# Purification and Characterization of Clavaminate Synthase from *Streptomyces clavuligerus*: An Unusual Oxidative Enzyme in Natural Product Biosynthesis†

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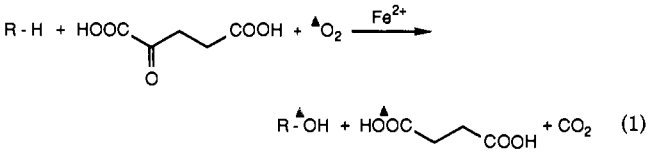
ABSTRACT: A pivotal step in the biosynthetic pathway to the β-lactamase inhibitor clavulanic acid is the conversion of proclavaminic acid to clavaminic acid in a reaction requiring $Fe^{2+}$, α-ketoglutarate, and oxygen [Elson, S. W., Baggaley, K. H., Gillett, J., Holland, S., Nicholson, N. H., Sime, J. T., & Woroniecki, S. R. (1987) *J. Chem. Soc., Chem. Commun.*, 1736–1738]. Clavaminate synthase, the enzyme that catalyzes this oxidative cyclization/desaturation, has been purified to homogeneity from clavulanic acid producing cells of *Streptomyces clavuligerus* (ATCC 27064). The enzyme behaved as a monomer during gel filtration and migrated with $M_r$ 47 000 during denaturing gel electrophoresis. After ion-exchange FPLC two active forms of the protein were resolved that differed slightly in kinetic constants and apparent molecular weight. Kinetic comparisons with the four possible diastereomers of proclavaminate confirmed the absolute configuration of the substrate to be 2*S*,3*R*. The stoichiometry of the overall transformation was determined to be proclavaminate + 2(α-ketoglutarate) + $2O_2$ → clavaminate + 2(succinate) + $2CO_2$ + $2H_2O$. In the absence of proclavaminate a slow decarboxylation of α-ketoglutarate to succinate and $CO_2$ was observed in an uncoupled reaction which resulted in enzyme inactivation. Steady-state kinetic studies were undertaken for an initial description of the enzyme's catalytic cycle. The double-reciprocal plot with α-ketoglutarate as the variable substrate was linear; this supports the proposal that two stepwise oxidations of proclavaminate occur, each with the consumption of α-ketoglutarate and oxygen and the release of succinate, $CO_2$, and $H_2O$. The intersecting initial velocity plots obtained from pairwise variation of substrate concentrations were consistent with a sequential kinetic mechanism for the first oxidation. Similarities observed between clavaminate synthase and α-ketoglutarate-dependent dioxygenases argue for a common mechanism of oxygen activation. However, the nature of the interactions of the substrates in the active site of clavaminate synthase apparently redirects the conventional hydroxylase activity of dioxygenases to the construction of a strained bicyclic skeleton driven by the overall reduction of dioxygen.

Clavulanic acid (**3**) is a natural product produced by *Streptomyces* spp. with potent β-lactamase inhibitory properties (Howarth et al., 1976; Reading & Hepburn, 1979). The biosynthesis of **3** proceeds from ornithine and glycerate (Townsend & Ho, 1985) through the intermediacy of proclavaminic acid (**1**) and clavaminic acid (**2**) (Scheme I; Elson et al., 1987b). The transformation of **2** into **3** has been shown to involve an unusual oxidative deamination and subsequent reduction accompanied by configurational inversion at carbons 3 and 5 to generate clavulanic acid (Townsend & Krol, 1988). The four-electron oxidative cyclization/desaturation of proclavaminic acid (**1**) to clavaminic acid (**2**) was shown by Elson's group at Beecham to be catalyzed in *Streptomyces clavuligerus* by a single enzyme, clavaminate synthase (CS),[1] in a complex reaction requiring $Fe^{2+}$, molecular oxygen, and α-ketoglutarate (Elson et al., 1987a). These cofactor requirements are characteristic of a class of dioxygenase enzymes that carry out hydroxylation reactions at unactivated carbon centers with concomitant decarboxylation of α-ketoglutarate to succinate, for example, the posttranslational modifications of procollagen by prolyl and lysyl hydroxylases [for a review see Kivirikko and Myllylä (1980)]. The overall reaction stoichiometry of these transformations may be generalized for a substrate RH as shown in eq 1. In these enzyme reactions one atom of dioxygen (▲) is incorporated into succinate while the other appears in the hydroxylated product. In a significant departure from this pattern the C-3 hydroxyl (*) of proclavaminate (**1**) was found to be retained exclusively in the formation of the oxazolidine ring of **2** with no incorporation of molecular oxygen into the bicyclic β-lactam product (Krol et al., 1989). However, we have shown very recently that C–O bond formation does occur with retention of configuration (Basak et al., 1990) in common with other dioxygenases whose hydroxylation stereochemistry has been examined (Fujita, 1964; Townsend & Barrabee, 1984; Englard et al., 1985; Stubbe, 1985).



(1)

It is increasingly clear from current research that quite distinct biochemical pathways exist to the principal β-lactam antibiotic families of penicillins/cephalosporins [for reviews see Queener and Neuss (1982) and Baldwin and Abraham (1988)], nocardicins/monobactams (Hosoda et al., 1977; O'Sullivan et al., 1982; Townsend & Brown, 1983; Townsend et al., 1983; Wilson et al., 1988), carbapenems (Williamson et al., 1985; Bycroft et al., 1988), and clavulanic acid/clavams (Elson & Oliver, 1978; Townsend & Ho, 1985; Elson et al.,

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[1] Abbreviations: CS, clavaminate synthase; α-KG, α-ketoglutaric acid (2-oxopentanedioic acid); IPNS, isopenicillin N synthase; DAOCS, deacetoxycephalosporin C synthase; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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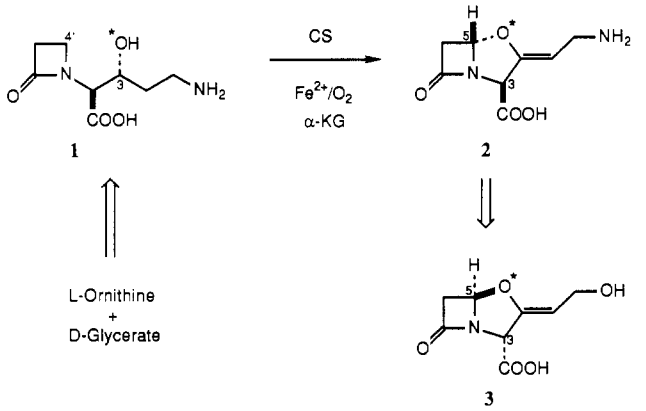
Scheme I




1987b; Krol et al., 1989). Nevertheless, the diversion of conventional hydroxylase activity to oxidative cyclization chemistry in clavaminate synthase bears important similarities to the central carbon–sulfur bond-forming transformations of penicillin and cephalosporin biosynthesis and, indeed, to biochemical sulfur insertion reactions into C–H bonds generally [for a review see Parry (1983)]. For example, isopenicillin N synthase (IPNS) (Hollander et al., 1984; Pang et al., 1984; Jensen et al., 1986) catalyzes the double oxidative cyclization of δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine (**4**) to isopenicillin N (**5**) (see Scheme II). A second enzyme, deacetoxycephalosporin C synthase (DAOCS) (Kohsaka & Demain, 1976; Hook et al., 1979; Dotzlaf et al., 1987; Baldwin et al., 1987; Rollins et al., 1988), mediates the oxidative ring expansion of penicillin N (**6**) to deacetoxycephalosporin C (**7**). These latter two enzymes have been isolated and studied from several fungal and bacterial sources, including *S. clavuligerus*.

In this paper we describe the purification to apparent homogeneity of two very similar forms of clavaminate synthase from *S. clavuligerus*. The four possible stereoisomers of proclavamininic acid have been synthesized to determine the absolute configuration of the natural substrate. Examination of the overall reaction stoichiometry suggests that two discrete oxidation reactions occur in each enzyme turnover. Like the oxidative cyclizations catalyzed by IPNS and DAOCS, molecular oxygen that would otherwise be expected to appear in product is instead reduced to water. The significance of uncoupled turnovers for enzyme stability is discussed in conjunction with results from steady-state kinetic studies to arrive at an initial description of the enzyme's catalytic cycle.

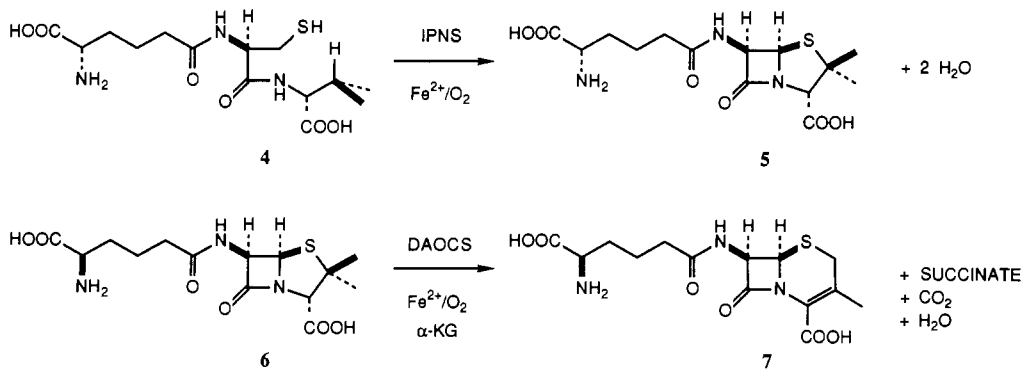
## Materials and Methods

The following were obtained from Sigma Chemical Co.: PMSF, benzamidine hydrochloride, soybean trypsin inhibitor, leupeptin, streptomycin sulfate, ammonium sulfate (grade III), DTT, sodium ascorbate, DMSO-based ninhydrin reagent, Hyamine hydroxide, soy flour, BSA, and catalase (stock no. C-40). Bacto-agar, yeast extract, soluble starch, and tryptone were purchased from Difco Laboratories. Whole soybeans (Arrowhead Mills) were purchased from a local health food store. DEAE-Sepharose and Sephadex G-75 chromatography media as well as Mono-Q and Superose 12 columns were obtained from Pharmacia. [1-$^{14}$C]-α-Ketoglutarate was purchased from New England Nuclear. Permafluor V scintillation cocktail was supplied by Packard. Optically pure and racemic proclavaminic acid and the racemic erythro isomers of same were synthesized and correlated to L-glutamic acid by methods described elsewhere (Krol et al., 1990). Concentrations of proclavaminic acid solutions were determined with a manual ninhydrin assay (Moore & Stein, 1954) using β-alanine as a standard. Reagent-grade imidazole was recrystallized from benzene four times and then washed with ether. The imidazole reagent used for enzyme assays consisted of a 3 M aqueous solution of imidazole adjusted with HCl to pH 6.8 (Bird et al., 1982).

*Growth of Cells.* Seed medium contained per liter 5 g of soy flour, 5 g of yeast extract, 10 g of glycerol, 5 g of tryptone, and 0.2 g of $K_2HPO_4$ adjusted to pH 6.8. Fermentation medium contained 15 g of freshly ground soybeans, 47 g of soluble starch, 0.1 g of $KH_2PO_4$, and 0.2 g of $Fe_2(SO_4)_3 \cdot 7H_2O$ adjusted to pH 6.8 with NaOH. Agar plates containing 1% yeast extract, 1% glucose, and 2% Bacto-agar were inoculated from a spore suspension of *S. clavuligerus* (ATCC 27064) and grown at 28 °C. Several 500-mL Erlenmeyer flasks each containing 100 mL of seed medium were inoculated from the agar plates and incubated at 28 °C in a rotary shaker (300 rpm) for 2 days. Four 2.8-L baffled flasks each containing 500 mL of fermentation medium were each inoculated with 20 mL from a seed flask and shaken at 300 rpm and 28 °C. Formation of clavulanic acid was monitored by HPLC on a Whatman Partisil 10 PAC column eluted isocratically with 0.1% trifluoroacetic acid/acetonitrile/methanol (78:12:5). Cells were harvested by centrifugation during production phase (3 days). The cell paste was resuspended in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM PMSF and pelleted again by centrifugation. The pelleted cells and insoluble soybean residue (97 g from 2 L of broth) were frozen in liquid nitrogen and stored at −70 °C.

*Assays.* Assays for specific activity during the purification procedure contained 50 mM sodium MOPS buffer, pH 7.0, 0.5 mM DTT, 0.1 mM sodium ascorbate, 1 mM α-ketoglutarate, 1 mM *rac*-proclavaminate, and 25 μM ferrous ammonium sulfate. For activity measurements of crude enzyme (prior to DEAE-Sepharose) the assays were conducted in glass test tubes in a final volume of 200 μL. Separate controls were run for each sample containing the same amount of extract but omitting the proclavaminate and including 0.2 mM EDTA

Scheme II



throughout the incubation. After a 5-min incubation at room temperature, the assays were terminated by the addition of 10 μL of 4 mM EDTA. The assay tubes were immersed in a boiling water bath for 30 s and then rapidly cooled. An aliquot of 180 μL was transferred from each test tube to a plastic microfuge tube containing 90 μL of imidazole reagent. After a 20-min incubation at 40 °C, 270 μL of water was added to each tube and the protein precipitate was pelleted by centrifugation. The absorbance of the supernatant fluid at 312 nm, corrected for the absorbance of the control, was used to compute turnover assuming an extinction coefficient of 26 900 $M^{-1}$ $cm^{-1}$ for the α,β-unsaturated acyl imidazole derivative of clavaminate (Bird et al., 1982). Assays in the latter part of the purification procedure containing much less protein were conducted similarly, except that boiling and individual controls were not required.

The standard assay used elsewhere in this paper was carried out in a final volume of 390 μL containing 50 mM sodium MOPS, pH 7.0 buffer, 0.5 mM DTT, 0.1 mM sodium ascorbate, and specified amounts of ferrous ammonium sulfate, α-ketoglutarate, and proclavaminate at 22 °C. With the exception of experiments examining the stereospecificity of the enzyme, all assays used racemic proclavaminate. Assays were initiated by the addition of enzyme and terminated after 2 min with 10 μL of 4 mM EDTA. This time period is short relative to inactivation that occurs during turnover. The samples were derivatized with 200 μL of imidazole reagent as described above. The absorbance at 312 nm was corrected for the absorbance of a control containing all assay components except enzyme. In experiments with α-ketoglutarate as a variable substrate an additional correction was applied, using $\epsilon_{312} = 25.6$ $M^{-1}$ $cm^{-1}$ experimentally determined for this acid. In experiments with $Fe^{2+}$ as a variable substrate, an additional correction was applied, using $\epsilon_{312} = 4.17$ $mM^{-1}$ $cm^{-1}$ for the Fe/EDTA complex. The concentrations of α-ketoglutarate stock solutions were calibrated enzymatically by using glutamate–pyruvate transaminase and lactate dehydrogenase as coupling enzymes. The concentration of stock iron solutions was determined spectrophotometrically by using Ferrozine (Stookey, 1970). Substrate consumption was always less than 10% for proclavaminate and oxygen and less than 20% for α-ketoglutarate. Modified computer programs of Duggleby (1984) were used to fit kinetic data to eq 2, 3, or 4 as appropriate. In eqs 2–4, $V$ is the maximal velocity, $A$ and $B$

$$v = VA(V/K_a)/[V + (V/K_a)A] \quad (2)$$

$$v = VA^2/(K_1 + K_2A + A^2) \quad (3)$$

$$v = VAB/(K_{ia}K_b + K_bA + K_aB + AB) \quad (4)$$

are substrate concentrations, $K_a$ and $K_b$ are the Michaelis constants for the subscripted substrates, $K_{ia}$ is the dissociation constant for $A$, and $K_1$ and $K_2$ are saturation constants for a substrate that binds twice with no intervening irreversible step.

For the radioactive assay measuring carbon dioxide formation the assay components were identical with those of the standard assay described above except for the substitution of [1-$^{14}$C]-α-ketoglutarate diluted with cold material to a specific activity of 0.4 μCi/μmol. Incubations were performed in 8-mL glass vials sealed with rubber serum stoppers and fitted with plastic center wells (Kontes). Each center well contained a piece of filter paper impregnated with 25 μL of 1 M Hyamine hydroxide in methanol. The assays were initiated by syringe addition of enzyme and terminated by injection of 50 μL of 50% trichloroacetic acid. After a 1-h incubation at 37 °C, the center wells were transferred to scintillation vials for counting in 10 mL of Permafluor V cocktail. Corrections were applied for nonenzymatic decarboxylation of α-ketoglutarate.

Each assay under oxygen-limiting conditions was conducted in a 1-mL glass micro reaction vial with conical bottom (Wheaton). The reaction mixture was air-equilibrated at 25 °C before dispensing 200-μL aliquots and micro stir bars into the vials. After the remaining volume was filled with thoroughly argon-sparged silicone oil (Aldrich), the vials were sealed with open-topped screw caps fitted with septa. The assays were initiated by syringe addition of 3 μL of enzyme (~125 μg), stirred briefly, and allowed to stand for various time periods. Each was terminated with 2 μL of deoxygenated 25 mM EDTA added via syringe. Aliquots of 100 μL were derivatized with 50 μL of imidazole reagent as described above and then diluted with 400 μL of water to measure the absorbance at 312 nm.

*Protein Purification.* All steps were conducted at 0–4 °C. "Tris buffer" refers to 50 mM Tris adjusted with HCl to pH 7.5 at room temperature. *S. clavuligerus* cell paste (50 g) was suspended in 100 mL of Tris buffer containing 2 mM DTT, 0.5 mM EDTA, 1 mM benzamidine, 1 μM leupeptin, 5 mg/L soybean trypsin inhibitor, and 1 mM PMSF. The cells were broken by sonication (Heat Systems-Ultrasonics Model 225R) for 5 min at 140 W on 50% duty cycle, and insoluble material was removed by centrifugation for 20 min at 20000*g*. One-fifth volume of a 5% solution of streptomycin sulfate was added to the stirred extract over 10–15 min. After an additional 15 min of stirring, the precipitate was removed by centrifugation for 20 min at 20000*g*. Solid ammonium sulfate was added over 10 min to 40% of saturation (242 g/L). After an additional 20 min of stirring, precipitated protein was removed by centrifugation at 20000*g* for 20 min. The supernatant fluid was then brought to 70% of saturation by the addition of solid ammonium sulfate over 15 min (202 g/L). The mixture was stirred for an additional 30 min, and the precipitated protein was collected by centrifugation at 20000*g* for 30 min.

The protein pellet was suspended in 1 mL of Tris buffer containing 1 mM DTT, 1 mM benzamidine, 0.2 mM PMSF, and 10 μM EDTA ("dialysis buffer") and dialyzed against the same buffer for several hours. After dialysis nondiffusable material (8 mL) was diluted 3-fold with dialysis buffer and centrifuged at 39000*g* for 10 min to remove undissolved precipitate. After further dilution to 60 mL, the protein was loaded on a 2.5 × 16 cm (80-mL) DEAE-Sepharose column equilibrated in dialysis buffer. The column was eluted with a linear gradient of NaCl from 0 to 250 mM in a total volume of 800 mL of dialysis buffer; 8-mL fractions were collected. Clavaminate synthase activity eluted at approximately 175 mM NaCl. Fractions 69–75 were pooled and concentrated to approximately 2 mL in Amicon ultrafiltration cells fitted with PM10 membranes. The concentrated protein was loaded on a 1.5 × 90 cm (160-mL) Sephadex G-75 column equilibrated in Tris buffer containing 1 mM DTT, 0.2 mM PMSF, and 10 μM EDTA. The column was eluted with this same buffer at approximately 5 mL/h; 1-mL fractions were collected. Fractions 60–67 were pooled and concentrated by ultrafiltration. For storage at this point, the protein was exchanged into 50 mM sodium MOPS (pH 7.0) buffer containing 25% glycerol, 1 mM DTT, 10 μM EDTA, and 0.1 mM PMSF. Aliquots were frozen in liquid $N_2$ and stored at −70 °C. The enzyme prepared in this way was stable for at least several months.

Additional purification of clavaminate synthase was accomplished at room temperature by using a Pharmacia FPLC apparatus equipped with a Mono-Q (anion-exchange) column. The column was preequilibrated in 50 mM Tris-HCl (pH 7.5)

Table I: Purification of Clavaminate Synthase from 50 g of *S. clavuligerus* Cell Paste[a]

| fractionation step | total protein (mg) | total activity (μmol/min) | specific activity [μmol/(min·mg)] | % recovery | purification (*x*-fold) |
|---|---|---|---|---|---|
| sonicated extract | 930 | 5.8 | 0.0062 | (100) | (1) |
| streptomycin sulfate | 610 | 5.4 | 0.0089 | 93 | 1.4 |
| 40–70% $(NH_4)_2SO_4$ | 400 | 5.6 | 0.014 | 97 | 2.3 |
| DEAE-Sepharose | 19 | 4.4 | 0.23 | 76 | 37 |
| Sephadex G-75 | 7.0 | 2.8 | 0.40 | 48 | 65 |
| FPLC on Mono-Q[b] | | | | | |
| peak 1 | 0.094 | 0.034 | 0.36 | 17[c] | 58 |
| peak 2 | 0.225 | 0.128 | 0.57 | 64[c] | 92 |

[a] Figures containing column elution profiles are included in the supplementary material. [b] 0.5 mg of protein was loaded from the previous step. [c] Recovery calculated for this step alone.

containing 20% v/v glycerol, and proteins were eluted with a gradient of the same buffer containing in addition 0.25 M KCl. The inclusion of glycerol was necessary in order to obtain good resolution and acceptable recovery of the highly purified enzyme. Protein from the gel filtration step (0.5 mg) was loaded onto the column and eluted at 0.5 mL/min; 1-min fractions were collected. Two major protein peaks eluted at around 130 mM KCl. Fractions containing each peak were pooled, and DTT and PMSF were added to 1 and 0.2 mM, respectively. The pooled protein solutions were stored at −70 °C.

*Other Methods.* The molecular weight of the native enzyme was estimated by gel filtration using the Pharmacia FPLC equipped with a Superose 12 column. The column was equilibrated in 50 mM Tris-HCl (pH 7.5) containing 0.15 M KCl. Proteins were eluted at 0.5 mL/min. β-Amylase ($M_r$ 200 000), alcohol dehydrogenase ($M_r$ 150 000), bovine serum albumin ($M_r$ 66 000), carbonic anhydrase ($M_r$ 29 000), and cytochrome *c* ($M_r$ 12 400) were used to calibrate the column. For amino acid analysis, samples of the highly purified clavaminate synthase (15 μg) were carboxymethylated and then hydrolyzed in 6 M HCl according to standard protocols (Perham, 1978). Amino acids were derivatized with phenyl isothiocyanate prior to analysis on a Waters PICO-TAG amino acid analysis system. Protein assays were performed by the method of Bradford (1976), using bovine serum albumin as the standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed with the buffer system of Laemmli (1970).

## Results and Discussion

*Growth of Bacteria and Enzyme Purification.* Clavulanic acid is one of several β-lactam-containing secondary metabolites produced by *S. clavuligerus* in response to changes in available nutrients (Romero et al., 1984). Crude extracts of *S. clavuligerus* produce clavaminate when $Fe^{2+}$ and α-ketoglutarate are added without exogenous proclavaminate (Elson et al., 1987a; S. P. Salowe, unpublished results), suggesting that a significant metabolic pool of proclavaminate exists and that the clavaminate synthase reaction is rate-limiting for the biosynthetic pathway. Since a correlation might thus be expected between the concentration of clavulanic acid in the medium and the intracellular level of this enzyme, we investigated two different media for clavulanic acid production. Although yields of clavulanic acid from fermentations were quite variable, the complex soybean and starch medium was found to be generally superior to the defined glycerol/sucrose/proline/glutamate medium of Romero et al. (1984). The specific activity of the enzyme in a partially purified crude extract of bacteria grown on the complex medium was severalfold higher than that in an extract of bacteria grown on the defined medium. Hence, the former medium was used as the primary source of material for enzyme purifications.

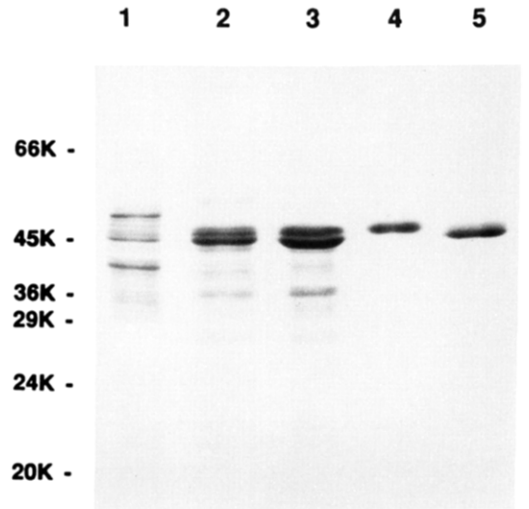



FIGURE 1: SDS–PAGE analysis of clavaminate synthase purification on a 12% gel stained with Coomassie brilliant blue. Lane 1, 40–70% $(NH_4)_2SO_4$ cut; lane 2, DEAE-Sepharose pool; lane 3, Sephadex G-75 pool; lane 4, first active peak from Mono-Q; lane 5, second active peak from Mono-Q.

The purification of clavaminate synthase employed ammonium sulfate fractionation, ion-exchange chromatography, and gel filtration, as summarized in Table I. The most effective step was chromatography on DEAE-Sepharose, after which clavaminate synthase was the major protein band when the pooled eluate was examined by denaturing gel electrophoresis (Figure 1). Subsequent purification on a column of Sephadex G-75 provided highly purified material in good overall yield that was used for the stability and kinetic studies described later in this paper.

The enzyme was additionally purified to electrophoretic homogeneity by using FPLC with a Mono-Q column. Recovery of protein was initially very poor, apparently due to irreversible aggregation. Addition of glycerol to the elution buffer improved both recovery and resolution. Surprisingly, enzyme activity was found associated with two protein peaks. Analysis of the pooled regions by SDS–PAGE showed each peak to be essentially homogeneous (Figure 1). The first peak comprised a single polypeptide with an estimated $M_r$ of 47 000, while the second peak contained a protein of estimated $M_r$ 46 000 (a small amount of material from the first peak can be seen to be carried over to the second from the gel). The proportion of these two enzyme forms varied in different enzyme preparations. While the two proteins may be isoenzymes, the simplest explanation is that the lower molecular weight material is derived from the larger protein by proteolysis either in vivo or as an artifact of the purification. Proteolytic inhibitors were kept present throughout the preparation, and the ratio of the two proteins, as judged by staining on a gel, did not change during the purification; rapid limited proteolysis

Table II: Amino Acid Analysis of Clavaminate Synthase

| amino acid | content (mol %)[a] | amino acid | content (mol %)[a] |
|---|---|---|---|
| Asx | 9.38 ± 0.25 | Tyr | 3.03 ± 0.07 |
| Glx | 8.70 ± 0.10 | Val | 5.80[c] |
| Thr | 7.25 | Met | 1.27 ± 0.02 |
| Ser | 5.30[b] | Cys | 1.38 ± 0.05[d] |
| Gly | 8.04 ± 0.15 | Ile | 1.27[c] |
| His | 2.97 ± 0.02 | Leu | 12.33 ± 0.11 |
| Arg | 9.22 ± 0.07 | Trp | ND[e] |
| Thr | 7.25[b] | Phe | 2.55 ± 0.02 |
| Ala | 13.01 ± 0.16 | Lys | 1.86 ± 0.005 |
| Pro | 7.53 ± 0.09 | | |

[a] The results [±SEM ($n$ = 3) where appropriate] were obtained from single analyses of 24-, 36-, and 48-h hydrolysates. [b] The values for threonine and serine were obtained by linear extrapolation to zero hydrolysis time. [c] Isoleucine and valine were determined from the 48-h hydrolysate. [d] Determined as (carboxymethyl)cysteine. [e] ND, not determined.

on breaking open the cells cannot be ruled out though. Peak 2 had a higher specific activity than peak 1 (Table I), and further kinetic analysis revealed that peak 1 had an approximately 1.4-fold higher $K_m$ and 1.2-fold lower $V_{max}$ than peak 2 when proclavaminate was the variable substrate with fixed 0.25 mM α-ketoglutarate and 10 μM $Fe^{2+}$. While in principle a mixture of enzymes catalyzing the same reaction with different kinetic parameters can give nonlinear double-reciprocal plots (Segel, 1975), the slight catalytic differences between the two forms of clavaminate synthase are not great enough to give detectable deviation from linearity in kinetic studies using the protein without FPLC purification.

*Physical Characteristics.* As indicated above, the molecular weight of purified clavaminate synthase was 47 000 ± 2000 as determined by SDS–PAGE, in good agreement with values of 49 200 and 47 000 reported by Elson et al. (1987a), determined by SDS–PAGE and native gel electrophoresis, respectively. The molecular weight estimation of 38 000 ± 5000 from the Superose 12 FPLC column indicates that the native enzyme is a monomer in solution. The lower $M_r$ obtained by gel filtration as compared to SDS–PAGE may indicate nonspecific interactions between the protein and the column matrix.

The optical spectrum of the enzyme revealed a typical protein absorbance around 280 nm in the ultraviolet with no evidence of longer wavelength chromophoric prosthetic groups. The amino acid composition (Table II) is typical of many globular proteins, although amino acids that utilize codons rich in A and T (for example, lysine and phenylalanine) are present only in low mole percentages. This may be explained by the high G-C content of *S. clavuligerus* DNA (approximately 70% G-C), which would tend to favor G-C-rich codons where possible, e.g., arginine instead of lysine.

*Stereospecificity.* Proclavaminic acid contains two asymmetric carbon centers. The four possible diastereomers were synthesized in order to identify the configuration of the natural substrate for clavaminate synthase. The racemic erythro isomers (2*S*,3*S* and 2*R*,3*R*) were not substrates for the production of clavaminate ($V_{max}$ < 5% that of racemic threo isomers). The stereospecificity of the enzyme for the threo isomers was investigated by comparison of the kinetic parameters for racemic substrate with optically pure 2*S*,3*R* compound, which was anticipated to be the true substrate from the configuration of the product. For a racemic substrate, S, in which only one enantiomer is processed by the enzyme and the other does not bind at all in the active site, the $k_{cat}$ (first-order rate constant as [S] goes to infinity) should be identical with that of the active enantiomer. The apparent $k_{cat}/K_m$ (the second-order rate constant as [S] goes to zero) for racemic compound, however, should only be half of the $k_{cat}/K_m$ for the active enantiomer since $v = (k_{cat}/K_m)[E][S]$ at low [S] and only half of the S present is the true substrate. Kinetic data for clavaminate synthase with (2*S*,3*R*)- and *rac*-proclavaminates as substrates are presented in Table III. It can be seen that the $k_{cat}/K_m$ ratio for optically pure to racemic material is very near to 2:1 as expected for only one enantiomer being a substrate. Since a competitive inhibitor present in constant ratio to a variable substrate has no effect upon the slope ($k_{cat}/K_m$) of a double-reciprocal plot but does influence the position of the intercept ($k_{cat}$) (Cleland et al., 1973), the slightly higher $k_{cat}$ for the pure 2*S*,3*R* isomer over the racemate can be rationalized by very weak binding ($K_i$ > 2 mM) of the 2*R*,3*S* enantiomer. The configuration of the natural proclavaminate substrate is thus 2*S*,3*R*, in confirmation of the stereochemical assignment reported elsewhere (Baggaley et al., 1988).

Table III: Kinetic Comparison of Racemic and Optically Pure Proclavaminates

| | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ ($min^{-1}$ $M^{-1}$) | $K_m$ (μM) |
|---|---|---|---|
| (2*S*,3*R*)-proclavaminate | 45.9 ± 0.5 | (2.43 ± 0.04) × $10^5$ | 190 ± 4 |
| *rac*-proclavaminate | 43.5 ± 0.3 | (1.16 ± 0.02) × $10^5$ | 376 ± 5 |

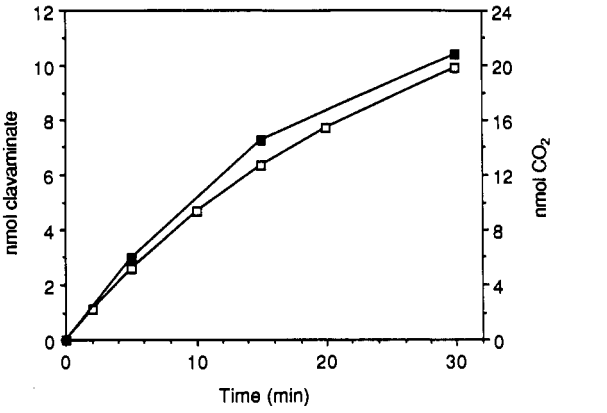
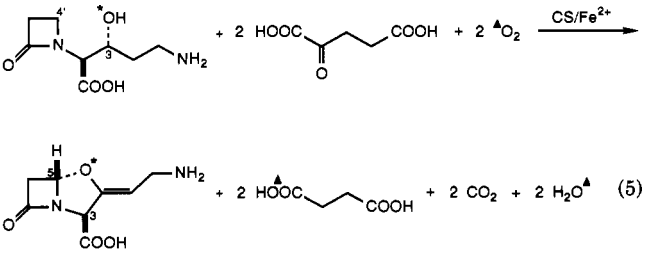



FIGURE 2: Stoichiometry of $CO_2$ formation. Parallel reactions under standard conditions containing 10 μM $Fe^{2+}$, 1 mM proclavaminate, and 1 mM α-ketoglutarate or [1-$^{14}$C]-α-ketoglutarate were assayed for clavaminate formation by the spectrophotometric assay (□) or for $CO_2$ formation by the radiochemical assay (■).

*Reaction Stoichiometry.* The stoichiometry of the overall reaction was investigated in two experiments. Figure 2 shows the time courses for parallel reactions monitored by the imidazole assay for clavaminate and the radiochemical assay for $^{14}CO_2$ released from [1-$^{14}$C]-α-ketoglutarate. As anticipated, 2 mol of carbon dioxide was formed for each mole of clavaminate produced. This agrees with the initial finding of Elson et al. (1987a) that 2 equiv of α-ketoglutarate was required for complete turnover of proclavaminate. The production of succinate was confirmed qualitatively (S. P. Salowe, unpublished results) by using a coupled enzymatic assay (Beutler, 1985). If the oxygen substrate required as electron acceptor in the reaction was completely reduced to water, the overall balanced equation would be represented by eq 5. The stoi-




(5)

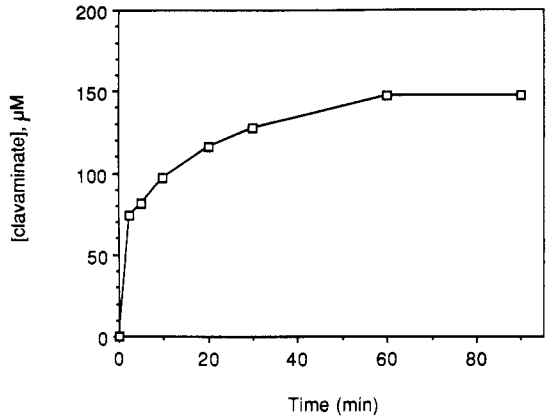



FIGURE 3: Stoiochiometry of oxygen consumption. Reaction mixtures under standard conditions contained 10 μM $Fe^{2+}$, 1 mM α-ketoglutarate, and 1 mM proclavaminate in sealed vials at 25 °C. Each point represents the average of at least two determinations of clavaminate concentration.

chiometry of eq 5 predicts that a reaction carried out in a closed vessel at 25 °C would reach an end point of 129 μM clavaminate, assuming a dissolved oxygen concentration of 258 μM (Hitchman, 1978). Experimentally, clavaminate production plateaued at 147 μM under oxygen-limiting conditions (Figure 3), within 15% of the theoretical value.[2] Given the uncertainty in the actual oxygen concentration in the reaction, this result confirms the requirement for two molecules of dioxygen in each enzymatic turnover as illustrated in eq 5. A comparison to the generalized stoichiometry of the hydroxylation reactions given in eq 1 indicates that the clavaminate synthase reaction involves two cycles of oxidation chemistry related to that performed by the α-ketoglutarate-dependent dioxygenases, but notably with the additional production of 2 equiv of water.

*Enzyme Stability during Catalysis.* Figure 2 reveals that enzymatic turnover was not linear with time. Several possibilities were considered for the origin of the curvature. Substrate depletion was ruled out, as only 5% of the initial proclavaminate and α-ketoglutarate were consumed after 30 min and the reaction was stirred to maintain constant dissolved oxygen. Since only micromolar concentrations of products were formed, it is unlikely that product inhibition accounted for the data. Futhermore, much higher concentrations of products have been generated in large-scale reactions for the examination of isotopically labeled clavaminates (Krol et al., 1989). Instability of the protein structure under assay conditions was also considered, but inclusion of the potentially stabilizing additives 0.2 mg/mL BSA, 25% glycerol, or 0.01% Triton X-100 detergent was without effect. Consequently, it was suspected that the loss of activity was related to the generation of reactive intermediates during the catalytic cycle. For example, enzyme inactivation could result if in some fraction of catalytic events a powerful oxidant formed at the active site oxidized a susceptible protein residue rather than the substrate. To estimate the frequency of these destructive turnovers, the data in Figure 2 were treated as a simple exponential decay of activity. From the rate constant for inactivation of 0.04 $min^{-1}$ ($t_{1/2} \sim$ 17min) and the initial turnover number of 27 $min^{-1}$ (assuming a single active site and $M_r$ 47 000) one may calculate that under the conditions of this experiment approximately 1 in 670 turnovers resulted in enzyme inactivation.

[2] The theoretical end point was calculated by using the solubility of oxygen in pure water in contact with air saturated with water vapor at 25 °C and a total pressure of 760 Torr. The true oxygen concentration is expected to differ somewhat since temperature was the only parameter affecting solubility that was controlled in the experiment.

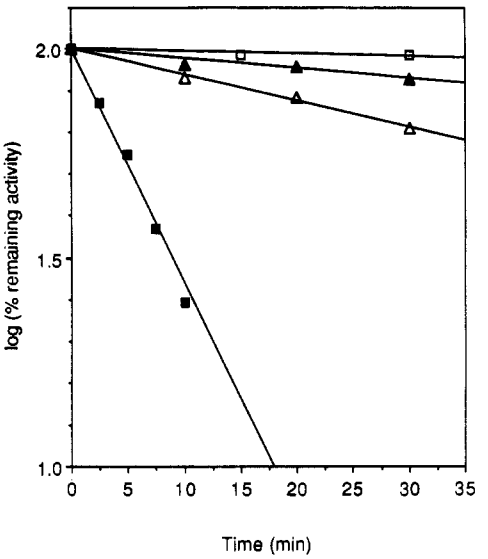



FIGURE 4: Enzyme at 1.3 mg/mL in 50 mM sodium MOPS buffer, pH 7.0, containing 0.5 mM DTT was incubated at 22 °C alone or with various additives: (□) no additions; (△) 10 μM $Fe^{2+}$ and 100 μM ascorbate; (■) same as (△) plus 1 mM α-ketoglutarate; (▲) same as (■) plus 0.1 mg/mL catalase. At various time points 10 μL was withdrawn for assay by the standard spectrophotometric method using 10 μM $Fe^{2+}$, 1 mM α-ketoglutarate, and 0.5 mM proclavaminate.

To further investigate this inactivation phenomenon, the enzyme stability was examined in incomplete reaction mixtures. As Figure 4 indicates, relatively concentrated enzyme is quite stable at room temperature in the absence of all substrates except oxygen ($t_{1/2} > 8$ h). The presence of $Fe^{2+}$ and ascorbate at assay concentrations, however, was found to cause a considerable decrease in stability ($t_{1/2} \sim 50$ min), while the additional inclusion of α-ketoglutarate resulted in rapid inactivation ($t_{1/2} \sim 5$ min). However, when 0.1 mg/mL catalase was present in the incubation mixture a dramatic increase in the half-life of the enzyme was seen ($t_{1/2} \sim 2$ h). The effect appeared to be specific to catalase, as substitution of BSA for catalase at the same concentration provided no protection against the inactivation (data not shown). Whether inactivation occurs by specific reaction of peroxide (or other reduced oxygen species) at the active site or by nonspecific oxidation in a more remote portion of the enzyme is not known. The linearity of proclavaminate turnover was not improved when catalase was included at 0.1 mg/mL, suggesting that free peroxide is not the cause of inactivation in the complete reaction.

The apparent formation of peroxide in the incomplete reaction mixture suggests that clavaminate synthase can catalyze a partial reaction in which oxidative decarboxylation of α-ketoglutarate occurs to form, e.g., a high-valent iron–oxo species. In the absence of proclavaminate the remaining oxidizing equivalents are discharged as partially reduced oxygen byproducts (such as peroxide or hydroxyl radical). To confirm that the enzyme was capable of this partial reaction in the presence of only $Fe^{2+}$, $O_2$, and α-ketoglutarate, the radiochemical assay was used for the detection of carbon dioxide. With 10 μM $Fe^{2+}$ and 1 mM α-ketoglutarate, the specific activity of the enzyme was 0.10 μmol of $CO_2$/(min·mg), or 5.8% of the 1.67 μmol of $CO_2$/(min·mg) observed in the presence of 1 mM proclavaminate.

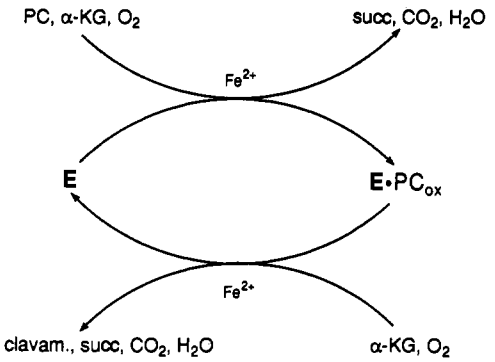
Uncoupled reactions have been observed previously for several α-ketoglutarate-dependent dioxygenases. Both prolyl

Table IV: Kinetic Constants for Substrates of the Clavaminate Synthase Reaction

| varied substrate[a] | fixed substrate[a] | pattern | apparent $K_m$ ($\mu$M) | | |
|---|---|---|---|---|---|
| | | | $Fe^{2+}$ | $\alpha$-ketoglutarate | proclavaminate |
| $Fe^{2+}$ vs $\alpha$KG | PC, 400 $\mu$M | intersecting | 0.95 ± 0.19 | 42.3 ± 9.5 | |
| $\alpha$KG vs PC | $Fe^{2+}$, 10 $\mu$M | intersecting | | 46.3 ± 10.8 | 335 ± 54 |
| $Fe^{2+}$ vs PC | $\alpha$KG, 100 $\mu$M | intersecting | 1.80 ± 0.24 | | 336 ± 51 |
| $Fe^{2+}$ vs PC | $\alpha$KG, 3 mM | parallel | 1.91 ± 0.1 | | 372 ± 35 |

[a] Abbreviations: PC, proclavaminate; $\alpha$KG, $\alpha$-ketoglutarate.

Scheme III[a]




[a] PC = proclavaminate, $\alpha$-KG = $\alpha$-ketoglutarate, succ = succinate, clavam. = clavaminate, and $PC_{ox}$ = enzyme-bound intermediate.

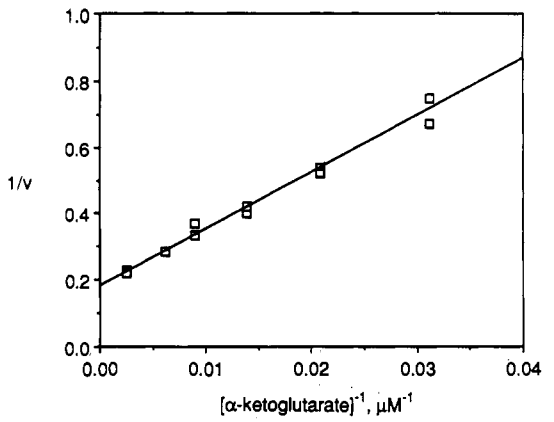



FIGURE 5: Double-reciprocal plot of the dependence of initial velocity upon $\alpha$-ketoglutarate concentration. Duplicate determinations were made at each concentration. The line shown was obtained from fitting of the data to eq 2. Velocity is expressed as $\mu$M clavaminate formed/min.

and lysyl hydroxylases catalyze slow decarboxylations (1–4% of maximal velocity) in the absence of their peptide substrates (Tuderman et al., 1977; Puistola et al., 1980a); the rates are enhanced in the presence of certain inhibitors (Counts et al., 1978; Rao & Adams, 1978). With thymine and $\gamma$-butyrobetaine hydroxylases the uncoupled reaction is seen exclusively with nonhydroxylatable substrate analogues (Holme et al., 1979, 1982; Hsu et al., 1981; Holme & Lindstedt, 1982; Wehbie et al., 1988). These enzymes share with clavaminate synthase the ability to harness the free energy of $\alpha$-ketoglutarate decarboxylation for the generation of a high-energy oxygen species that can perform a difficult oxidation when an appropriate substrate is bound. Formation of a ferryl oxidant, $[Fe{=}O]^{2+}$, has been proposed to occur in $\alpha$-ketoglutarate-dependent hydroxylation (Siegel, 1979). While as yet there is no direct evidence for the existence of such a species, both the kinetic isotope effect studies on $\gamma$-butyrobetaine hydroxylase (Blanchard & Englard, 1983) and the recent demonstration of olefin epoxidation by thymine hydroxylase (Thornburg & Stubbe, 1989) have been cited in support of ferryl participation.

*Steady-State Kinetics.* The stoichiometry of the clavaminate synthase reaction reveals the involvement of five substrates and a metal ion in the formation of five products. This is a particularly complex multisubstrate system for kinetic analysis, especially since there are two substrates ($\alpha$-ketoglutarate and oxygen) that bind twice during the reaction. Our working hypothesis for the mechanism proposes that two discrete oxidations of proclavaminate occur at one active site to form clavaminate. As presented in Scheme III, each half of the reaction is postulated to consume $\alpha$-ketoglutarate and oxygen and release succinate, carbon dioxide, and water. When a substrate binds twice and the points of addition are connected reversibly, a parabolic double-reciprocal plot is expected because of squared terms in the rate equation (Segel, 1975). If, however, the points of addition are separated by an irreversible step (such as product release or saturation with an intervening substrate), the double-reciprocal plots are linear as observed with carbamoyl-phosphate synthetase (Raushel et al., 1978) and acetolactate synthase (Schloss et al., 1985). Therefore, the linearity of the double-reciprocal plots with $\alpha$-ketoglutarate or oxygen as the variable substrate is a test of the postulated stepwise oxidation model of Scheme III. The velocity dependence upon the concentration of $\alpha$-ketoglutarate in the range 30–400 $\mu$M is shown in Figure 5. Data fitting to the simple hyperbolic Michaelis–Menten equation (eq 2) gave an entirely satisfactory fit with $K_m$ 93 ± 5 $\mu$M. Attempts to fit the more complex eq 3 failed to give a meaningful solution. The experimental evidence thus supports the stepwise mechanism of Scheme III.

With the mechanistically plausible assumption of stepwise oxidations the kinetic analysis simplifies to the consideration of a pseudoquadreactant system (three substrates plus metal ion). The kinetic mechanism of the first oxidation could be envisioned to occur in either a sequential or ping-pong fashion; in the latter case the oxidative decarboxylation forming the postulated ferryl species would precede binding of proclavaminate. The slow rate of the uncoupled reaction discussed above argues that a ping-pong mechanism is not kinetically competent. Direct evidence in favor of a sequential mechanism was obtained from initial velocity patterns. When the three components whose concentrations could be varied most easily ($Fe^{2+}$, $\alpha$-ketoglutarate, proclavaminate) were examined pairwise at nonsaturating levels of the third substrate, the double-reciprocal plots were all intersecting. The kinetic constants obtained from computer fitting to eq 4 are tabulated in Table IV. Since all of these experiments were conducted at a constant (ambient) oxygen tension, a definitive statement cannot be made that a fully sequential mechanism exists involving all four components in the first reaction of Scheme III. Formal kinetic mechanisms may be written with oxygen binding first or last and separated by an irreversible step from the additions of the other three substrates, but such schemes are not chemically reasonable. It is highly unlikely that oxygen would bind first to bare enzyme rather than coordinate to an already bound iron. It is also unlikely that an irreversible step follows addition of $Fe^{2+}$, $\alpha$-ketoglutarate, and proclavaminate

prior to oxygen binding as no other cofactor is present in the protein to act as an electron acceptor. Furthermore, the uncoupled reaction argues for the existence of a quaternary complex of enzyme with $Fe^{2+}$, $\alpha$-ketoglutarate, and oxygen. For the present, therefore, it is entirely satisfactory to write the fully sequential mechanism for the first oxidation of proclavaminate.

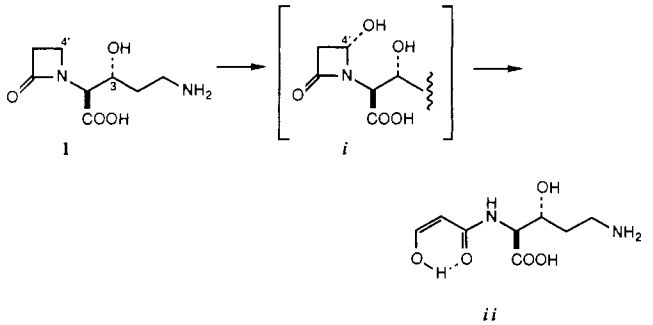
Sequential mechanisms may be ordered or possess varying degrees of randomness for substrate additions. When the fixed concentration of $\alpha$-ketoglutarate was raised, the initial velocity patterns for $Fe^{2+}$ and proclavaminate became less intersecting until at [$\alpha$-ketoglutarate] > $50K_m$ the data could be fit to a parallel pattern (Table IV). The simplest kinetic mechanism that can accommodate this observation, as well as the evidence for a quaternary complex without proclavaminate in the uncoupled reaction, is ordered binding in the sequence $Fe^{2+}$, $\alpha$-ketoglutarate, oxygen, and proclavaminate. However, preliminary inhibition studies designed to confirm the ordered sequence have been inconclusive.[3] There are cases in terreactant systems where both ordered and partially random pathways give a common set of initial velocity patterns (Viola & Cleland, 1982). It would seem entirely possible that similar overlap could also occur in quadreactant systems for which detailed theory is as yet unavailable. Although kinetic studies on prolyl, lysyl, and thymine hydroxylases have all been interpreted as supporting ordered sequential mechanisms (Holme, 1975; Myllylä et al., 1977; Puistola et al., 1980a,b), a similar caution regarding the potential for partial randomness may apply for these enzymes as well.

*Oxidative Cyclization in β-Lactam Biosynthesis.* The similarities between clavaminate synthase and other $\alpha$-ketoglutarate-dependent oxygenases argue for a common mechanism of oxygen activation with the potential formation of a ferryl oxidant. For the latter enzymes hydroxylation may occur by homolytic hydrogen abstraction and radical recombination with the formal equivalent of •OH as has been proposed for cytochromes P-450 (Guengerich & Macdonald, 1984; Ortiz de Montellano, 1986, 1987). Although no *net* incorporation of molecular oxygen takes place in the clavaminate synthase reaction, the transient participation of reactive hydroxylated intermediates cannot be strictly excluded on the basis of the information presently available. However, such intermediates can be readily visualized to lead to competing and unproductive chemical fates for proclavaminate[4] or to exchange reactions contrary to experimental results obtained with labeled materials (Krol et al., 1989). We have previously suggested that the 3-hydroxyl of the substrate **1** could serve as a ligand to iron to redirect hydroxylase activity to oxidative cyclization/desaturation chemistry. Such coordination is consistent with the observation of sequential kinetics and presumably would be enhanced during the actual catalytic event in which iron is proposed to attain the more electrophilic ferryl oxidation state. A number of detailed mechanisms can be advanced which invoke radical or, through electron transfer, ionic intermediates. Alternatively, the functional difference between clavaminate synthase and the $\alpha$-ketoglutarate-dependent dioxygenases could owe to the protein architecture of the synthase active site whose features may prevent oxygen transfer from occurring. Arguments of the latter kind have been put forth to rationalize, in part, differences in chemical reactivity among hemoproteins (Ortiz de Montellano, 1986). Clearly, further information from a range of experimental approaches will be required to distinguish among these possibilities.

The only other known enzyme requiring $Fe^{2+}$, $\alpha$-ketoglutarate, and oxygen that performs an oxidative cyclization/desaturation instead of typical hydroxylation chemistry is DAOCS, the enzyme responsible for the ring expansion of penicillin N (**6**) to deacetoxycephalosporin C (**7**). While some mechanistic investigation of this enzyme has been conducted (Baldwin & Abraham, 1988; Baldwin et al., 1989), a thorough study of its kinetics and uncoupling would be useful for comparison with clavaminate synthase. Oxidative cyclization also occurs in the conversion of the tripeptide **4** to isopenicillin N (**5**) catalyzed by IPNS. In this reaction $\alpha$-ketoglutarate is not required and molecular oxygen is used with maximum efficiency as an electron acceptor to give 2 equiv of water for each dioxygen consumed (White et al., 1982). An unusual feature of catalysis shared by clavaminate synthase and IPNS is the ability to mediate multiple stepwise oxidations without substantial release of an intermediate. This may be contrasted with thymine hydroxylase; although three successive oxidations of a methyl group are catalyzed by this enzyme, product dissociation occurs after each step (Liu et al., 1973). In the clavaminate synthase reaction the intermediate may be insufficiently stable for release from the active site. The structure of the putative intermediate and whether it arises from initial oxidation at C-3 or C-4′ are prominent mechanistic questions currently being addressed with substrate analogue and isotope effect studies. Although clavaminate synthase, IPNS, and DAOCS execute distinct cyclization/desaturation reactions, there are likely to be mechanistic details in common for coupling the exergonic reduction of dioxygen to the thermodynamically unfavorable conversion of acyclic or relatively unstrained substrates into bicyclic products of conserved or significantly higher strain energy. The ultimate release of this chemical potential in the active sites of target enzymes is fundamental to the efficacy of the β-lactam antibiotics as clinical agents.

[3] The interpretation of these experiments is complicated by the difficulty in distinguishing between simple dead-end inhibitors and compounds that induce substrate inhibition through partial reactions.

[4] This can be seen particularly, e.g., for hydroxylation at C-4′ where rapid opening of the resulting hydroxyazetidinone would be expected to occur:

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SUPPLEMENTARY MATERIAL AVAILABLE

Column elution profiles for protein purification on DEAE-Sepharose (Figure 1), Sephadex G-75 (Figure 2), and Mono-Q ion exchange (Figure 3) and double-reciprocal plots used to

calculate kinetic constants in Table IV (Figure 4) (3 pages). Ordering information is given on any current masthead page.

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# Base Stacking and Unstacking As Determined from a DNA Decamer Containing a Fluorescent Base†

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ABSTRACT: Time-resolved fluorescence decay of a single-stranded DNA decamer d(CTGAAT5CAG), where d5 is the fluorescent base 1-(β-D-2′-deoxyribosyl)-5-methyl-2-pyrimidinone, was measured and analyzed at several temperatures. The d5 base in the decamer is resolved into three states according to their fluorescence decay lifetime characteristics and temperature dependence of their associated amplitudes: fully extended and completely unstacked state, loosely associated state, and fully stacked state. These states are in slow exchange compared to their fluorescence decay rates. The population of the fully extended and completely unstacked state is small and decreases further with increasing temperature. The loosely associated state, whose fluorescence can still be efficiently quenched by other DNA bases, occupies a large portion of the conventionally defined unstacked state. Stacking enthalpy and entropy for the d5 base with thymine or cytosine bases in the DNA decamer are calculated to be −6.6 kcal/mol and −22 cal/mol·K, respectively. This work shows that fluorescent bases in DNA can be useful to the study of local conformations of bases.

Base stacking interactions are one of the driving forces for helix formation in double-stranded nucleic acids (Saenger, 1984). Although considerable effort has been made to quantitate the stacking interactions in ribonucleotides and to determine the number of states available for the bases, there is at present no consensus value for stacking enthalpy (Petersheim & Turner, 1983) and the issue of the number of states that a base can adopt during stacking and unstacking processes is not clearly understood (Powell et al., 1972; Lee & Tinoco, 1977; Baker et al., 1978; Reich & Tinoco, 1980; Olsthoorn et al., 1981). Thermodynamic parameters for stacking interactions are usually measured by UV absorption (Petersheim & Turner, 1983) or by NMR chemical shifts (Lee & Tinoco, 1977) based on a two-state model, or measured by calorimetry (Breslauer & Sturtevant, 1977). These methods can provide information about the macroscopic states of nucleobases but are limited in their ability to describe the local states of individual bases. These limitations are due either to sensitivity or to base motions occurring on the time scale of measurements.

Helix to coil transitions in DNA are usually monitored by UV hypochromicity changes in the DNA bases, which are interpreted as effects resulting from the disruption of stacked states and formation of unstacked states. This type of measurement can quantitate overall transitions in DNA structure but sheds little light on the local states of individual bases. NMR experiments on proton exchange show that base pairs open and close at finite rates, but recent results (Reid, 1987) question the lifetimes of the base-pair open states. Comparison of UV melting results with those from NMR proton exchange indicates that the open state in NMR may not be the unstacked state as assumed in UV hypochromicity (Benight et al., 1988). Thus the nature of the local states of bases at varying temperatures still remains to be accurately described.

Fluorescence emissions of DNA bases are sensitive to local environments and thus provide some information about the nature of local states. However, the fluorescence of the four

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