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Expression in *Escherichia coli* of a Clavaminic Acid Synthase Isozyme: A Trifunctional Oxygenase Involved in Clavulanic Acid Biosynthesis

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Abstract: Purification and sequencing of clavaminic acid synthase (CAS) activities from *S. clavuligerus* SC2 provided evidence for the existence of two CAS isozymes. One of these isozymes was cloned and expressed in *E. coli*. The recombinant CAS isozyme was shown to catalyse the hydroxylation of (2*S*)-5-guanidino-2-(2'-oxoazetidin-1'-yl)pentanoic acid, in addition to the production of clavaminic acid from dihydroclavaminic acid via proclavaminic acid, consistent with the trifunctional role proposed for clavaminic acid synthase in the biosynthesis of clavulanic acid. A preliminary purification and characterisation of the recombinant isozyme is reported.

INTRODUCTION

Clavulanic acid (**1**) is produced by *Streptomyces clavuligerus* and is currently the most important commercial inhibitor of β-lactamases.[1] *In vivo* biosynthetic studies have shown that the carbon skeleton of clavulanic acid is derived from the 3-carbon pool [2,3] and arginine[4]. Subsequent work established that clavaminic acid (**2**) is a precursor of clavulanic acid (**1**) and that clavaminic acid (**2**) is in turn produced from the monocyclic β-lactam proclavaminic acid (**3**). The conversion of **3** to **2** was shown to be mediated by an α-ketoglutarate and ferrous dependent oxygenase, clavaminic acid synthase (CAS), using purified extracts of *S. clavuligerus*.[5-7] Thus, CAS belongs to the family of 2-oxo acid dependent and related dioxygenases[8], several members of which are also involved in the penicillin and cephalosporin biosynthesis pathway[9]. A protocol for the preparation of purified CAS and its initial characterisation have also been reported by Townsend *et al.*[10] Two forms of the enzyme, differing slightly in kinetic properties and molecular weight, were isolated. It was speculated that the lower molecular weight protein was derived from the larger protein by proteolysis, possibly during the purification, although the possibility of isozymes was not ruled out. Both forms were reported to catalyse the conversion of proclavaminic acid (**3**) to clavaminic acid (**2**). Subsequently Marsh *et al* have reported the cloning and sequencing of genes encoding for two CAS isozymes from *S. clavuligerus*.[11]

In contrast to a study[12] reporting that there is no substantial release of an intermediate in the conversion of **3** to **2**, using partially purified CAS we were able to demonstrate the intermediacy of the saturated clavam dihydroclavaminic acid (**4**).[13] Recently we have shown that CAS from *S. clavuligerus* catalyses the hydroxylation of (2*S*)-5-guanidino-2-(2'-oxoazetidin-1'-yl)pentanoic acid (**5**) to give alcohol (**6**).[14] Based in part on this observation we proposed a trifunctional role for CAS in the biosynthesis of

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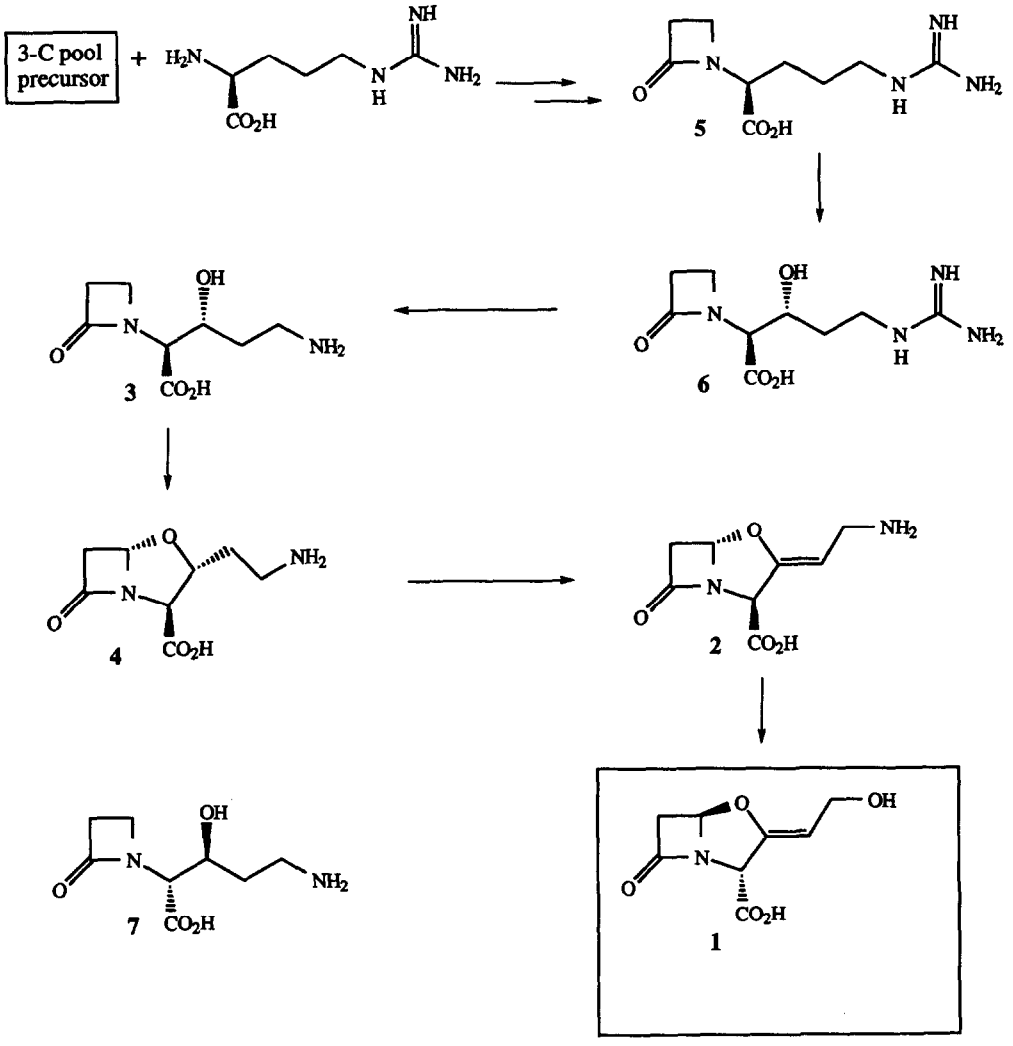
clavulanic acid (**1**) and predicted the presence of an amidino hydrolase capable of converting **6** to proclavaminic acid (**3**). Subsequently proclavaminic acid amidino hydrolase has been discovered[15] and a biosynthetic pathway to (**1**) has been proposed (Scheme 1). The stereospecificity of the hydroxylation has been investigated and shown to occur with stereospecific removal of the *pro*-R hydrogen at C-3 of **5**.[16] There remains two gaps in the pathway: the formation of **5** from a C-3 pool metabolite and arginine and the process by which **2** is converted into **1**, which must involve a double epimerisation.



Scheme 1, The Biosynthesis of Clavulanic Acid (1)

In order to unequivocally demonstrate that a single protein is capable of carrying out the conversion of of **5** to **6**, **3** to **4**, and **4** to **2** and to further investigate of the possibility of the existence of CAS isozymes we

embarked upon the purification and sequencing of the two forms of CAS from *S. clavuligerus*. Herein, we report the purification and *N*-terminal sequence data of the two forms of CAS from a reisolate of *S. clavuligerus* ATCC 27064 , denoted SC2, and the cloning of a *cas* gene from *S. clavuligerus* SC2 into *Escherichia coli*. The pT7 system of Tabor and Richardson[17,18], which has been previously used[19,20] for the expression of other streptomycete genes in *E. coli* was chosen for the expression of the *cas* gene. An initial purification and partial characterisation of CAS from the recombinant *E. coli* source is also reported.

EXPERIMENTAL PROCEDURES

Materials and methods: Chemicals used in this study were obtained from Sigma Chemical Co., unless otherwise stated. Concentrations of protein were determined by the method of Bradford using bovine serum albumin (BSA) as a standard.[21] Protein purification of CAS was in part carried out using a Pharmacia FPLC system. SDS-PAGE was performed using a Bio-Rad mini-protean II system using 12.5 % running gels and 3% stacking gels. The molecular weight of CAS was estimated (on SDS-PAGE) using the following standards: myosin (205 000 Da), β-galactosidase (116 000 Da), phosphorylase B (97 400), BSA (66 000 Da), egg albumin (45 000 Da), and carbonic anhydrase (29 000 Da). Molecular weight analysis by gel filtration was carried out on a Pharmacia FPLC system equipped with a Superose-12 HR column. The column was equilibrated with 50 mM Tris-HCl containing 150 mM KCl, pH 7.5, using the following standards: BSA (66 000 Da), egg albumin (45 000 Da), carbonic anhydrase (29 000 Da), and α-lactalbumin (14 200 Da). Mass spectrometric analysis of CAS was carried out using a VG-Bio Q triple quadrupole atmospheric mass spectrometer equipped with an electrospray interface. Samples (10 µl) were injected into the electrospray source via a loop injector as a solution, typically 10-20 pmol/µl in water: methanol: acetic acid [49:50:1 (v/v/v)] at a flow rate of 2µl per minute. Masses were calibrated with myoglobin (16 951.48 Da). Isoelectric focusing was carried out on a Pharmacia Phast-gel system, using Pharmacia LKB biotechnology standards pH 3-10.

Assays of Enzyme Activity: Assays for CAS activity, during protein purification procedures, were conducted in a final volume of 100µl in 5ml plastic bijoux. Protein extracts were incubated at ambient temperature (*ca.* 25°C), in the presence of 1 mM iron (II) sulfate, 1 mM DTT, 5 mM α-ketoglutarate and 5 mM (±)-*threo*-proclavaminic acid (**3**). [In our hands neither ammonium sulphate nor ascorbate were found to be beneficial for activity using either native or recombinant protein, both being modest inhibitors at >5mM concentrations.] After 5 minutes, the reaction mixture was derivatised with 25µl of 3.2M imidazole, pH 6.8, and incubated for a further 10 minutes. An aliquot of 25µl was analysed by h.p.l.c.[22](Rheodyne injector fitted with a 2 µl sample loop, Waters Associates chromatography pumps) using an ODS column (250 x 4.6 mm), pre-equilibrated with 100 mM phosphate buffer containing 6% methanol, pH 3.2. Derivatised clavaminic acid (**3**) was monitored at λ =312 nm, 0.1 AUFS using a Cecil CE 2112 detector and a JJ instruments CR 650A chart recorder. The amount of derivatised **2** produced was determined by reference to a standard curve of similarly derivatised synthetically prepared sample of the enantiomer of **2**, prepared from clavulanic acid.[23]

Kinetic analyses of CAS purified from recombinant *E. coli* cells were carried out (at least) in triplicate at 30 ± 0.1 °C (using a Luckman R300 incubator) and essentially the same assay as above except that samples

were analysed at 0.05 AUFS. Turnover of all substrates was always less than 10%. Data was analysed using the RS1 software using the Michaelis-Menten progress curve and the direct linear plot method of Cornish-Bowden and Eisenthhal[24,25].

Dihydroclavaminic acid (4) was isolated from the incubation of (+)-*threo*-proclavaminic acid (3) (0.6 mg) with partially purified preparations of CAS obtained from recombinant *E. coli* cells. Reactions were quenched after 90 minutes and no additional iron (II) sulphate or DTT was added. The resulting dihydroclavaminic acid (4) (*ca.* 100 μg) was isolated as before.[13] The ^{1}H NMR (500 MHz) spectrum was analogous to that previously reported for biosynthetic **4**, except for the lack of a deuterium label. m/z (electrospray) = 201 (100%), 202 (7).

Growth of S. clavuligerus SC2: S. clavuligerus SC2 was stored as spore suspensions in glycerol at - 80 °C between growths. Agar plates were made up using 1% dextrin and 2% agar (Oxoid no. 1), 0.4% (w/v) $CaCO_3$, 0.1% of KH_2PO_4, $MgSO_4$, NaCl, $(NH_4)_2SO_4$, and 0.1% of a trace element aqueous solution [containing 0.1% (w/v) of each of $FeSO_4.7H_2O$, $MnSO_4.4H_2O$ and $ZnSO_4.7H_2O$]. The agar plates were inoculated with thawed *S. clavuligerus* SC2 spores and incubated at 27 °C. Seed culture (100ml) contained in a 500ml flask was inoculated with *S. clavuligerus* SC2 using a well sporulated agar plate and incubated at 200 r.p.m. and 27°C at pH 7.0 on a gyrotary shaker for *ca.* 65 hours. Seed medium contained (per litre) 20g soya bean flour, 10g dextrin, 5g glycerol trioleate, and 0.6g KH_2PO_4. Seed culture (2 ml) was used to inoculate 60 ml of growth medium in a 500 ml flask, which was incubated under the same conditions between 48 to 55 hours. The fermentation medium contained (per litre) 35g soya meal, 50 g dextrin, 5 g glycerol, 0.7 g KH_2PO_4 and 10 ml of a trace element solution [(w/v) 1.0%$CaCl_2.2H_2O$, 1% $MgCl_2.2H_2O$, 1% NaCl, 0.35 $FeCl_3.6H_2O$, 0.05% $ZnCl_2$, 0.05% $CuCl_2.2H_2O$ and 0.05% $MnSO_4.4H_2O$.] Cells were harvested by centrifugation (17 200 *g* for 20 min at 4 °C) and stored at - 80 °C if required.

Purification and sequencing of CAS from S. clavuligerus SC2 (Table 1): Harvested *S. clavuligerus* was resuspended in 0.9% (w/v) NaCl and recentrifuged. The cells were then resuspended in 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 10% (v/v) glycerol, pH 7.5 and sonicated in 25 ml aliquots (4 x 10 seconds, 10 microns, MSE soniprep 150) with stirring for 30 seconds at 4 °C between bursts. DNA was removed by addition of streptomycin sulphate to a final concentration of 1% (w/v) and the resulting extract was centrifuged at 15 000 g for 20 min at 4 °C. The supernatant was concentrated using a 40 - 80% ammonium sulphate fractionation followed by centrifugation (15000g, 20 min, 4 °C). Two protocols for the further purification of CAS were used. Initially the fractionated protein was dialysed against 50 mM Tris-HCl pH 7.0 then applied to a column of DEAE-Sepharose (200 ml) equilibrated in the same buffer at 60 ml/hour. CAS was eluted with a gradient of 50-250 mM NaCl in Tris-HCl pH 7.0. Active fractions were pooled and applied to a column of Sephadex G75 (770 ml) equilibrated in 50 mM Tris-HCl pH 7.0 at 25 ml/hour. After pooling active fractions were concentrated and carboxymethylated with iodoacetic acid/mecaptomethanol before *N*-terminal sequencing by Edman degredation. Subsequently a more extensive purification protocol was developed. The pellet from ammonium sulphate fractionation was redissolved in the sonication buffer and loaded onto a LKB Ultra-gel ACA 54 gel filtration column (425 ml) and eluted in the same buffer at 20 ml/hour overnight. Active fractions were pooled, diluted with deionised water, and loaded onto a DEAE-trisacryl column (70 ml) equilibrated in 25 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 10% (v/v) glycerol, pH 7.5. The column was eluted using a 0-1 M gradient of NaCl in 1000 ml of the same buffer

at 4 °C overnight. Active fractions were pooled and stored at - 80 °C. Further purification was achieved by loading *ca.* 1 mg of the partially purified CAS onto a Mono-Q 5/5 anion-exchange column equilibrated in 50 mM Tris-HCl in 20% (v/v) glycerol at 4 °C using a Pharmacia FPLC system. The column was eluted using a 40-70% gradient of 250 mM KCl in the same buffer and fractions of 1 ml were collected. CAS activity was eluted at *ca.* 55% (138 mM) KCl and at ca. 65% (163 mM) KCl. A third protein was eluted in fractions collected immediately after those containing CAS activity. This protein had no apparent CAS activity (see results and discussion).

N-Terminal sequencing of partially purified CAS was carried out by further purification using SDS-PAGE (15% gel) followed by electro-blotting onto a Waters Immobilon membrane in a Bio-Rad mini-transblot cell at 0.3 A for 1 h in the presence of 10 mM CAPS in 50% methanol, pH 11. After staining with Coomassie Brilliant Blue R-250 the required bands were excised and placed in a blot cartridge for sequencing, by pulsed liquid-phase Edman degradation. Tryptic digestion and subsequent purifications using h.p.l.c. were carried out according to previously published protocols[26].

Molecular Biology (Figure 2): Plasmid pBROC413 was derived from pT7-7 by means of a 0.25 kb deletion between the *Lsp*I and *Bgl*II sites of pT7-7. pBROC413 is a non-mobilisable derivative of pT7-7 and was constructed to facilitate the large-scale fermentations of strains carrying recombinant plasmids. pBROC421 was constructed by the insertion of a 2.2 kb *Nru*I fragment containing the *cas* gene from pBROC44 into the *Sma*I site of pT7-7.[27] Plasmid pBROC423 was derived from pBROC421 by replacement of the 0.9 kb *Nde*I-*Pst*I fragment containing upstream sequences and the first 30 codons of the *cas* gene with a 0.1 kb *Nde*I-*Nco*I synthetic DNA fragment. Plasmid pBROC424 was constructed by the replacement of the polylinker *Nde*I-*Pst*I fragment in pBROC413 with a 1.4 kb *Nde*I-*Pst*I fragment containing the *cas* gene from pBROC423. Thus, plasmid pBROC424 is a non-mobilisable derivative of pBROC423.

Recombinant plasmids were initially introduced into *E. coli* DH1 (*supE*44 *hsdR*17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1) and the correct construct identified.[28] Plasmids were then introduced into *E. coli* BL21 (DE3) [*hsd*S, *gal* (cIts857 *ind*, *sam*7, *nin*5, *lac*UV5-T7 gene *1*)][29] by electroporation for expression studies. The recombinant strains were grown at 37 °C, except were otherwise specified on Luria agar or in Luria broth[30] supplemented with ampicillin at 50 μg ml^{-1}. To express the *cas* gene from the T7 promoter, 3 ml of overnight culture was inoculated into 200 ml of pre-warmed broth and incubated until OD_{600} reached 0.5-0.6 (*ca.* 2 h). The T7 RNA polymerase gene was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the culture was incubated for a further 3 hours, when the cells were harvested by centrifugation (17 200 g for 20 min at 4 °C).

Purification and Sequencing of Recombinant CAS (Table 2): *E. coli* BL21 (DE3)(pBROC424) was harvested by centrifugation and resuspended in 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 10% (v/v) glycerol, pH 7.5. Sonication (4 x 10 second bursts, 100W using a MSE Soniprep sonicator, with 100μ probe) was followed by removal of insoluble material by centrifugation (20 000g for 10 min.). DNA was precipitated by addition (over *ca.* 5 min.) of streptomycin sulphate to a stirred solution of the extract to give a final streptomycin concentration of 1% (w/v). The suspension was then centrifuged (15 000 g for 30 min.). Purification of CAS was carried out by loading protein extract (5.7mg in 250ml) onto a Superose-12 HR column, pre-equilibrated in 20 mM Tris-HCl pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 10% (v/v) glycerol, and 0.02% NaN_3. The column was eluted with the same buffer at 0.6 ml min^{-1}, and 1 ml fractions collected.

The three fractions containing CAS activity were pooled and loaded onto a Mono-Q 5/5 anion exchange column, equilibrated in the same buffer and 1 ml fractions collected. The column was eluted with a 20-65% gradient of 400 mM NaCl over 15 ml. The three fractions found to contain CAS activity were eluted at *ca.* 45% NaCl (180 mM). A sample of CAS was subjected to N-terminal sequencing after SDS-PAGE, using a similar protocol to that used for the sequencing of the native proteins.

RESULTS AND DISCUSSION

Our second and preferred purification of CAS from sonicated extracts of *S. clavuligerus* SC2 followed a similar strategy to that previously reported by Salowe *et al.* (Table 1)[10]. The final stage in the purification was also achieved using Mono-Q anion-exchange chromatography. Three closely running proteins (I, II, and III) at M_r *ca.* 45 000, were eluted in order of decreasing M_r, as judged by SDS-PAGE. The protein with the highest apparent molecular weight (I) was well separated from the other two proteins by this procedure, whilst the other two proteins (II and III) were separated less efficiently. CAS activity was found to reside predominantly in the first (I) and second (II) protein fractions to be eluted. Specific activities (for 3 to 2) of between 0.4 and 0.6 $IUmg^{-1}$ protein were obtained for these two bands.

Purification step	Specific activity /IU mg^{-1}	Total activity /IU	% recovery
'crude'	0.0035	9.5	100
AcA 54 pool	0.145	4.0	42
DEAE-trisacryl			
Mono-Q 1	0.304	0.67	7
Fraction 2	0.248	0.55	6

Table 1: Purification of CAS from *S. clavuligerus* SC2; The final step of the purification was achieved using Mono-Q chromatography - see text.

(a)	(i)	G	L	P	V	E	A	D	A	D	L	T	P	T
		P	S	S	T	P	A	P						
	(ii)	A	L	D	E	V	T	E	A	V	Y	L	E	P
		G	D	L	L	I	V	D	N	F	R			
(b)		A	S	P	I	V	D	C	T	P	Y	R	(A)	(E)
		(L)	(L)											
(c)		(D)	S	P	I	V	D	(Q)	T	P				
(d)		L	S	S	E	T	S	E	T	L	L			
(e)		T	S	V	D	C	T	A	Y	G	P	E	L	-
		A												
(f)		A	S	P	I	V	D	C	T	P	Y			

Table 2: Amino acid sequence data obtained from the purification of CAS activities from *S. clavuligerus* SC2: (a) (i) and (ii) = *N*-Terminal sequences of peptides isolated after tryptic digestion of band I; (b) *N*-terminal sequence of band II; (c) *N*-Terminal sequence of CAS from preliminary purification; (d) *N*-Terminal sequence of degredation fragment; (e) *N*-Terminal sequence of band III; (f) N-Terminal sequence of the recombinant CAS isozyme. Uncertain amino acid analyses are in parentheses.

These results are largely consistent with the results of Salowe *et al.*[10], who observed two proteins, of Mr 47, 000 and 46, 000 as estimated by SDS-PAGE. Each of the three proteins (I, II, and III) were subjected

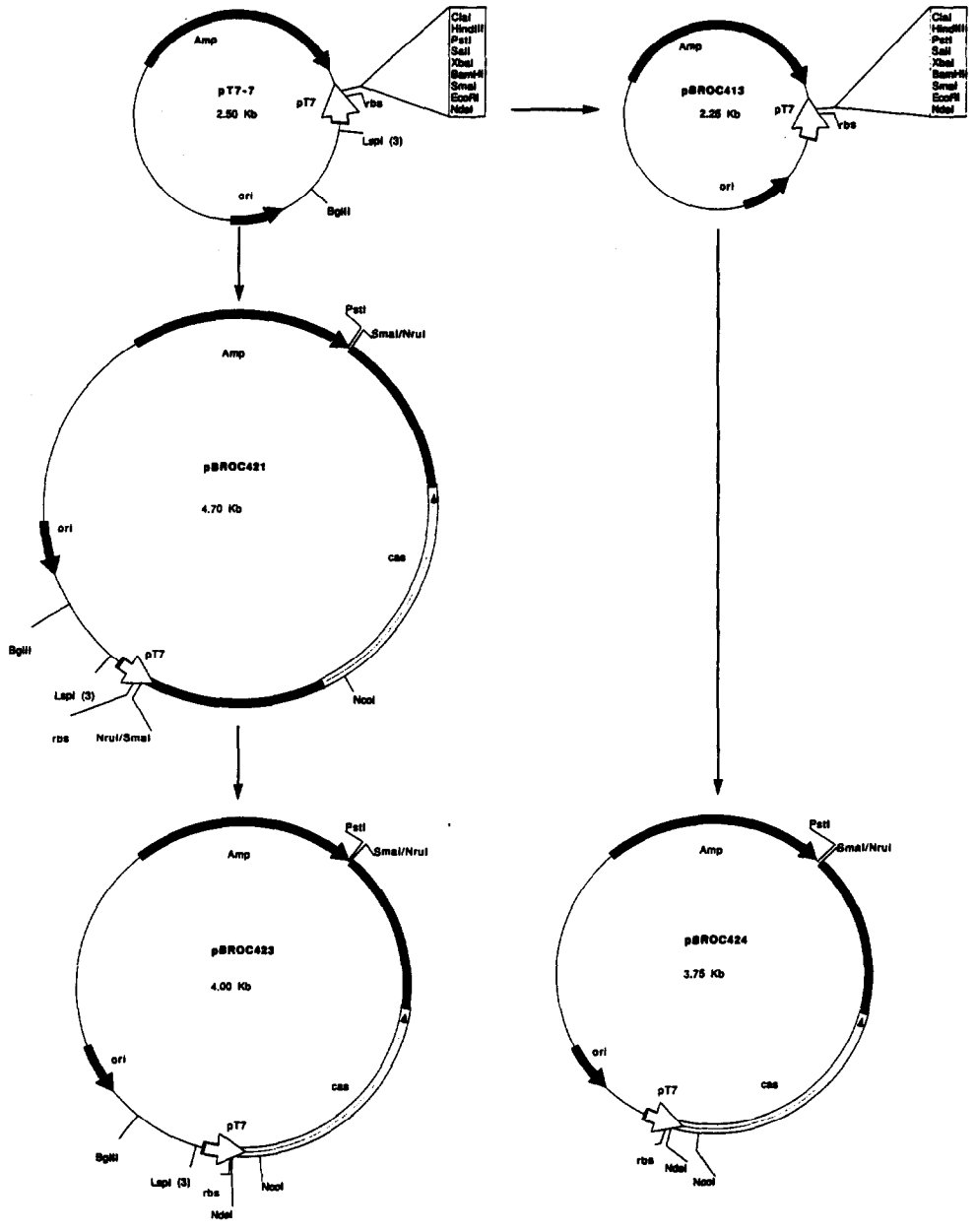
to sequence analysis. The first protein (I) to elute failed to give an *N*-terminal sequence and was assumed to be blocked at the *N*-terminus. Tryptic digestion of this protein (I) was followed by sequencing of selected fragments (Figure 3a). The *N*-terminal sequence of the second protein (II) to elute was identical to that derived from a CAS isozyme subsequently expressed in recombinant *E. coli* cells (*vide infra*) (Figure 3b). The *N*-terminal sequence of CAS purified from *S. clavuligerus* SC2 according to our initial protocol was also consistent with the N-terminal sequence obtained for protein II (Figure 3c). The fraction containing the second closely running protein (II) to elute was also found to contain a small amount of a protein of lower Mr (*ca.* 17 500 by DS-PAGE) (Figure 3d). This fragment was also subjected to *N*-terminal analysis and was subsequently recognised as a degradation fragment of protein II. The third closely running protein (III) was also sequenced, but is probably not responsible for the CAS catalysed reaction (Figure 3e).

Comparison of the *N*-terminal sequence of CAS purified from *S. clavuligerus* according to our initial purification protocol, with the predicted amino acid sequence from the nucleic acid sequence was used to identify the open reading frame of a CAS isozyme in pBROC44. The plasmid pBROC44 was constructed by the inserion of a 6.8kb *Sph* I fragment[31] of *S. clavuligerus* DNA in to the streptomycete plasmid pIJ702[30]. Although pBROC44 contains the entire *cas* gene, it is not suitable for expression in *E. coli*, because the *S. clavuligerus* promoter sequence is unlikely to be efficiently recognised in *E. coli*. Thus, three plasmids based on the pT7 system of Tabor and Richardson[17,18] were constructed (Figure 2). The initial plasmid constructed was pBROC421 which contained the *cas* gene together with 0.7 kb upstream and 0.5 kb downstream DNA flanking sequences. In this construct the *cas* gene is transcribed by T7 RNA polymerase from the Ø10 promoter, and translation is presumed to initiate at the *S. clavuligerus* ribosome binding site adjacent to the *cas* gene on the cloned DNA. CAS activity was detectable in crude cell free extracts of *E. coli* BL21 (DE3)(pBROC421), but no obvious expression of the *cas* gene was observed by SDS-PAGE.

(a)		M	A	S	P	I	V	D	C	T	P
(b)	T	ATG	GCT	TCT	CCG	ATC	GTT	GAC	TGC	ACC	CCG
(c)			C			A					
(a)		Y	R	D	E	L	L	A	L	A	S
(b)		TAC	CGT	GAC	GAA	CTG	CTG	GCT	CTG	GCT	TCC
(c)			C		G		C	G	C		
(a)		E	L	P	E	V	P	R	A	D	
(b)		GAA	CTG	CCG	GAA	GTT	CCG	CGT	GCT	GAC	CTC
(c)		G	T	C	G	G		C	G		

Table 3: Codon changes at the 5' end of the *cas* gene: (a) The deduced amino acid sequence, with the experimentally determined sequence of the recombinant protein underlined; (b) Nucleotide sequence of the upper strand of the synthetic DNA used in the construction of pBROC423; (c) The nucleosides where the native sequence differed from the synthetic sequence.

In an attempt to increase the level of expression, the plasmid pBROC423 was subsequently constructed in which the sequences upstream of the CAS gene had been deleted. In this construct translation initiates from the vector ribosomal binding site, which is adjacent to the *Nde*I site. In pBROC423 the first 30 codons of the CAS gene were also deleted and were replaced by a synthetic DNA sequence encoding identical



Scheme 2: Construction of plamids expressing the *cas* gene

amino acids, but using codons more commonly found in *E. coli* [32](Figure 4). These changes resulted in an significant increase in the level of the expression of the *cas* gene and a heavy band was observed at M_r 45 000 on SDS-PAGE in strains displaying CAS activity. Detailed consideration has been given to a number of possible factors, including codon usage, which may influence the level of expression of streptomycete genes in *E. coli*.[20] The nucleotide changes carried out by us, however, resulted in a reduction in the GC content of the first 30 codons of the *cas* gene from 70 to 61mol% as well as the conversion to synonymous codons. Thus, it is not possible to conclude that the observed increase in expression level is due to greater translational efficiency or another factor.

In *E. coli* BL21 (DE3)(pBROC423) CAS was found to comprise *ca* 10% of the total cell protein. Plasmid pBROC424 is a non-mobilisable plasmid that is identical in other respects to pBROC423, which was constructed to facilitate large-scale fermentations of CAS. The level of CAS expression in *E. coli* BL21 (DE3)(pBROC424) was also *ca.* 10% of the total cell protein.

The recombinant protein corresponding to the band at *ca.* 45 000 Da (by SDS-PAGE) was confirmed to be the CAS isozyme corresponding to protein II from *S. clavuligerus*. Thus, N-terminal sequencing of the recombinant isozyme (Figure 4) gave an identical sequence to that obtained for protein II. Comparison of the predicted sequence for the expressed enzyme with the sequence obtained for protein I showed significant homology with regions of the cloned CAS, but clear differences were apparent. Thus, there are two CAS isozymes in *S. clavuligerus*, corresponding to proteins I and II isolated during purification of the two native CAS activities. Comparison of the amino acid sequences reported herein with the recently published DNA predicted amino acid sequences reveals that protein I corresponds to CS1 and protein II to CS2 of Marsh *et al*[11]. It is of interest that the N-terminal amino acid sequence of protein III is identical to that predicted for CS1. Hence, protein III appears to be an N-terminally unblocked derivative of protein I. Protein III has a lower molecular weight than protein I and is not thought to retain significant CAS acitvity. Hence, it is likely that proteolysis of protein I to give protein III probably involves removal of the C-terminal amino acids in addition to cleavage of the N-terminal methionine. Comparison of the N-terminal amino acid sequence obtained for the degradation fragment reveals identity with both isozymes and may reflect the short half life reported for CAS from *S. clavuligerus*.[10]

Purification step	Specific activity/IUmg^{-1}	Total activity/IU	% recovery
crude	0.024	0.14	100
Superose-12[a]	0.077	0.169	120
Mono-Q:			
Fraction A	0.11	0.029	2.2
Fraction B	0.37	0.216	57
Fraction C	0.183	0.109	14

Table 4: Purification of recombinant CAS.[a] Active fractions were pooled.

An initial purification of recombinant CAS has been performed (Table 2). Glycerol was found to stabilise the protein as judged by assay with (±)proclavaminic acid (3). After the Mono-Q step typical purifications via this protocol gave CAS of >95% purity as judged by SDS-PAGE with specific activities of

0.3-0.5 IUmg^{-1}. The Mr for the recombinant CAS isozyme as judged by SDS-PAGE was consistent with that observed for protein II from the *S. clavuligerus* preparation. The isoelectric point for the recombinant CAS isozyme was determined to be 5.70 ± 0.05, the same as that previously reported for native CAS[5].

Analysis of the recombinant CAS isozyme by electrospray mass spectroscopy (ESIMS) indicated a molecular mass of 35 667 ± 1.95 Da, which is close to the calculated value (35 665 Da) of the protein without the N-terminal methionine (predicted value with N-terminal methionine = 35 801 Da). ESIMS analysis of purified protein II showed a molecular mass of 35 666 ± 4.85 Da. It is striking that the apparent mass obtained from SDS-PAGE analysis differs significantly from the observed ESIMS value and that predicted from the gene sequence. Gel filtration of the recombinant CAS gave an estimated mass of 38 000 ±500 Da identical to that obtained for protein II.

Incubation of proclavaminic acid (**3**) with the recombinant CAS isozyme on a preparative scale led to the isolation of clavaminic acid (**2**), but the availability of larger quantities of CAS allowed for the first time the isolation and characterisation of a pure (>95%, by 500 MHz NMR) sample of dihydroclavaminic acid (**4**). The ^{1}H NMR (500 MHz) spectrum and ESIMS of (**4**) were identical to those obtained for its enantiomer, which was obtained by synthesis from **1**.[13] The isolated dihydroclavaminic acid (**4**) was re-incubated with the purified recombinant CAS and shown to be converted to clavaminic acid (**2**). Previously, we had used 3-[^{2}H$_1$]-proclavaminic acid (**2a**) with a mixture of the native CAS isozymes to facilitate the isolation of 3-[^{2}H$_1$]-dihydroclavaminic acid (**4a**).[13] The isolation of **4** from incubations of fully protiated **3** with CAS demonstrates that its production is not dependent upon the substitution of the 3-H with deuterium or tritium and can thus be classified as an intermediate rather than a shunt metabolite.

Preliminary kinetic analysis of Mono-Q purified recombinant CAS gave the following apparent values: for L-*threo*-proclavaminic acid (**3**), K_m = 0.44 mM, k_{cat} = 14.6 min^{-1} and for (±)-*threo*-proclavaminic acid (**3**), K_m = 0.44 mM, k_{cat} = 8.2 min^{-1}, k_{cat}/K_m = 1.9 x 10^4 min^{-1} M^{-1}. Marked deviation from steady-state kinetics was observed with (±)-(**3**) using direct linear plots. This indicates that D-*threo*-proclavaminic acid (**7**) is probably behaving as a competitive inhibitor of the CAS reaction. Salowe *et al.* also noted an increase in the K_m and a decrease in k_{cat}/K_m for (±)-*threo*-proclavaminic acid (**3**) compared to enantiomerically pure proclavaminic acid (**3**), using a mixture of the native CAS isozymes. For 2-oxoglutarate (using **3** as a substrate) the following values were obtained: K_m = 0.26 mM, k_{cat} = 3.72 min^{-1}, k_{cat}/K_m = 1.4 x 10^4 min^{-1} M^{-1}.

In summary, purification and sequencing of CAS activities from *S. clavuligerus* SC2 provided evidence for the expression of two CAS isozymes, both of which can catalyse the formation of clavaminic acid (**3**) from proclavaminic acid (**2**). One of these isozymes has been cloned and expressed in *E. coli* and demonstrated to catalyse the conversion of proclavaminic acid (**3**) to clavaminic acid (**2**), via dihydroclavaminic acid (**4**), confirming that a single isozyme is capable of catalysing both the CAS cyclization and desaturation reactions and that **4** is a free (*i.e.* does not remain enzyme bound) intermediate in the conversion of **3** to **2**. The purified recombinant CAS was also shown to catalyse the hydroxylation of **5** to **6**[14,33], unequivocally demonstrating that a single CAS isozyme is capable of mediating three separate transformations in the biosynthesis of clavulnic acid (**1**). The most remarkable observation we have made in our studies on the role of CAS in the biosynthesis of clavulanic acid (**1**) is the dependence of the type of oxidative chemistry catalysed by CAS, *i.e.* hydroxylation (**5** to **6**) or desaturative cyclisation/desturation (**3** to

4, 4 to 2), on the side chain (amino or guanidino) of the substrate. The availability of recombinant CAS should facilitate the study of these interesting transformations.

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33. Full details of this transformation will be described elsewhere.

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