) had only a minor effect on MBP−CrpE activity. Thus, the reconstituted MBP−CrpE reaction included Fer, FNR, MBP−CrpE, NADPH, and O_2. Maximal enzyme activity occurred in 50 mM sodium phosphate buffer, pH 6.4, at 25 °C (Figure S3).

Analysis of MBP−CrpE Substrate Tolerance. Compounds used to assess the substrate specificity of MBP−CrpE (Cr-438, Cr-424, Cr-442, Cr-430, and Cr-537, see Figure 1) were synthesized chemoenzymatically (Figure S4) from the corresponding *N*-acetylcysteamine (SNAC) *seco*-cryptophycin. The structure of each compound was confirmed by ^{1}H NMR, ^{13}C NMR, and MS analysis (Supporting Information). In the final step, CrpD TE was used to catalyze macrocyclization of *seco*-cryptophycins[19] to provide Cr-3, Cr-4, Cr-17, Cr-43, and Cr-538, respectively. Two additional members in the library included *SNAC*-Unit A as a linear CrpE substrate and Cr-B as a cyclic substrate (Figure 1).

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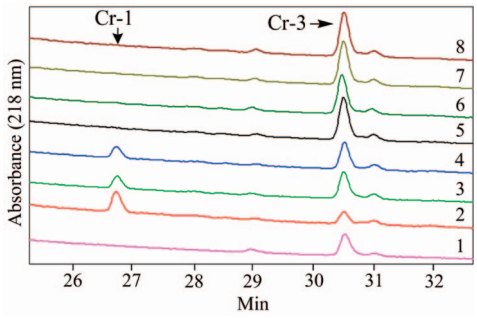

Figure 2. Investigation of MBP−CrpE reaction system components. The standard enzyme reaction with Cr-3 as substrate contained MBP−CrpE, spinach ferredoxin (Fer), spinach ferredoxin NADP$^+$ reductase (FNR), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (G6PDH) in 100 μL of storage buffer and was analyzed by HPLC-UV. Lane 1, standard Cr-3; lane 2, standard enzyme reaction; lane 3, standard reaction with CrpE replacing MBP−CrpE; lane 4, standard reaction omitting G6PDH; lane 5, standard reaction with *E. coli* flavodoxin (Fld) and NADPH flavodoxin reductase (Fpr) instead of Fer and FNR; lane 6, standard reaction with rat NADP$^+$ P450 reductase instead of Fer and FNR; lane 7, standard reaction omitting Fer; lane 8, the standard reaction omitting FNR. Only Fer and FNR were accepted by MBP−CrpE to produce Cr-1 from Cr-3. The presence of an NADPH regenerating system was not necessary for P450 activity. CrpE was capable of producing Cr-1 from Cr-3, although a decrease in overall production was observed. Thus, subsequent studies utilized MBP−CrpE as the enzyme source.

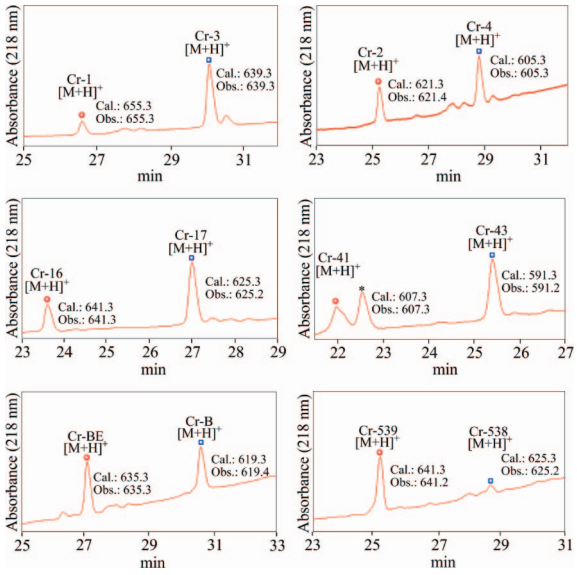

Figure 3. HPLC UV and MS analyses of MBP−CrpE reactions with Cr-3, Cr-4, Cr-17, Cr-43, Cr-B, and Cr-538 as substrates. All substrates and products yielded the expected [M + H]$^+$ values. The blue squares and the red circles represent substrates and products, respectively. A single contaminant (labeled with a dark star) was found in all reactions and is attributed to DMSO used to dissolve all substrates.

MBP−CrpE substrate selectivity was investigated with the above 12-member library that was designed to probe the affect of structural variation in Unit B and Unit C on P450 catalysis and binding. None of the linear compounds were converted to product, while all cyclic cryptophycin analogues were epoxidized by MBP−CrpE (Figure 3). Products of Cr-1, Cr-2, and Cr-16 in the reactions gave *m*/*z* values of 655.3, 621.4, and 641.3, respectively, consistent with their calculated molecular weights (Figure 3). Cr-41 (the product of MBP−CrpE conversion of Cr-43) represents a new cryptophycin analogue, not observed previously in nature. MBP−CrpE was also able to utilize unnatural cyclic cryptophycins as substrates to provide Cr-BE and Cr-539, respectively, as demonstrated by MS analysis (Figure 3). The stereochemistry and position of the β-epoxide in Cr-2 produced from Cr-4 has been confirmed previously by ^{1}H NMR analysis (Table S1).[12] Additionally, the two unnatural substrates, Cr-B and Cr-538, were treated with *m*CPBA, a reagent known to provide nonselective epoxidization,[33] in order to generate both the α- and β-epoxide stereoisomers, for comparison with the CrpE-catalyzed reaction product (Figure S5). The β-epoxide products from *m*CPBA reactions had the same retention times as those from enzyme reactions in HPLC analysis, thus confirming that, in each case, CrpE generated a single cryptophycin product that contained the desired β-epoxide configuration.

Table 1. Substrates, Slopes, *R*, and k_{cat}/K_m Values in Kinetic Analysis of MBP−CrpE[a]

substrate	slope	*R*	k_{cat}/K_m
Cr-3	0.396	0.9999	0.099
Cr-4	0.116	0.9981	0.029
Cr-17	0.121	0.9965	0.031
Cr-43	0.320	0.9973	0.080
Cr-B	0.242	0.9999	0.061
Cr-538	1.086	0.9956	0.272

[a] All experiments were performed in duplicate. The final MBP−CrpE concentration was 0.2 μM in all experiments. The volume of all reaction here was 100 μL. The units of slope and k_{cat}/K_m are pmol·μM^{-1}·min^{-1} and μM^{-1}·min^{-1}, respectively.

Kinetic Analysis of MBP−CrpE with Different Substrates. Further studies of MBP−CrpE were conducted to establish the kinetic parameters for epoxidation. In these experiments, all plots of v (pmol·min^{-1}) versus [S] (μM) were linear with 100 μM as the highest substrate concentration (Figure S6). Thus, the expected K_m values are much larger than the final cyclic substrate concentrations ([S]) in the assays. In this study, the Michaelis–Menten equation was converted into $v = k_{cat} \times [E] \times [S]/K_m$, giving slopes in the plots equal to $k_{cat}/K_m \times [E]$. The data (Table 1) show that MBP−CrpE most efficiently epoxidized Cr-538, whose k_{cat}/K_m value is 0.272 μM^{-1}·min^{-1}, while Cr-4 had the lowest k_{cat}/K_m value at 0.029 μM^{-1}·min^{-1}. Among the group of natural substrates, Cr-3 was most efficiently epoxidized by CrpE, consistent with Cr-1 as the predominant cryptophycin isolated from *Nostoc* sp. ATCC 53789. Structural modifications at Unit B and Unit C revealed significant affects on MBP−CrpE catalytic efficiency, although substrate solubility ultimately limited kinetic analysis.

Binding Analysis of MBP−CrpE. The characteristics of MBP−CrpE were assessed further by analyzing spectral changes caused by substrate binding to the enzyme. Each cyclic depsipeptide substrate generated a type I binding spectrum with a peak at ~388 nm and a trough at ~422 nm (Figure 4). The initial plot of ΔA versus S for most substrates could not be fit to the $\Delta A = \Delta A_{max} S/(K_d + S)$ equation. Instead, an equation $\Delta A = \Delta A_{max} S^n/(K_d^n + S^n)$ was used to generate sigmoidal curves. Here n is the Hill coefficient, a measure that normally indicates substrate cooperativity. By this equation, ΔA_{max}, K_d, and the n value of each cyclic substrate were generated (Table 2). All substrates had n values larger than 1, indicating cooperative binding to MBP−CrpE. Interestingly, the calculated Hill coefficient values varied according to specific structural modifications on cryptophycin Unit B and Unit C, suggesting the importance of these subunits in binding of substrates in the

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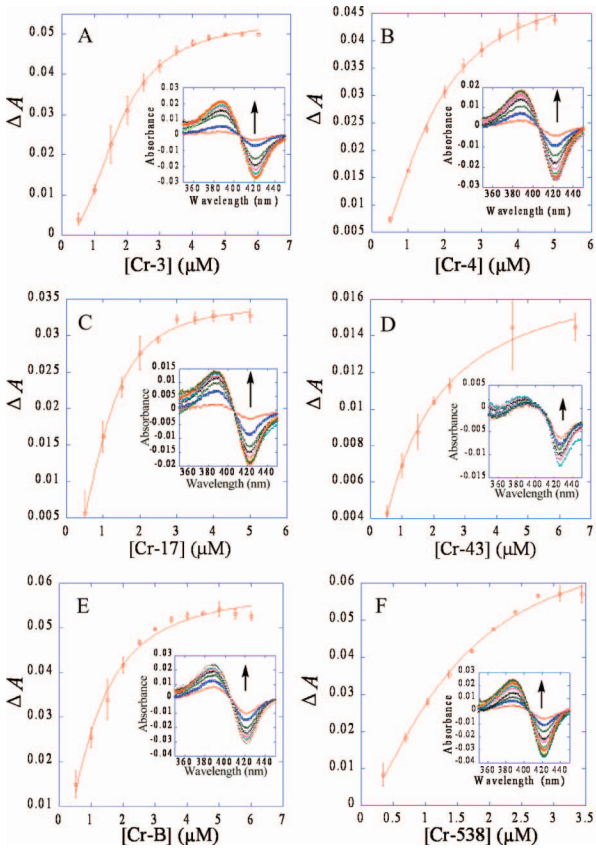

Figure 4. Spectral titration of 0.3 μM MBP−CrpE with Cr-3 (0.5–6 μM) (A), Cr-4 (0.5–5 μM) (B), Cr-17 (0.5–5 μM) (C), Cr-43 (0.5–6 μM) (D), Cr-B (0.5–6 μM) (E), and Cr-538 (0.35–3.5 μM) (F). The difference spectra resulting from different substrates are shown as insets (A−F). The direction of spectral shift upon substrate addition is shown (arrows). Absorbance changes were determined by subtracting A388 with A422 and were fitted to the equation $\Delta A = \Delta A_{max} S^n/(K_d^n + S^n)$.

Table 2. Binding Analysis of MBP−CrpE with Cyclic Substrates[a]

substrates	ΔA_{max} ($\mu M^{-1} \cdot cm^{-1}$)	K_d (μM)	n
Cr-3	0.0536 ± 0.0007	1.72 ± 0.03	2.37 ± 0.10
Cr-4	0.0511 ± 0.0014	1.57 ± 0.07	1.66 ± 0.09
Cr-17	0.0343 ± 0.0004	1.06 ± 0.03	2.20 ± 0.12
Cr-43	0.0179 ± 0.0015	1.50 ± 0.27	1.11 ± 0.16
Cr-B	0.0590 ± 0.0021	1.13 ± 0.07	1.56 ± 0.16
Cr-538	0.0744 ± 0.0047	1.43 ± 0.14	1.53 ± 0.13

[a] All experiments were performed in triplicate.

enzyme active site pocket. As shown in Eadie−Hofstee plots, all macrocyclic substrates except Cr-43 displayed cooperativity curves (Figure S7).

The K_d values of all CrpE substrates evaluated in this study were very similar. Cr-17 had the tightest binding with the K_d value of 1.06 ± 0.03 μM, while Cr-3 had the highest K_d value of 1.72 ± 0.03 μM, indicating that the interaction between substrates and the enzyme are substantial. This result also revealed that the structural variations among these substrates do not significantly affect substrate binding affinity. Maximal absorbance changes caused by different substrate binding varied from 0.0179 ± 0.0015 μM^{-1} cm^{-1} of Cr-43 to 0.0744 ± 0.0047 μM^{-1} cm^{-1} of Cr-538. Five *seco*-cryptophycin analogues and the linear *SNAC*-Unit A were also assessed in binding analysis (data not shown). Up to 100 μM of each linear substrate was added to the protein solution, but none induced a spectral change, indicating the importance of the cryptophycin macrocyclic core for substrate binding to MBP−CrpE. As a control, GroEL was substituted in place of MBP−CrpE (Figure S8), and when titrated with Cr-538, no meaningful spectral change was observed.

Discussion

Vinca alkaloids and taxol are two widely used chemotherapeutic agents in cancer treatment.[9,10] They target tubulin to inhibit microtubule dynamics in malignant cell division and, as molecular probes, established that tubulin is a valuable target for anticancer drug development. However, both vinca alkaloids and taxol are susceptible to P-gp and/or MRP, thus stimulating a search for new small molecules with a similar mechanism of action.[34–36] Cryptophycins were found to block G2/M phase transition in the cell cycle at picomolar concentrations by stimulating cellular microtubule instability, inhibiting microtubule assembly, and inducing tubulin self-association.[6] The in vivo potency of Cr-1 is 100–1000 times greater than that of taxol and vinblastine.[37] A chemically synthesized Cr-1 analogue, Cr-52 (LY 355073), evaluated in phase II clinical trials was effective against multi-drug-resistant cancer cells, suggesting that cryptophycin might be an effective chemotherapeutic agent.[37,38] Extensive SAR studies of Cr-1 were performed based on its promising activity.[14,39] Recently, Cr-309 and Cr-249 were developed as second-generation clinical candidates with improved stability and water solubility. The enhanced efficacy of these compounds compared to that of Cr-52 (LY 355073)[40] has motivated new efforts to move these compounds into clinic trials.

Over 6000 P450s have been identified from animals, plants, fungi, and bacteria. They function in both primary and secondary metabolic pathways through degradation of xenobiotic drugs[41] and production of bioactive natural products. CrpE catalyzes epoxidation at the C2′−C3′ position of the cryptophycin A subunit to produce highly potent metabolites. In this paper, we report a detailed in vitro analysis of CrpE and its ability to catalyze epoxidation of a series of natural and unnatural cryptophycin analogues. These substrates were used to investigate enzyme tolerance, stereoselectivity, kinetic parameters, substrate binding ability, and cooperativity.

Among the >25 natural cryptophycin analogues, ~14 include functional group variations such as Cl versus H, OCH_3 versus OH in Unit B, or CH_3 versus H in Unit C.[12] To assess the specificity of CrpE for Unit B and Unit C structural variation, 12 substrates were synthesized. Each of the six cyclic substrates was effectively epoxidized by the enzyme. Low solubility of cryptophycins led us to determine k_{cat}/K_m values for each molecule, in an approach similar to our previous work with

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CrpD TE.[19] Functional group modifications in Unit B resulted in complex effects on CrpE reaction efficiency. Although the presence of chlorine in Unit B of Cr-3 results in four times greater CrpE catalytic efficiency compared to Cr-4, the chlorine in Cr-17 (bearing tyrosyl OH) decreases efficiency about 3-fold compared to Cr-43 (Table 1). A significant increase in reaction efficiency was observed for Cr-538 that includes an *O*-methyl in Unit B (Table 1), with a k_{cat}/K_m value about three times greater than Cr-3. Interestingly, ~2-fold increased enzyme reaction efficiency was exhibited toward Cr-B bearing a C6 *gem*-dimethyl group compared to the C6 methyl group in Cr-4. However, the modifications on Unit B and Unit C do not significantly change substrate binding to CrpE (Table 2).

In order to determine the difference in substrate binding relative to catalytic efficiency, we probed the observed K_m and K_d parameters for CrpE. Using the steady state assumption, $K_d = k_{-1}/k_1$ while $K_m = (k_{-1} + k_2)/k_1$. If $k_{-1} >> k_2$, K_m approximates K_d. On the other hand, $K_m > K_d$ if k_2 is in the range of k_{-1}. In this study, the putative K_m values of all CrpE cyclic substrates were significantly greater than 100 μM, which is beyond the substrate solubility limit. In contrast, the observed K_d values of all substrates were below 2 μM, indicating that all k_2 values are over 50-fold greater than k_{-1} values. From this point, CrpE is highly efficient in transition from the enzyme–substrate complex formed in substrate binding to free enzyme and product. In some enzyme kinetic models,[42] K_m is expressed with an additional series of rate constants. We believe that the observed K_d parameters for CrpE are the accumulated binding affinity values of at least two substrate molecules due to cooperativity. Thus, it is difficult to ascertain which rate constant(s) contributes to the $K_m/K_d > 50$. Although the ratio of K_m/K_d for CrpE, as determined in this study, is unusually large, it is not rare to observe other P450 enzymes with K_m/K_d larger than 1. Both coumarin 7-hydroxylase mutant, P450coh C6L, and P450EryF double mutant I174F/A245T, which used 7-benzyloxyquinoline as substrate, had a ratio of K_m/K_d about 5 and over 2, respectively.[43,44]

The catalytic efficiency of CrpE was evaluated by comparing its kinetic parameters to other reported P450 epoxidases. In converting epothilone D to epothilone B, EpoK has a calculated k_{cat}/K_m value of 1.75 $\mu M^{-1}\cdot min^{-1}$. A similar catalytic efficiency ($k_{cat}/K_m = 1.44$ $\mu M^{-1}\cdot min^{-1}$) is displayed by PimD during the generation of pimaricin from 4,5-desepoxypimaricin.[20,31] Human P450 CYP3A4 and CYP3A7 each catalyzes the 10,11-epoxidation of carbamazepine during drug metabolism.[45] CYP3A4 has k_{cat}/K_m values of 0.256 and 0.095 $\mu M^{-1}\cdot min^{-1}$ by fitting the data to a two-site equation, whereas CYP3A7 has a value of 0.0007 $\mu M^{-1}\cdot min^{-1}$. The k_{cat}/K_m values of CrpE determined in this study range between 0.029 and 0.272 $\mu M^{-1}\cdot min^{-1}$ for the six cyclic substrates. Thus, CrpE efficiency is approximately 6- to 60-fold lower than EpoK and PimD, but similar to CYP3A4, and at least 40-fold better than CYP3A7.

Both homotropic and heterotropic cooperativity has been observed in CYP3A4 studies, reflecting a complicated ligand−enzyme interaction.[46] In addition to CYP3A4, cooperativity has also been observed in human P450s 2C9, 1A2, and 2B6, indicating this characteristic is relatively common among multiple human P450s.[47–49] P450EryF involved in the biosynthesis of erythromycin is the only bacterial P450 reported to have cooperativity toward unnatural substrates; however, none of them are analogues of 6-deoxyerythronolide B.[50–52] Two molecules of both androstenedione and 9-aminophenanthrene were observed by X-ray crystallography to occupy the relatively large substrate binding pocket of P450EryF, with the former positioned closer to the heme iron.[53] On the basis of several crystal structures and allied studies, two prevailing hypotheses which could explain the mechanism of P450 cooperativity have emerged.[54–58] First, it has been proposed that multiple substrates bind to the same large binding pocket of the enzyme, whereby the presence of the additional ligand is required for effective catalysis by inducing tight binding of the first molecule.[59–61] Alternatively, it has been suggested that P450s provide one additional site for substrate or effector binding. Consistent with this hypothesis, Williams et al. identified two binding sites in the CYP3A4 X-ray structure.[56] One molecule of progesterone was located in the peripheral binding site, which was separated from the heme iron.

To our knowledge, all identified human P450s with cooperativity are responsible for the degradation of endogenous and exogenous chemicals. Relatively few studies have been conducted on P450 enzymes involved in the biosynthesis of natural products, and none have been shown to include substrate cooperativity. However, a recent study by Waterman et al. demonstrated that two flaviolin molecules were found in substrate binding pockets of both CYP158A1 and CYP158A2 from *Streptomyces coelicolor* A3(2).[62,63] Furthermore, substrate binding analysis yielded a Hill coefficient value of approximately 1.6 for CYP158A2. In this case, both flaviolin molecules serve as substrates in oxidative coupling reactions catalyzed by these P450s and therefore do not involve homotropic cooper-

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ativity. Thus, CrpE is the first identified biosynthetic P450 with homotrophic cooperativity, which is involved in natural product tailoring reactions.

In the current study, cyclic substrates cooperatively bind (with the exception of Cr-43 with an n value of 1.11 ± 0.16) to CrpE with n values larger than 1. Both Cr-3 and Cr-17 have n values larger than 2, while the n values of Cr-4, Cr-B, and Cr-538 were between 1.53 and 1.66. Although an X-ray structure of CrpE is currently not available, it is anticipated that one large substrate binding pocket will be present, occupied by at least two molecules of a specific cryptophycin analogue.

The β-epoxide functional group in cryptophycin natural products is crucial for biological activity,[14] and relates directly to their promise as anticancer agents. In this study, we have demonstrated that the CrpE P450 catalyzes efficient, stereospecific introduction of the functional group and provides a possible alternative to current chemical methods. The enzyme is able to accommodate variation in B and C unit structures resulting in the generation of new cryptophycin analogues. Finally, we have determined that, in addition to stereospecific epoxidation, CrpE P450 represents the first example of substrate cooperativity involving a family of natural product molecules with high therapeutic promise.

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Supporting Information Available: General chemicals, DNA subcloning, and bacterial strains used in study and protocols for heterologous overexpression and purification of MBP−CrpE, removal of GroEL from MBP−CrpE, spectral analysis of MBP−CrpE, the lists of all authors in refs 26, 40, and 63, and table of ^{1}H NMR data of Unit A of Cr-1, Cr-38, and enzyme–product with Cr-4 as substrate. This material is available free of charge via the Internet at http://pubs.acs.org.

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