Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 7, pp 1059–1064, 1996

PII: S0968-0896(96)00088-0
0968-0896/96 $15.00+0.00

A Single Monomeric Iron Center in Clavaminate Synthase Catalyzes Three Nonsuccessive Oxidative Transformations

Robert W. Busby and Craig A. Townsend*
Department of Chemistry, The Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218-2685, U.S.A.

Abstract—The trifunctional oxygenase clavaminate synthase 2 (CS2) catalyses a hydroxylation reaction and two coupled oxidative reactions, a cyclization and a desaturation, in a nonsuccessive manner. A series of experiments was performed to elucidate the number of CS2 catalytic site(s) utilized in the three oxidative transformations. The stoichiometry of Fe^{II} required by CS2 was determined to be one ion per catalytically active enzyme molecule for the cyclization/desaturation reactions, and an affinity label, modeled after the substrate for the hydroxylation reaction, was synthesized and effectively inactivated CS2. The kinetics of this process showed concentration dependence and substrate protection consistent with active site direction. In addition, when this affinity label was incubated with CS2, the enzyme showed the same first-order rate of activity loss over time in *both* the hydroxylation activity assay and the cyclization/desaturation activity assay. These results support the view that all of the reactions catalysed by CS2 occur in a single catalytic site containing one Fe^{II}. Copyright © 1996 Elsevier Science Ltd

Introduction

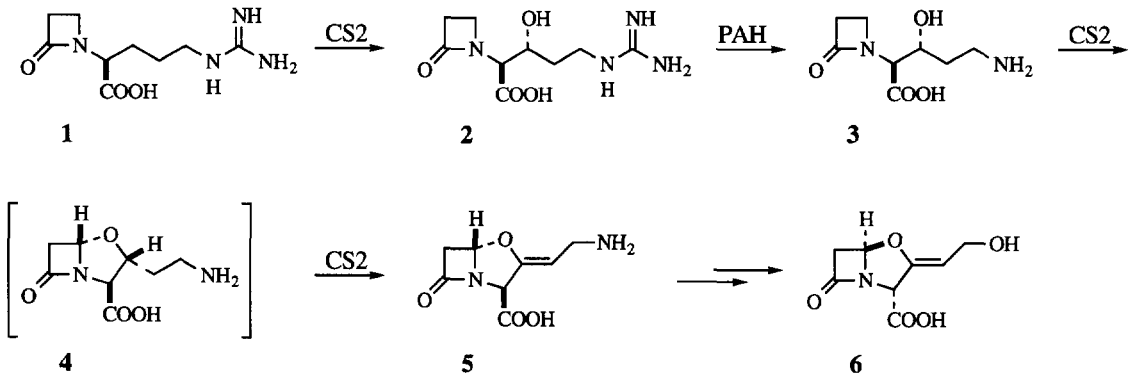
The biosynthesis of the clinically important[1] β-lactamase inactivator[2,3] clavulanic acid (**6**)[4] proceeds through the agency of a remarkable enzyme, clavaminate synthase.[5,6] Two isozymes of this protein, CS1 and CS2, have been isolated and characterized,[7,8] the latter of which resides in the gene cluster responsible for the biosynthesis of **6**.[9] Each is a nonheme Fe^{II}, α-KG-dependent oxygenase[5,7] capable of three distinct oxidative transformations (Scheme 1).[10,11] The isolated intermediates deoxyguanidinoproclavaminic acid (**1**)[12] and proclavaminic acid (**3**)[5,13,14] are both substrates for this enzyme as is the transient intermediate dihydroclavaminate (**4**).[15,16] By means of conventional α-KG-dependent hydroxylase chemistry,[17] CS2 converts **1** to **2**,[12] a reaction believed to proceed through an activated oxygen species generated by the reaction of α-KG with molecular oxygen.[18,19] One atom of molecular oxygen is incorporated into **2**[20] and the other into succinate.[17,21] Guanidinoproclavaminic acid (**2**), however, is not a substrate for CS2[22] and hydrolysis of the guanyl group by proclavaminate amidino hydrolase (PAH) is a requirement for further catalysis by CS2 (Scheme 1).[12] The conversion of **3** to clavaminic acid (**5**) requires two oxidative steps, cyclization to afford dihydroclavaminic acid (**4**)[16,23] and, finally, desaturation to give **5**.[7,13,24] Although one atom of molecular oxygen is likely incorporated into α-KG and released as succinate, the other is released as H_2O,[7,25] in sharp contrast to the hydroxylation reaction. No molecular oxygen is incorporated into clavaminate in these latter two transformations.[6] This enzyme catalyses three nonsuccessive reactions encompassing both typical and nonconventional dioxygenase activities. In this paper we present evidence that CS2 has a single active site containing one ferrous ion utilized for all of these oxidations.

[1]Clavaminate synthase, first abbreviated CS,[6] has been denoted clavaminic acid synthetase[5] and subsequently clavaminic acid synthase (CAS). We believe the first of these is preferable in view of conventional biochemical nomenclature.

Results

Despite the apparent diversity in the overall chemical transformations of **1** to **5**, all the oxidations catalysed by clavaminate synthase require identical cofactors and there is precedent for each of these reactions occurring by the same mechanism in so far as the activation of molecular oxygen in the presence of iron(II).[18,19] If this activation occurs in the same active site, then it would follow that there should be one metal ion per enzyme molecule. Determination of the stoichiometry of Fe^{II} in the CS2 active site(s) is, therefore, important to the identification of the number of these catalytic sites.

The titration of recombinant CS2[26,27] with Fe^{II} was accomplished using **3** as the substrate at a fixed concentration (0.8 mM) and keeping the amount of enzyme used to initiate the assays constant as well. The amount of Fe^{II} in the reaction was varied from 0.25 to 5.0 equiv relative to the concentration of CS2 present (1.25 μM), and the rate of production of **5** was used to calculate the velocity at each Fe^{II} concentration. Figure 1 shows that the slopes of the velocity at substoichiometric amounts of iron and V_{max} intersect at approximately one equiv of Fe^{II}. A reciprocal plot of these data is shown in Figure 2 from which a V_{max} of 0.69 μmol min^{-1} mg^{-1} was calculated and a binding constant for Fe^{II} was determined to be slightly less than 1 μM. These results indicate that, at least for the cyclization and desaturation reactions, there is one ferrous ion per CS2 molecule.



Scheme 1.

Small-molecule chemical modification has been useful in preliminary characterization of the CS2 catalytic site.[27] In order to further characterize the nature of the CS2 active site, an additional approach to chemical modification was sought in the creation of an affinity label that would covalently modify the enzyme active site. Paradoxically, this enzyme, which has three natural substrates, has been notoriously selective in accepting derivatives of **3** as inactivators or alternate substrates[28,29] with the exception of the γ-lactam analogue of **3**.[30] A substrate analogue of **1**, *N*-α-acetyl-L-arginine, was turned over by CS2 to efficiently yield the hydroxylated product.[31] A potential affinity label was sought in a modification of this analogue to incorporate an α-halocarbonyl moeity, *N*-bromoacetyl-L-arginine (**7**). The L-enantiomer would presumably serve as a better affinity label due to the fact that the 2*S* stereochemistry is required for natural or alternate substrate turnover.[14,30–34]

The synthesis of **7** was accomplished using a modified procedure of McKay and Plummer, who synthesized *N*-bromoacetyl-D-arginine.[35] The synthetic route to **7** outlined in Scheme 2 starts with the synthesis (**a**) of

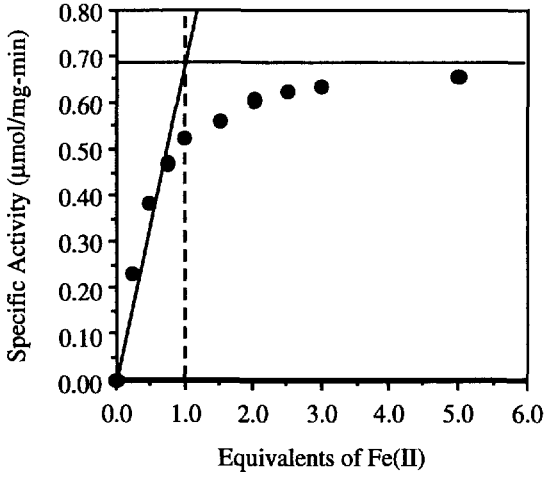


Figure 1. Titration of CS2 with Fe^{II}. CS2 cyclization/desaturation activity was determined using the indicated equivalents of Fe^{II} at 23 °C in 50 mM MOPS buffer, pH 7.0.

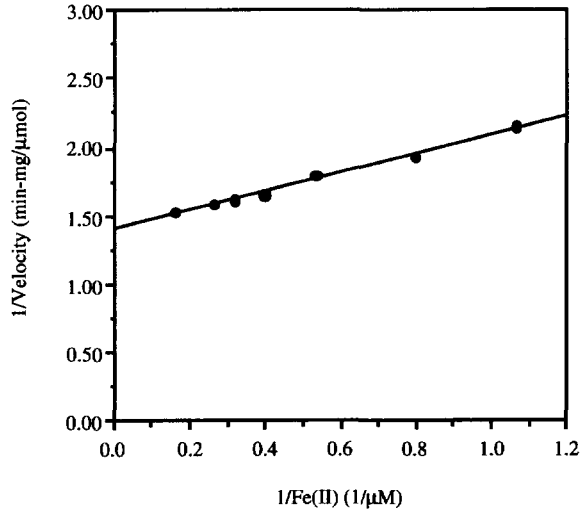
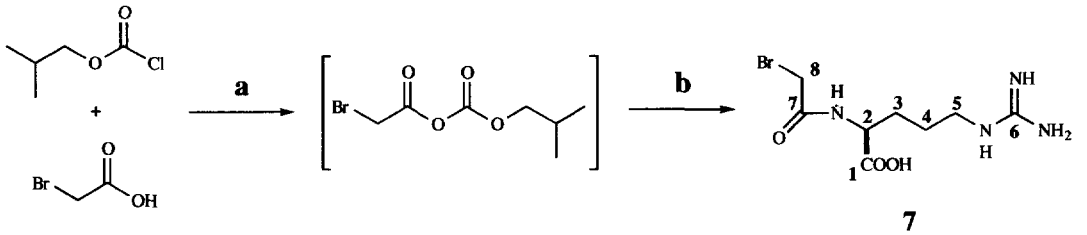


Figure 2. Reciprocal plot of CS2 velocity determined from cyclization/desaturation activity at the indicated Fe^{II} concentrations from the titration experiment shown in Figure 1.



Scheme 2.

the mixed anhydride by the reaction of bromoacetic acid, isobutylchloroformate, and triethylamine (1.0, 1.1, and 1.2 equivalents, respectively) in anhydrous tetrahydrofuran for 20 min. The mixed anhydride was then added (**b**) to 0.9 equiv of aqueous L-arginine containing excess sodium bicarbonate (pH 9) to afford **7** after 90 min. Following HPLC purification, **7** was tested as a possible inactivator of CS2. In independent tests incubation of **7** with CS2 resulted in irreversible, time-dependent inactivation of not only the hydroxylation of **1** to **2** (monitored by HPLC), but also the oxidative cyclization/desaturation reactions in the conversion of **3** to **5** (Fig. 3). The activity of CS2 in the presence of 7.5 mM of **7** was plotted over time (Fig. 3) and the decay in activity was determined to be logarithmic with a half-life ($t_{1/2}$) of 11.2 min at 30 °C.

Using the method of Bird[36] to measure the production of bicyclic products in the turnover of proclavaminate,[7] the rates of inactivation were determined over several concentrations of **7**. The resulting Kitz and Wilson plot[37] showed the inactivator exhibited saturation kinetics. The data were evaluated using the kinetic analysis programs of Cleland[38] and the K_i was determined to be 38.3 ± 3.6 mM and the rate constant at saturation (k_{inact}) was 0.32 ± 0.03 min^{-1}. Substrate and cofactor protection were observed as shown in Figure 4. The kinetic behavior of **7** in all these experiments is, therefore, consistent with active site inactivation.

If there is a single active site, an affinity label as **7** should inactivate all CS2 activities comparably. The rate of inactivation of CS2 by **7** was determined simultaneously for all reactions by the incubation of **7** with recombinant CS2 and using aliquots from this mixture to initiate the two different assays as a function of time. *For all activities*, CS2 was inactivated by 7.5 mM of **7** at the same logarithmic rate with a $t_{1/2}$ of approximately 11 min at 30 °C (Fig. 5). The parallel rates of CS2 inactivation with either substrate demonstrates that covalent modification is occurring in the same active site.

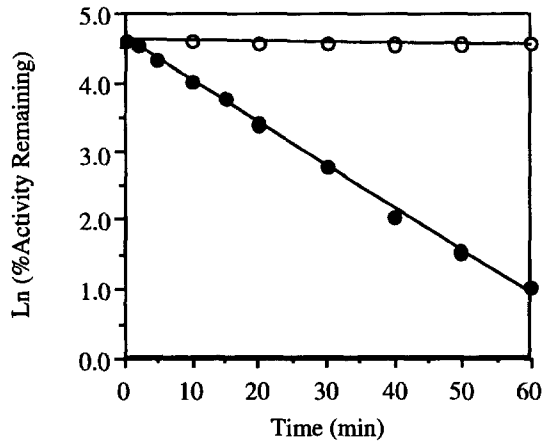


Figure 3. Time-dependent inactivation of CS2 by **7** as measured in the cyclization/desaturation assay. The natural logarithm of remaining enzyme activity versus time is shown for CS2 incubated at 30 °C with 7.5 mM **7** (●), and without **7** (○).

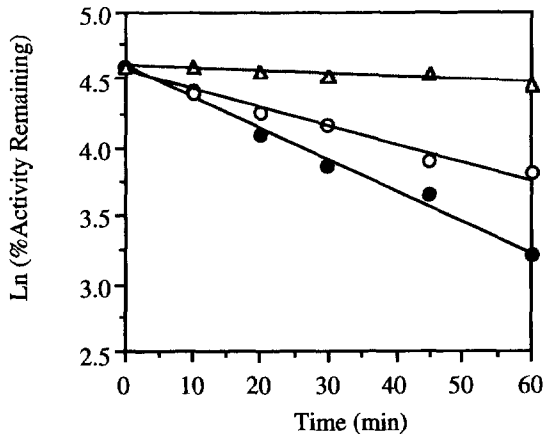


Figure 4. The presence of substrate and cofactors slow the rate of CS2 inactivation by **7**. The natural logarithm of remaining enzyme cyclization/desaturation activity versus time is shown for CS2 incubated at 30 °C with the following: 2.5 mM of **7** with no substrate or cofactors present (●), 2.5 mM of **7** and 5 mM or **3** ($>10\times K_m$), 0.5 mM α-ketoglutarate ($>10\times K_m$) and 10 μM $CoCl_2$ ($>10\times K_i$) (○), and a control of CS2 at 30 °C in the absence of **7** (Δ).

Discussion

CS2 is an exceptional enzyme that not only catalyses three reactions, but also is involved in two nonsuccessive steps in the biosynthesis of clavulanic acid. Enzymes that catalyse two reactions are uncommon and trifunctional enzymes are rarer still. Thymine 7-hydroxylase from *Neurospora crassa* is an example of a trifunctional enzyme that catalyses three successive oxidations (Scheme 3).[39,40] This enzyme oxidizes thymine (**8**) to 5-hydroxymethyluracil (**9**), 5-formyluracil (**10**), and 5-carboxyuracil (**11**) all coupled to the Fe^{II}/O_2-dependent decarboxylation of α-ketoglutarate.[39-42] All these transformations are presumably examples of the same oxidative chemistry, i.e., hydroxylation, and all reactions are consecutive with the first two products also serving as substrates for the subsequent reaction. Clavaminate synthase, in contrast,

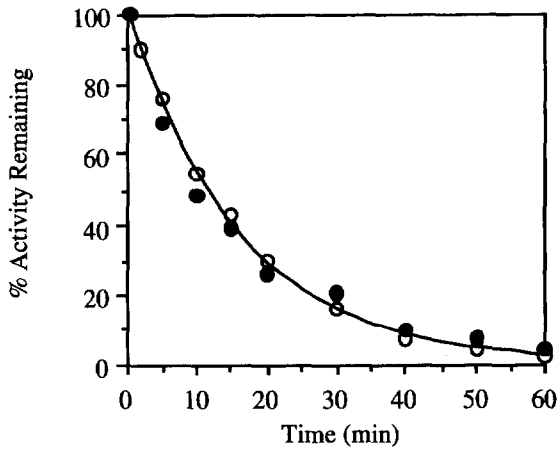
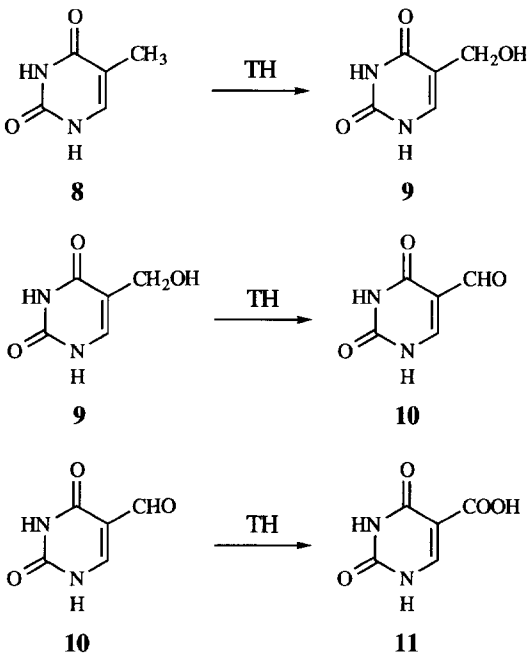


Figure 5. Inactivation of CS2 by **7** as measured for all CS2-catalyzed activities. The decrease in enzyme activity versus time is shown for CS2 incubated with the affinity label **7**. Aliquots were removed and used to initiate assays detecting hydroxylation activity (●) and cyclization/desaturation activity (○).



Scheme 3.

performs three *different* oxidative reactions with only a single product serving as a substrate, and requires the agency of an intervening enzyme (PAH) in order to carry out all three.

The important question of whether each of these reactions occurs at the same active site has until now remained unaddressed. In addition to the likelihood of a common oxygen activation mechanism for each reaction, the substrate for CS hydroxylation inhibits the turnover of **3**, the substrate for cyclization, and vice versa.[43] This outcome would be expected if both substrates bind in the same active site. The results of the determination of ferrous ion stoichiometry indicate a single Fe^{II}/CS2 molecule, consistent with other Fe^{II} oxygenases such as IPNS. Clavaminate synthase has shown many similarities to IPNS in the types of oxidations it catalyses[44] and that both enzymes have active site histidines.[27,45,46] Further evidence for the existence of a single catalytic site was obtained with the synthesis and testing of the effective affinity label **7**. This compound was modeled after the hydroxylation substrate but irreversibly inactivated the oxidative cyclization/desaturation activities as well as the hydroxylase activity of CS2. Finally, **7** inactivates all CS2 activities in an identically time-dependent manner. Taken together, these experiments constitute strong evidence that all CS2-catalysed transformations occur within a single active site harboring a single ferrous ion.

Experimental

Materials and methods

The following were obtained from Sigma (St Louis, MO): 1,4-dithio-D,L-threitol (DTT), sodium ascorbate and sodium α-ketoglutarate. L-Deoxyguanidinoproclavaminate (**1**) was synthesized as described elsewhere[22] as was *rac*-proclavaminic acid (**3**).[11,14,16] The imidazole reagent used for enzyme assays consisted of a 3 M aqueous solution of imidazole (recrystallized three times from benzene) adjusted with concd HCl to pH 6.8.[36] The Partisil 5 μm, ODS 3, 250 × 4.6 mm HPLC column used for the hydroxylase assay was purchased from Phenomenex (Torrance, CA), and the Partisil 10 μm, ODS 3, 250 × 9.4 mm HPLC column used in the purification of **7** was purchased from Whatman (Hillsboro, OR). The following instruments were used: Waters 600 HPLC and 490 Programmable Multiwavelength Detector (Milford, MA), a Beckman DU-70 UV–vis spectrophotometer (Fullerton, CA), a DuPont-Sorvall RC5 Superspeed Refrigerated Centrifuge (Wilmington, DE), an Eppendorf 5402 Refrigerated Centrifuge (Fremont, CA), and a Controlled Environment Incubator Shaker (model G-25) purchased from New Brunswick Scientific (Edison, NJ).

Preparation of recombinant clavaminate synthase

The isozyme CS2 was overexpressed and purified as previously described.[27]

Protein concentration determination

Protein concentrations were determined by the method of Bradford[47] using bovine serum albumin as the standard.

Cyclization/desaturation assay

Clavaminate synthase specific activity assays were conducted as previously described[7] using the Bird imidazole method[36] to measure the conversion of **3** to **5** by UV spectrophotometry. In a total volume of 200 μL, the substrate concentration was 0.8 mM *rac*-proclavaminate, the reaction temperature was 23 °C, and the reactions were initiated by the addition of CS2 to 1.25 μM. Incubation times ranged from 1.0 min for the iron(II) titration to 2.0 or 2.25 min for all other experiments prior to quenching by the addition of 10 μL of 4 mM EDTA. The imidazole derivatization was carried out by the addition of 100 μL of 3.0 M imidazole reagent, followed by a 20 min incubation at 40 °C. The corrected absorbance at 312 nm (adjusted for a blank containing no substrate) was used to compute the velocity assuming an extinction coefficient of 26,900 $M^{-1}\,cm^{-1}$ for the α,β-unsaturated acyl imidazole derivative of clavaminic acid.[36]

Determination of iron(II) stoichiometry

The titration of CS2 with Fe^{II} was carried out by monitoring the conversion of **3** to **5** as above. The substrate concentration was 0.8 mM *rac*-proclavaminate and reactions contained 50 mM MOPS, pH 7.0, 1.0 mM α-KG, 0.5 mM DTT, 0.1 mM ascorbic acid and variable concentrations of ferrous ammonium sulfate (FeAS). Ferrous ammonium sulfate, standardized by the method of Stookey,[48] was provided at the following

final concentrations: 0.313, 0.625, 0.938, 1.25, 1.88, 2.50, 3.13, 3.75, and 6.25 μM. The reactions were derivatized as above and the velocity plotted against the number of equiv of Fe^{II}.

Synthesis of *N*-bromoacetyl-L-arginine (7). The method used was similar to that of McKay and Plummer.[35] Bromoacetic acid (306 mg, 2.20 mmol) and triethylamine (370 μL, 2.65 mmol) in 10 mL of freshly distilled THF under argon were cooled to −15 °C in a dry ice/glycerol bath and isobutylchloroformate (235 μL, 2.42 mmol) was added dropwise. After the addition was complete, the mixture stirred for 20 min at −15 °C (Scheme 2, step **a**). This solution was then added to an equal volume of aqueous L-arginine (349.1 mg, 2.00 mmol) at 0 °C containing an excess of sodium bicarbonate (558.7 mg, 6.65 mmol) to maintain pH 9.0. The solution was stirred for 90 min at 0 °C to afford **7** (Scheme 2, step **b**). The THF was removed in vacuo at 0 °C and the compound was purified by reverse-phase HPLC (Whatman Partisil 10 μm, ODS 3; 250 × 9.4 mm; detection at 220 nm; mobile phase: H_2O, 2.0 mL min^{-1}; $t_R = 11.5$ min). ^1H NMR (400 MHz, D_2O): δ 1.56 (m, 2H, H-4), 1.70 (m, 1H, H-3α), 1.81 (m, 1H, H-3β), 3.15 (t, $J_{4-5} = 6.8$ Hz, 2H, H-5), 3.86 and 3.92 (ABq, $J_{AB} = 11.8$, 2H, H-8), 4.14 (dd, $J = 7.9$, 5.1 Hz, 1H, H-2) ppm; ^{13}C NMR (100 MHz, D_2O/dioxane): δ 24.7 (C-4), 28.1 (C-8), 28.6 (C-3), 40.5 (C-5), 55.0 (C-2), 156.6 (C-6), 169.2 (C-7), 177.9 (C-1) ppm; CIMS: *m/z* 297, 295 (MH^+), 296, 294 (M^+), 215 ($MH^+ - HBr$), 173 [(MH^- - HBr) - H_2CCO], 82, 80 (HBr).

Determination of inactivation kinetic constants K_i and k_{inact}

The inactivation kinetic constants were determined by measuring the rate of inactivation of CS2 at 1.25, 1.63, 2.5, 5.0, and 7.5 mM **7**. For each concentration of **7**, CS2 (494 μg) was incubated at 30 °C in 50 mM MOPS, pH 7.0. Aliquots of 20 μL were withdrawn at various time points and used to initiate reactions containing 1.0 mM *rac*-proclavaminate, 1.0 mM α-KG, 0.5 mM DTT, 0.1 mM ascorbic acid and 25 μM FeAS in 50 mM MOPS, pH 7.0. The reactions proceeded for 2 min before quenching with EDTA and derivatizing with imidazole as above. The loss of enzyme activity over time was used to calculate the first-order rate constant $t_{1/2}$ (min^{-1}) for each concentration of **7**. The K_i and k_{inact} were obtained by plotting $t_{1/2}$ versus the inverse concentration of **7**.

Protection of CS2 from inactivation by 7

The rate of inactivation of CS2 was measured in the presence and absence of the substrate **3**, the cofactor α-KG, and the competitive inhibitor CoCl2.[27] Except in the control, 2.5 mM **7** of was added to CS2 (494 μg) and incubated at 30 °C in 50 mM MOPS, pH 7.0. In order to test for protection, 5 mM of **3**, 10 μM $CoCl_2$, and 500 μM α-KG were included in certain incubation mixtures. Aliquots of 20 μL were withdrawn at various time points and used to initiate reactions as above except the concentration of FeAS was doubled to 50 μM. The final concentration of racemic **3** in all reactions was 1.0 mM. The reactions proceeded for 2 min before quenching with EDTA. The reactions were derivatized as above and the activity plotted over time.

Simultaneous inactivation of CS2 by 7

CS2 (858 μg) in 50 mM MOPS, pH 7.0, was inactivated by 7.5 mM **7** in a final volume of 260 μL. The enzyme solution was incubated at 30 °C and aliquots of 10 μL were withdrawn at indicated time points and used to initiate either the cyclization/desaturation assay (described above) or analyzed by HPLC for hydroxylase activity (described below). In each case the reactions contained 50 mM MOPS, pH 7.0, 1.0 mM α-KG, 0.5 mM DTT, 0.1 mM ascorbic acid and 25 μM FeAS.

HPLC hydroxylation assay

The reactions for the hydroxylation assay were prepared as the UV cyclization/desaturation assay with the following exceptions: 1.5 mM of **1** in a total volume of 200 μL was used as the substrate and the reactions proceeded at 30 °C for 3 min prior to quenching with 0.20 mM EDTA. Samples were individually thawed, filtered through a 0.22 μm filter and analyzed by reverse-phase HPLC (Partisil 5 μm, ODS 3; 250 × 4.6 mm; detection at 220 nm; mobile phase: H_2O, 1.0 mL min^{-1}; $t_R = 6.4$ min for **2**, 10.5 min for **1** and 12.5 min for **7**).

Acknowledgments

We thank Drs Janos Pitlik and Todd Houston for productive discussions and the synthesis of substrates used in the enzyme assays. We are grateful to the NIH (AI14937) for financial support of this research and for major funding that made possible the purchase of the NMR instrumentation used (RR04794 and RR06468).

References

1. Todd, P. A.; Benfield, P. *Drugs* **1990**, *39*, 264.

2. Fisher, J.; Charnas, R. L.; Knowles, J. R. *Biochemistry* **1978**, *17*, 2180.

3. Charnas, R. L.; Fisher, J.; Knowles, J. R. *Biochemistry* **1978**, *17*, 2185.

4. Howarth, T.; Brown, A.; King, T. *J. Chem. Soc., Chem. Commun.* **1976**, 266.

5. Elson, S. W.; Baggaley, K. H.; Gilett, J.; Holland, S.; Nicholson, N. H.; Sime, J. T.; Woroniecki, S. R. *J. Chem. Soc., Chem. Commun.* **1987**, 1736.

6. Krol, W. J.; Basak, A.; Salowe, S. P.; Townsend, C. A. *J. Am. Chem. Soc.* **1989**, *111*, 7625.

7. Salowe, S. P.; Marsh, E. N.; Townsend, C. A. *Biochemistry* **1990**, *29*, 6499.

8. Marsh, E. N.; Chang, M. D.-T.; Townsend, C. A. *Biochemistry* **1992**, *31*, 12648.

9. Ward, J. M.; Hodgson, J. E. *FEMS Microbiol. Lett.* **1993**, *110*, 239.

10. Baldwin, J. E.; Lloyd, M. D.; Wha-Son, B.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H.; Nicholson, N. H. *J. Chem. Soc., Chem. Commun.* **1993**, 500.

11. Townsend, C. A. *Biochem. Soc. Trans.* **1993**, *21*, 208.

12. Elson, S. W.; Baggaley, K. H.; Davidson, M.; Fulston, M.; Nicholson, N. H.; Risbridger, G. D.; Tyler, J. W. *J. Chem. Soc., Chem. Commun.* **1993**, 1212.

13. Elson, S. W.; Baggaley, K. H.; Gilett, J.; Holland, S.; Nicholson, N. H.; Sime, J. T.; Woroniecki, S. R. *J. Chem. Soc., Chem. Commun.* **1987**, 1738.

14. Krol, W. J.; Mao, S.-S.; Steele, D. L.; Townsend, C. A. *J. Org. Chem.* **1991**, *56*, 728.

15. Baldwin, J. E.; Adlington, R. M.; Bryans, J. S.; Bringhen, A. O.; Coates, J. B.; Crouch, N. P.; Lloyd, M. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H.; Cassels, R.; Nicholson, N. H. *Tetrahedron* **1991**, *47*, 4089.

16. Salowe, S. P.; Krol, W. J.; Iwata-Reuyl, D.; Townsend, C. A. *Biochemistry* **1991**, *30*, 2281.

17. Hanauske-Abel, H. M.; Günzler, V. *J. Theor. Biol.* **1982**, *94*, 421.

18. Siegel, B. *Bioorg. Chem.* **1979**, *8*, 219.

19. Kivirikko, K. I.; Myllylä, R. In *The Enzymology of Post-Translational Modification of Proteins*; Freedman, R. B.; Hawkins, H. C., Eds.; Academic: New York, 1980; pp 53–104.

20. Townsend, C. A.; Krol, W. J. *J. Chem. Soc., Chem. Commun.* **1988**, 1234.

21. Hayaishi, O.; Nozaki, M.; Abbott, M. In *Enzymes*; Boyer, P. D., Ed.; Academic: New York, 1976; 3rd edn, pp 119–189.

22. Wu, T. K.; Busby, R. W.; Houston, T. A.; McIlwaine, D. B.; Egan, L. A.; Townsend, C. A. *J. Bacteriol.* **1995**, *177*, 3714.

23. Baldwin, J. E.; Adlington, R. M.; Bryans, J. S.; Bringhen, A. O.; Coates, J. B.; Crouch, N. P.; Lloyd, M. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H.; Cassells, R.; Nicholson, N. *J. Chem. Soc., Chem. Commun.* **1990**, 617.

24. Baldwin, J. E.; Adlington, R. M.; Crouch, N. P.; Drake, D. J.; Fujishima, Y.; Elson, S. W.; Baggaley, K. H. *J. Chem. Soc., Chem. Commun.* **1994**, 1133.

25. Basak, A.; Salowe, S. P.; Townsend, C. A. *J. Am. Chem. Soc.* **1990**, *112*, 1654.

26. Lawlor, E. J.; Elson, S. W.; Holland, S.; Cassels, R.; Hodgson, J. E.; Lloyd, M. D.; Baldwin, J. E.; Schofield, C. J. *Tetrahedron* **1994**, *50*, 8737.

27. Busby, R. W.; Chang, M. D.-T.; Busby, R. C.; Wimp, J.; Townsend, C. A. *J. Biol. Chem.* **1995**, *270*, 4262.

28. Elson, S. W.; Baggaley, K. H.; Holland, S.; Nicholson, N. H.; Sime, J. T.; Woroniecki, S. R. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1503.

29. Iwata-Reuyl, D.; Basak, A.; Silverman, L. S.; Engle, C. A.; Townsend, C. A. *J. Nat. Prod.* **1993**, *56*, 1373.

30. Baldwin, J. E.; Adlington, R. M.; Bryans, J. S.; Schofield, C. J.; Baggaley, K. H.; Cassels, R. *J. Chem. Soc., Chem. Commun.* **1992**, 877.

31. Baldwin, J. E.; Lee, V.; Lloyd, M. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H. *J. Chem. Soc., Chem. Commun.* **1993**, 1694.

32. Baggaley, K. H.; Nicholson, N. H.; Sime, J. T. *J. Chem. Soc., Chem. Commun.* **1988**, 567.

33. Baggaley, K. H.; Elson, S. W.; Nicholson, N. H.; Sime, J. T. *J. Chem. Soc., Perkin Trans. 1* **1990**, 1513.

34. Baggaley, K. H.; Elson, S. W.; Nicholson, N. H.; Sime, J. T. *J. Chem. Soc., Perkin Trans. 1* **1990**, 1521.

35. McKay, T. J.; Plummer, T. H. Jr *Biochemistry* **1978**, *17*, 401.

36. Bird, A. E.; Bellis, J. M.; Gasson, B. C. *Analyst* **1982**, *107*, 1241.

37. Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245.

38. Cleland, W. W. In *Methods in Enzymology*; Purich, D. L., Ed.; Academic: New York, 1979; pp 103–138.

39. Holme, E.; Lindstedt, G.; Lindstedt, S.; Tofft, M. *Biochim. Biophys. Acta* **1970**, *212*, 50.

40. Holme, E.; Lindstedt, G.; Lindstedt, S.; Tofft, M. *J. Biol. Chem.* **1971**, *246*, 3314.

41. Liu, C. K.; Shaffer, P. M.; Slaughter, R. S.; McCroskey, R. P.; Abbott, M. T. *Biochemistry* **1972**, *11*, 2172.

42. Bankel, L.; Lindstedt, G.; Lindstedt, S. *Biochim. Biophys. Acta* **1977**, *481*, 431.

43. Busby, R. W.; Townsend, C. A. Unpublished results.

44. Baldwin, J. E.; Abraham, E. P. *Nat. Prod. R.* **1988**, *5*, 129.

45. Roach, P. L.; Clifton, I. J.; Fülöp, V.; Harlos, K.; Barton, G. J.; Hajdu, J.; Andersson, I.; Schofield, C. J.; Baldwin, J. E. *Nature (London)* **1995**, *375*, 700.

46. Ming, L.-J.; Que, L, Jr; Kriauciunas, A.; Frolik, C. A.; Chen, V. J. *Inorg. Chem.* **1990**, *29*, 1111.

47. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.

48. Stookey, L. L. *Anal. Chem.* **1970**, *42*, 779.

(*Received in U.S.A.* 21 *December* 1995; *accepted* 1 *April* 1996)