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Research Article

Probing the penicillin sidechain selectivity of recombinant deacetoxycephalosporin C synthase

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Abstract. Deacetoxycephalosporin C synthase from *Streptomyces clavuligerus* catalyses the conversion of the five-membered penicillin ring to the unsaturated six-membered cephem ring of deacetoxycephalosporin C. The effects on enzyme activity of the penicillin substrate sidechain and various cofactors were investigated using a continuous spectrophotometric assay. The conversion of penicillin G to phenylacetyl-7-aminodeacetoxycephalosporanic acid (G-7-ADCA) was confirmed, and further details of the reaction were elucidated. The conversion of ampicillin to cephalexin was faster than that of acetyl-6-APA to acetyl-7-ADCA $k_{cat} = 0.120 \pm 0.001\ s^{-1}$ versus $0.035 \pm 0.001\ s^{-1}$, but they had similar K_m values: 4.86 ± 0.12 and 3.28 ± 0.26 mM, respectively. Amoxycillin and penicillin V were also converted at low levels. Conversion was not detected for penicillanate, 6-aminopenicillanate, carbenicillin, temocillin, ticarcillin or benzylpenicilloic acid, suggesting that the enzyme has a relatively strict selectivity for the sidechain of the penicillin substrate.

Key words. Cephem antibiotic biosynthesis; enzyme inactivation; iron(II); 2-oxoglutarate-dependent oxygenase; spectrophotometric assay.

Introduction

Since their discovery more than 60 years ago, β-lactam antibiotics have gained in importance to become the most widely used antibacterial agents. However, the continuous challenge exerted by emerging β-lactam-resistant bacterial strains, principally mediated by β-lactamases [1], has required the discovery of new and more efficient β-lactam derivatives. Because of their structural complexity, these important antibiotics are industrially produced from materials derived from the fermentation of production strains. However, the most clinically useful cephalosporins and penicillins are made by expensive and environmentally damaging synthetic manipulations of fermentation-derived materials. Therefore, there is substantial interest in the development of efficient and cost-effective systems to produce β-lactam antibiotics (especially those with hydrophobic sidechains, e.g. phenylacetyl) and their precursors both in vivo and in vitro. In recent years, this objective has been facilitated by the development of recombinant production strains [2].

Deacetoxycephalosporin C synthase (DAOCS) catalyses the committed step in the biosynthesis of cephamycin C in *Streptomyces clavuligerus*, the expansion of the five-membered ring of penicillin N (1a) to the unsaturated cephem ring of deacetoxycephalosporin C (1b) (fig. 1)

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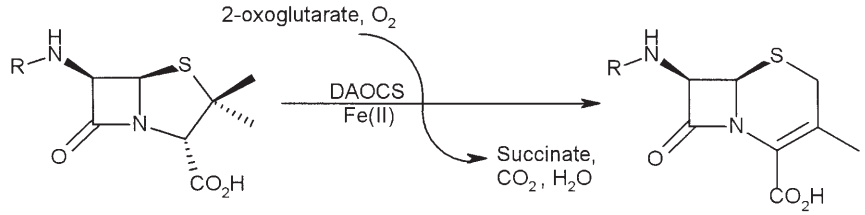

	R	Penicillins **(a)**	Cephalosporins **(b)**
1	D-(α-aminoadipoyl) -	penicillin N	DAOC
2	phenylacetyl -	penicillin G	G-7-ADCA
3	R-(2-amino)phenylacetyl -	ampicillin	cephalexin
4	acetyl -	acetyl-6-APA	acetyl-7-ADCA

Figure 1. Reactions catalysed by DAOCS.

[3]. DAOCS is an iron(II), 2-oxoglutarate-dependent oxygenase, which requires reducing agents such as dithiothreitol (DTT) and ascorbate for maximum in vitro activity [4]. It belongs to a subfamily of enzymes which are related by sequence homology, and includes deacetylcephalosporin C synthase (DACS) (which catalyses the subsequent hydroxylation step in the same pathway), isopenicillin N synthase (IPNS, the enzyme responsible for the formation of the penicillin nucleus), and DAOC/DAC synthase (the bifunctional enzyme which catalyses the ring-expansion and hydroxylation reactions in the biosynthesis of cephalosporin C in *Cephalosporium acremonium*) [5]. The structure and function of DAOCS have been partially elucidated by a series of site-directed mutagenesis experiments [6–8] and complementary X-ray crystal structures [3, 9].

A number of assays for DAOCS and other oxygenases have been used in the past, including monitoring production of succinate [10, 11] or carbon dioxide [6] from 2-oxoglutarate, and bioactivity [12], high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) assays [3, 13] for production of cephems. The inherent drawback of these methods is that they are discontinuous and only yield one point on the time course curve describing the enzyme kinetic reaction. Accurate characterisation of the kinetic properties of an enzyme requires several such points, especially if the studied reaction presents some complexity such as biphasic kinetics, progressive inactivation or product inhibition. Moreover, these assays are labour intensive and therefore cumbersome when large numbers of sample are to be analysed. In addition, all of these assays have specific disadvantages. Assays for 2-oxoglutarate conversion are limited by the fact that many oxygenases can convert 2-oxoglutarate without concomitant oxidation of the other ('prime') substrate [4], and this uncoupled reaction can be significantly enhanced with some unnatural substrates or in mutant enzymes [14]. The bioactivity assay for DAOCS is only semiquantitative and antibiotic activity is dependent on the structure of the product [especially of the sidechain; see e.g. ref. 13 for comments on the limitation of this assay]. HPLC-based assays are time-consuming and often necessitate developing new elution conditions for each potential product, which can be difficult in the absence of authentic standards. Moreover, these approaches are limited in their ability to analyse the properties of a large number of mutants in vitro and to look for novel products. There also appears to be a discrepancy between the in vitro results and the in vivo production of cephems; e.g. penicillin G (2a) is a poor substrate for recombinant DAOCS in vitro [3], but was not expanded at all to the phenylacetyl-7-aminodeacetoxycephalosporanic acid (G-7-ADCA) (2b) in recombinant *Penicillium chrysogenum* [2]. A spectrophotometric assay for DAOCS has previously been described [10] but its potential uses were never developed. This paper reports a novel direct spectrophotometric assay for DAOCS based on continuous monitoring of absorbance at 260 nm due to the production of the deacetoxycephem ring. It has been used to probe the kinetics and substrate/cosubstrate selectivity of recombinant DAOCS, and to characterise several previously unknown substrates, including acetyl-6-APA and ampicillin. This assay will facilitate more detailed kinetic analysis of DAOCS and its functional mutants, but is not suitable for analysis of reactions producing cepham products [e.g. 3 β-hydroxy-3-methylcepham; ref. 15] since they lack an absorbance at 260 nm.

Materials and methods

Materials were obtained from Sigma-Aldrich or Merck unless otherwise specified and were of analytical grade or higher. Acetyl-6-APA was synthesised as described elsewhere [16]. Wild-type enzyme was purified by anion-exchange and gel filtration chromatographies as previously described [3], except that a 0.1–0.3 M NaCl gradient over 800 ml was used to elute Q-sepharose column.

Isolation of deacetoxycephem products

Incubations for the isolation of products were carried out as previously described [3, 13] using 10 mM sodium ampicillin and purified DAOCS [5.86 mg, 0.16 IU/mg, 0.99 IU (μmol/min), assayed with penicillin G] and yielded ca. 1% substrate conversion. The freeze-dried products were separated by HPLC on a C4 Hypersil column (Phenomenex, 250 × 4.6 mm) using isocratic elution in 25 mM NH_4HCO_3 and 10% (v/v) methanol with a retention volume of 26 ml. The ^{1}H-NMR spectrum (500 MHz [17] and HPLC profile of the freeze-dried product (800 μg) were identical to that of commercial cephalexin (Melford) [m/z (+ve ESI MS) 348 (100%, M-H$^+$), (–ve ESI MS) 346 (100%, M-H$^+$]. Acetyl-6-APA (4a, 12.75 mM) and purified DAOCS (11.50 mg, 0.16 IU/mg, 1.84 IU, assayed with penicillin G) were incubated and about 10% conversion was obtained. The freeze-dried products were seperated by HPLC on a C4 Hypersil column (Phenomenex, 250 × 4.6 mm) at 1.5 ml min using 25 mM NH_4HCO_3 with 2% (v/v) methanol for 5 min and a 2–10% (v/v) methanol gradient over 10 min. Acetyl-7-ADCA (4b, 71 mg) eluted at 2.72–4.16% (v/v) methanol with a retention volume of 7 ml [^{1}H-NMR: 500 MHz, D_2O) δ_H 1.95 (3H, s, CH_3), 2.10 (3H, s, CH_3CO), 3.25 (1H, d, J = 18 Hz, CH_2) (other proton obscured by coeluting impurity with multiplets at 3.50–3.60, 3.60–3.70 and 3.80–3.85), 3.35 (s, residual methanol), 5.10 (1H, d, J=3.7 Hz, H-6), 5.60 (1H, d, J=4.5 Hz, H-7, m/z (–ve ESI MS) 255 (100%, M-H$^+$].

HPLC assays of DAOCS activity

The reaction mixtures (20- or 50-μl samples) were analysed by reverse-phase HPLC performed on a Merck Lichrospher RP18 100-5 μm column (250 × 4 mm). A linear elution gradient at 1 ml/min was used with acetonitrile in 25 mM potassium phosphate buffer pH 6.8: 2% (v/v) acetonitrile for 1 min, 2–30% (v/v) over 14 min, 30–80% (v/v) over 2 min, 80% (v/v) for 2 min. Under these conditions, the expanded cephem product elutes just before the penicillin substrate. Retention times for penicillin G and G-7-ADCA were about 18.5 and 17.5 min, and about 4.5 and 7.5 min for 6-APA and 7-ADCA, respectively. UV detection was at 215 and 260 nm.

Spectrophotometric DAOCS assays with penicillins

UV spectrophotometric measurements were made in 0.2- or 1-cm lightpath quartz cuvettes using a Beckman DU8 instrument interfaced with a computer for data collection or with a Hewlett-Packard HP8452A diode array spectrophotometer.

The assay solution was made by dilution of stock solutions of buffers, cofactors and other components to the following final concentrations: 50 mM HEPES-NaOH, 50 mM ammonium sulphate, 1 mM Tris(carboxyethyl)-phosphine (TCEP), 100 μM ascorbate and 100 μM 2-oxoglutarate. The pH was adjusted to 7.5 at room temperature and the penicillin concentration varied from 0.2 to 10 mM. In practice, 2- to 3-ml mixes at each penicillin concentration were made and kept on ice. Before assaying, a small volume of concentrated ferrous ammonium sulphate solution was added (25 μM final concentration) and the mixture was incubated at 30 °C for up to 3 min. A sample (450 μl) was withdrawn, supplemented with concentrated DAOCS enzyme solution (4–8 μM final concentration), placed in a cuvette and the absorbance at 260 mm monitored. During the measurement, the temperature was maintained at 30 °C by a Peltier system. Under these conditions, initial rates were proportional to the enzyme concentration and were calculated from the slope of the linear part of the reaction time course at the start of the reaction. A blank assay with no enzyme was similarly recorded and if a significant linear change in absorbance was observed in this blank assay, the values obtained in the presence of enzyme were corrected accordingly. The rate values in the text and figures were obtainded from at least four independent measurements and one blank assay for each condition or concentration tested. The assay was modified in some experiments as described in the figure legend. The steady-state kinetic parameters were obtained by non-linear regression fitting of the data to the Henri-Michaelis kinetic model.

Results and discussion

Development of the spectrophotometric assay

The conditions of the spectrophotometric assay were derived from preliminary analyses of the importance of the various cofactors by HPLC analysis (table 1). Ammo-

Table 1. The effect of cofactors on DAOSs activity.

Cofactors	Relative activity (%)
DTT/ascorbate/$FeSO_4$/2-oxoglutarate	100
Without ascorbate	40 ± 6
Without DTT	174 ± 14
Without ascorbate and DTT	43 ± 8
TCEP/ascorbate (0.05 mM)/$FeSO_4$/ 2-oxoglutarate	123 ± 7
TCEP/ascorbate (2 mM)/$FeSO_4$/ 2-oxoglutarate	240 ± 25
Without ascorbate	80 ± 7
Without $FeSO_4$	< 1
Without ascorbate and $FeSO_4$	< 1
Without 2-oxoglutarate	< 1
Without 2-oxoglutarate and ascorbate	< 1

Standard assay conditions were Tris.HCl (50 mM) pH 7.5, $(NH_4)_2SO_4$ (50 mM), penicillin G (10 mM), DTT (2 mM) or TCEP (2 mM), ascorbate (1 mM, 2-oxoglutarate) (1 mM), $FeSO_4$ (2 mM), DAOCS (2.7 μM). 100% activity of wild-type enzyme corresponds to 0.044 IU/mg.

nium sulphate at low concentrations acts as a stabilising agent for the *S. clavuligerus* DAOCS enzyme [3] by preventing its aggregation and precipitation in solution. Tris-HCl buffer is often used in these assays for historical reasons rather than for its intrinsic qualities. In fact, Tris possesses some undesirable properties such as its reactive primary amine [18] and its ability to chelate metals, which may not be optimal for studying biological systems. Moreover, β-lactams, and especially penicillins, may be hydrolysed by Tris or other cationic buffers. Phosphate buffer was avoided in this assay as it dramatically increases the ferrous iron oxidation rate [19, 20], and can also mediate β-lactam cleavage [21]. Thus, the anionic buffer HEPES was chosen (pKa = 7.5 at 25 °C) because it lacks a reactive primary amine and metal-binding properties.

Standard assay conditions were first optimised with penicillin G (fig. 2) or ampicillin (data not shown) using a diode array spectrophotometer to follow the DAOCS-catalysed reaction. Similar results were obtained in both cases. The end-products of the reaction were also analysed by HPLC. Authentic cephalexin coeluted with the cephem product derived from ampicillin (data not shown), and the identity was confirmed by isolation of the product and characterisation by ^{1}H-NMR and ESI MS (Materials and methods).

In a subsequent experiment, a 3-ml assay mixture was equilibrated at 30 °C, iron (II) was added to 25 µM and the reaction was started by rapidly mixing with DAOCS (5 µM final concentration). A 450-µl sample from this mixture was used to monitor the absorbance variation at 260 nm. The remaining solution was incubated at 30 °C and 120-µl samples were progressively removed, the reaction stopped by addition of 5 µl of 4 mM *o*-phenanthroline [a strong iron (II) chelator] and kept on ice. These samples were subsequently analysed by HPLC. The reaction displayed a short burst followed by a quasilinear increase in absorbance at 260 nm, correlating well with the increase in the amount of G-7-ADCA determined by HPLC analysis (fig. 3). Incubation of the reaction mixture for 2 min at 30 °C prior to adding the enzyme eliminated the burst phase.

To calculate initial rate values from the spectrophotometric assay, and to verify the validity of the method, the difference molar absorption coefficient at 260 nm ($\Delta\varepsilon_{260}$) associated with the expansion of penicillin G to G-7-ADCA was determined. A 2-ml assay solution containing 0.4 or 0.8 mM penicillin G and 100 µM ascorbate was supplemented with 25 µM iron (II) and kept on ice. DAOCS was added (7.4 µM) to 450-µl samples with rapid mixing and the absorbance recorded until no further change was observed. Blank assays devoid of enzyme were utilised as controls. The total variation in absorbance at 260 nm determined from duplicate measurements at each penicillin concentration was 0.119 and 0.157, respectively. At the end of the reaction, 200-µl samples were withdrawn from these assays. 1 M hydrochloric acid (20 µl) was added

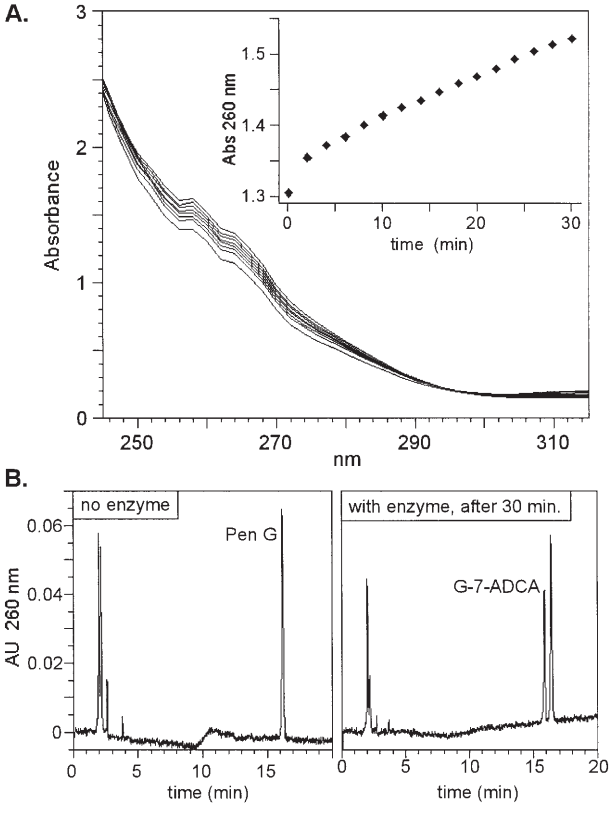

Figure 2. Spectrum recorded on a diode array spectrophotometer at 4-min intervals of a 450-µl sampe containing penicillin G (4 mM), HEPES-NaOH (50 mM), $(NH_4)_2SO_4$ (50 mM), 2-oxoglutarate (4 mM), TCEP (1 mM), iron(II) (25 µM), DAOCS (5 µM), pH 7.5, 30 °C. *Inset* Replat of the absorbance values at 260 nm versus time. (*B*) HPLC chromatograms of the assay mixture with no enzyme present and at the end of the reaction.

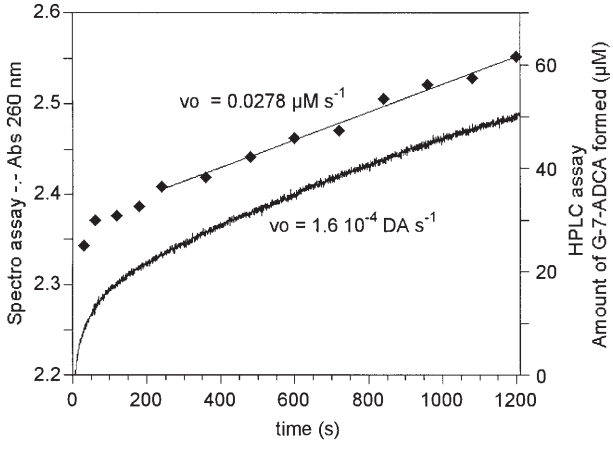

Figure 3. Spectrophotometric assay of DAOCS with penicillin G. A linear regression of the values taken between 4 and 20 min gave a similar rate of 1.5×10^{-4} µmol s^{-1} mg$_E^{-1}$ for both methods. The assay conditions are the same as in figure 2, except that 5 mM penicillin G and 10 mM 2-oxoglutarate were used.

and the samples were incubated for 60 min at 37 °C to destroy any remaining penicillin G [22]. The optimum experimental conditions were verified by HPLC. The solution was neutralised by addition of 100 mM sodium phosphate pH 8.0 (180 µl) and stored on ice. The amount of G-7-ADCA produced in the initial 450-µl assays was determined by the DD-peptidase inactivation assay [23] and found to be 19.5 and 25.6 µM. From these results, a $\Delta\varepsilon_{260}$ value of 6100 $M^{-1}\,cm^{-1}$ was calculated, which is in good agreement with the values of 6120 $M^{-1}\,cm^{-1}$ and 6110 $M^{-1}\,cm^{-1}$ obtained from the difference absorption at 260 nm for solutions (pH 7.0) of ampicillin and cephalexin and of 6-APA and 7-ADCA, respectively.

Importance of iron (II) and 2-oxoglutarate in the DAOCS reaction

Preliminary analyses of the cofactor requirements of recombinant DAOCS by the standard HPLC assay are summarised in table 1. As expected, iron(II) and 2-oxoglutarate are absolutely required for activity. Under the conditions of the spectrophotometric assay, the concentration of iron(II) does not have a considerable influence on the reaction rate (fig. 4). The optimum ferrous iron concentration was 25–50 µM, representing a 5–10 molar excess relative to the enzyme. High concentrations of ferrous iron are undesirable because the hydrated form rapidly oxidises at pH 7.5 to produce insoluble polymeric ferric hydroxyl complexes, which interfere with the assay by clouding the solution and causing a high positive drift in absorbance.

Assays conducted at various 2-oxoglutarate concentrations revealed an interesting kinetic pattern (fig. 5). 2-oxoglutarate concentrations of up to 200 µM resulted in an increase in initial rate. However, substrate inhibition was observed at 2-oxoglutarate concentrations above this level. This phenomenon has been previously observed with another 2-oxoglutarate-dependent oxygenase, thymidine 7-hydroxylase, where an uncompetitive type of substrate inhibition was attributed to a putative dead-end complex [24]. For subsequent assays, an optimal 2-oxoglutarate concentration of 100 µM was chosen.

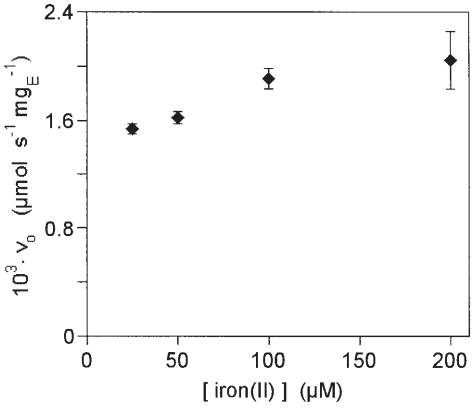

Figure 4. Requirement of DAOCS for iron(II). Spectrophotometric assay, conditions were as in figure 2 except that 2 mM penicillin G and 100 µM 2-oxoglutarate were used.

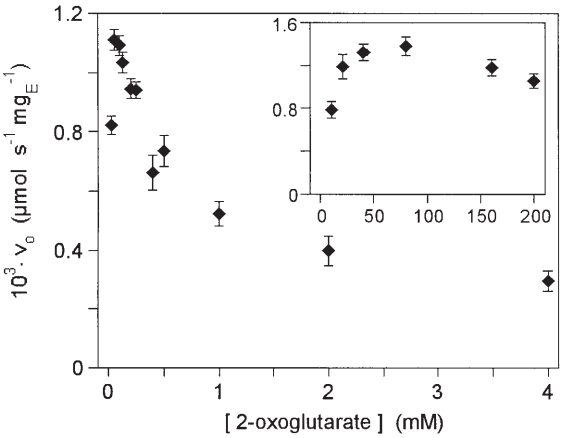

Figure 5. Dependence of DAOCS activity on the 2-oxoglutarate concentration. Spectrophotometric assay, conditions were as in figure 2 except that the substrate was 1 mM penicillin G. *Inset* Assay with lower (µM) concentrations of 2- oxoglutarate.

The effect of dioxygen and carbon dioxide

The dioxygen cosubstrate, and the CO_2 coproduct are dissolved gases and this complicates kinetic analyses since it is difficult to control their concentrations in solution. The solubility of molecular oxygen (21% in air at 0.1 MPa) at 30 °C in water is about 7.6 mg/ml (238 µM), but this solubility decreases as the temperature rises. Moreover, the continuous exchange of gases between the atmosphere and solution makes it difficult to estimate variations in oxygen concentration during the assay. The solubility of CO_2 is difficult to estimate due to the presence of a temperature- and pH-dependent equilibrium between carbonic acid, hydrogen carbonate (bicarbonate) and carbonate, and the formation of carbamates with other basic/nucleophilic components of the reaction. Complexation of iron by these species is likely to be inhibitory, although the magnitude of the effect is difficult to estimate.

Importance of reducing agents

The presence of reducing agents in the assay buffer has generally been observed to enhance the in vitro reaction of 2-oxoglutarate-dependent oxygenases including DAOCS. There are seven cysteinyl residues in DAOCS, but no intramolecular disulphide bridges, and no sulphydryl group involved in the binding of the active-site iron or the substrate [9]. The reducing agents are thought to fulfill three roles: to keep the pool of ferrous iron in a reduced state, to deactivate reactive oxygen species (ROS) (either enzyme bound or in solution) and to reduce potential faulty disulphide bridges. Iron (II) is very susceptible to oxidation by molecular oxygen at neutral pH va-

lues and this oxidation generates ROS via Fenton-type chemistry, which may cause oxidative damage to biomolecules [20, 25]. However, reducing agents can have some detrimental effects since they also replenish the source of the ROS by recycling the oxidised iron [26].

Removal of DTT enhances DAOCS activity, whilst removal of ascorbate decreases it (table 1), two apparently contradictory results. In fact DTT, a commonly used reducing agent for oxygenases, is known to ligate iron in a bidentate manner and this complex favours the rapid metal-catalysed auto-oxidation of the dithiol in solution [19]. This auto-oxidation has two adverse effects on the assay: it consumes oxygen, a coreactant in DAOCS catalysis, and it generates ROS and reactive thyil radicals [26]. TCEP, a soluble non-volatile phosphine, does not interact with iron in solution, is more stable to oxidation in solution and is a better reducing agent at pH values below 7.5 [27, 28]. The replacement of DTT by TCEP, especially in the presence of ascorbate, enhanced considerably enzyme activity (table 1), probably due to the removal of the unfavourable effects of DTT whilst retaining a favourable reducing environment.

Ascorbate is the principal obstacle to the direct observation of DAOCS activity at 260 nm because of its strong absorption at this wavelength (ε_{265}=16,550 $M^{-1}\,cm^{-1}$ at pH lower than or equal to 6.8) [29] and the millimolar concentrations often used in the earlier assays. Ascorbate is apparently devoid of the negative effects of DTT since it activates the enzyme, possibly by keeping the iron in the ferrous state. It has also been proposed to reduce the ferryl species during uncoupled turnover, e.g. in prolyl-4-hydroxylase [14]. However, ascorbate is known to have pro-oxidant activities in the presence of iron(II) [30].

The spectrophotometric and HPLC assays were used to measure the effect of ascorbate on DAOCS activity. The results (fig. 6) indicate that ascorbate has a definite effect on the activity of the enzyme. Without ascorbate, the increase at 260 nm is not linear, and the reaction rate appears to decrease with time. This is probably due to progressive enzyme inactivation as the amount of substrate consumed is too low to produce such a decrease in the reaction rate. The addition of ascorbate has two marked effects: (i) the reaction rate or yield increases to a maximum value at an ascorbate: enzyme ratio of 10–20 (50–100 µM) and then decreases rapidly (fig. 7); (ii) at concentrations greater than 100 µM, ascorbate apparently accelerates enzyme inactivation, and the total change in absorbance (i.e. the yield of the reaction) decreases drastically. As the concentration of ascorbate influences the observed initial rates, assays must be conducted at a defined ascorbate concentration to achieve proper comparison between results. For subsequent assays, an optimal ascorbate concentration of 100 µM was chosen.

Thus, reducing agents in the DAOCS reaction buffer have two opposing effects: stimulation of activity and slow in vitro inactivation of the enzyme. The exact mechanism(s) of inactivation have not been totally elucidated, but are likely due to competing non-catalytic reactions. Others have observed inactivation of DAOCS during catalysis [31] and similar behaviour has been observed with other non-haem oxygenases such as IPNS [A. Dubus, unpublished data] and 1-aminocyclopropane-1-carboxylate oxidase [32]. Cell-free extracts of *Streptomyces* DRS-1 converted ampicillin to cephalexin in the presence of 2-oxoglutarate, suggesting that in vitro production of cephems may be considered. However, some control over

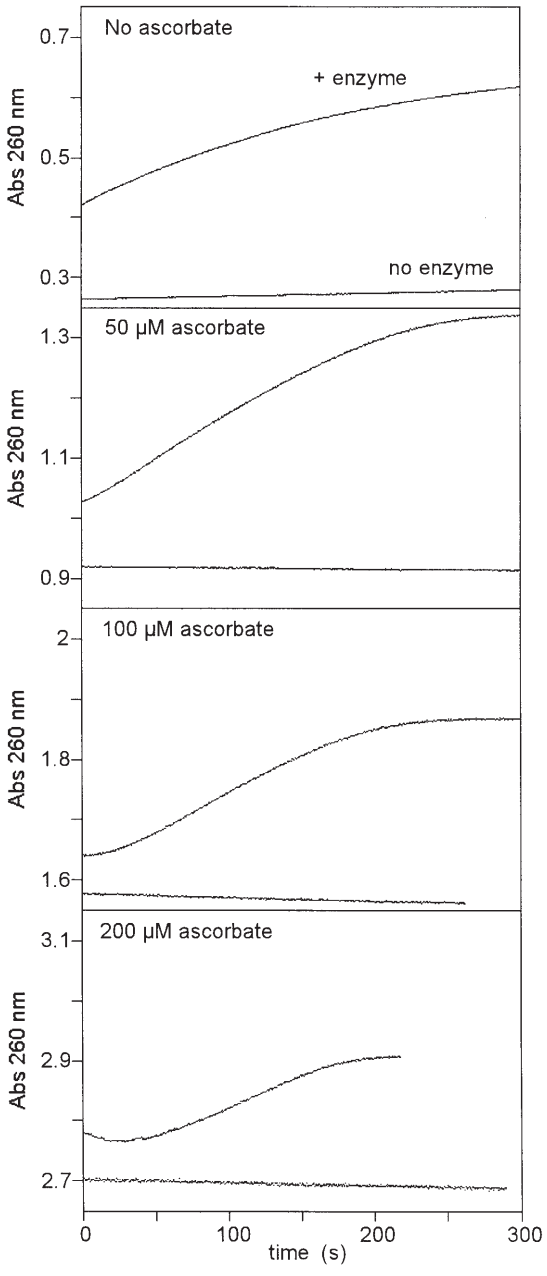

Figure 6. Time course of DAOCS activity at different ascorbate concentrations. Assay conditions as in figure 2 except that 50 µM iron(II), 1 mM penicillin G and 100 µM 2-oxoglutarate were utilised.

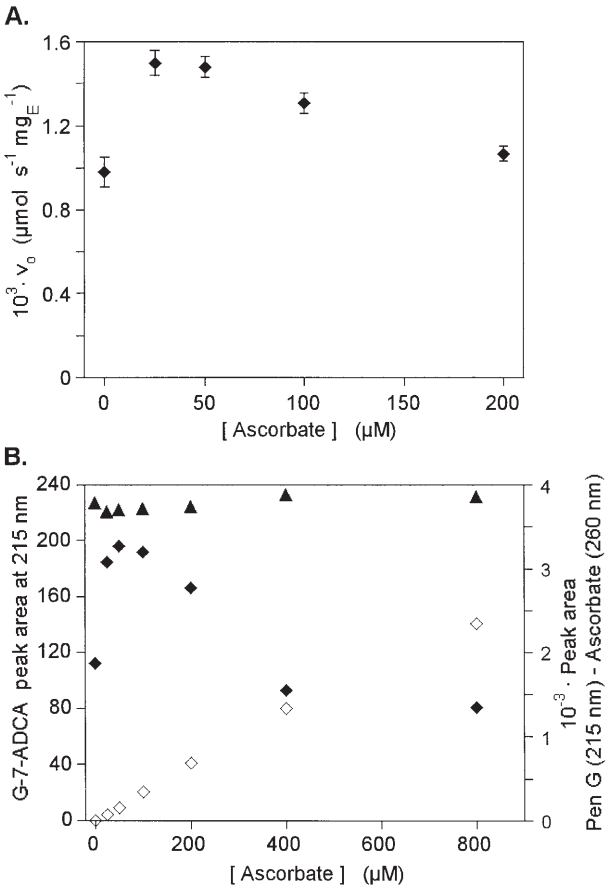

Figure 7. Dependence of DAOCS activity on ascorbate concentrations. (*A*) Spetrophotometric assay; the initial rates were calculated from experiments similar to those in figure 6. (*B*) HPLC assay under the same conditions as in figure 4. The assay volume was 400 µL, 20-min incubation and the reaction was quenched by addition of 5 µl *o*-phenanthroline (20 mM). ▲ penicillin G; ◆ G-7ADCA, ◇ ascorbate.

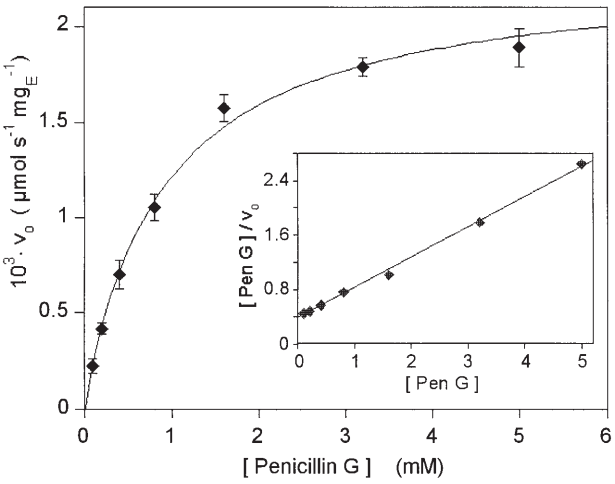

Figure 8. Steady-state rate measurements as a function of penicillin G concentration. Assay conditions: 50 mM HEPES-NaOH, 50 mM $(NH_4)_2SO_4$, 100 µM 2-oxoglutarate, 100 µM ascorbate, 1 mM TCEP, pH 7.5 25 µM iron(II), 5 µM DAOCS, 30 °C. The curve results from non-linear regression fitting of the data to the Henri-Michaelis equation with the following parameters: k_{cat} = 0.079 ± 0.001 s^{-1}, K_m = 0.89 ± 0.02 mM. *Inset* Linear regression on the data rearranged according to the Hanes-Woolf equation.

the competing reactions leading to enzyme inactivation will be necessary for such processes to be viable.

Substrate selectivity of DAOCS

A lag phase was observed when submillimolar concentrations of penicillin substrates were used (data not shown). This lag decreased as the concentration of penicillin was increased and was barely noticeable above 1 mM. The cause of this lag phase remains to be investigated, but may be due to aggregation of DAOCS at high concentrations [3, 8]. The conversion of the enzyme from its aggregated form to the active monomer will be promoted by dilution of the enzyme at the start of the assay, but would also be promoted by the binding of the penicillin substrate.

The steady-state rates exhibited simple saturation kinetics when penicillins were used as the variable substrate [e.g. for penicillin G (fig. 8)]. The derived values are not true kinetic constants since only one substrate concentration was varied in the experiment. The apparent kinetic parameters for penicillin G (at 100 µM 2-oxoglutarate) were K_m = 0.89 ± 0.02 mM; k_{cat} = 0.079 ± 0.001 s^{-1}; specific activity = 0.137 IU/mg.

These values are in reasonably good agreement with those previously reported [3] measured using a HPLC assay: K_m = 2.03 ± 0.12 mM; k_{cat} = 0.062 ± 0.001 s^{-1}; specific activity = 0.108 IU/mg. However, they are surprisingly similar if one considers that the latter values were obtained under some experimental conditions that the present work shows to be unfavourable for DAOCS activity: 50 mM Tris-HCl, pH 7.5, 50 mM ammonium sulphate, 1 mM 2-oxoglutarate, 2 mM DTT, 1 mM ascorbate, 2 mM $FeSO_4$, 2.7 µM DAOCS. These parameters were derived using a discontinuous assay with a 5-min incubation period. The high iron and reducing agent concentration will probably result in non-optimal enzyme activity (and possibly inactivation) during this assay period. Moreover, the non-linear conditions in this assay result in under-estimation of initial rates, with lower k_{cat} and higher K_m values. In contrast, the continuous spectrophotometric assay allows direct observation of the enzymatic reaction and more reliable values of initial rates and derived kinetic parameters can be obtained.

Our analysis also showed that DAOCS successfully catalyses the in vitro conversion of ampicillin to cephalexin with the following apparent kinetic parameters: K_m = 4.86 ± 0.12 mM; k_{cat} = 0.120 ± 0.001 s^{-1}; specific activity = 0.209 IU/mg. For the first time, DAOCS was also shown to expand acetyl-6-APA to its cephem product with the following parameters: K_m = 3.28 ± 0.26 mM; k_{cat} = 0.035 ± 0.001 s^{-1}; specific activity = 0.060 IU/mg.

Several other penicillins and other β-lactam compounds were tested for their ability to be converted by the recombinant enzyme. Low levels of conversion to cephem products were observed when amoxycillin and penicillin V were used as substrates. No products were observed with penicillanate, 6-APA, carbenicillin, temocillin, ticarcillin or benzylpenicilloic acid. This last compound did not behave as an inhibitor in a competition assay using penicillin G as substrate. Thus, it appears that DAOCS can convert a number of penicillins with aromatic sidechains (penicillin G and V, ampicillin and amoxycillin) with varying degrees of efficiency. Short D-2-amino compounds such as leucine, glutamic acid, norleucine, norvaline, 2-aminobutyrate and 2-aminoadipate acid were used in conjunction with 6-APA to investigate whether they could facilitate expansion by occupying the side-chain-binding pocket. No evidence for conversion of 6-APA to 7-ADCA was obtained.

The enzyme was also examined for its ability to hydroxylate the C3-methyl group of the deacetoxycephem product. Cephalexin and 7-ADCA were incubated with the enzyme under the optimised assay conditions and the products were examined by HPLC using deacetyl-cephaloglycin and 7-aminodeacetylcephalosporanic acid as standards. No evidence for the hydroxylation reaction was obtained, consistent with the results of incubations of recombinant DAOCS with penicillin N and DAOC [M. D. Lloyd, unpublished results], penicillin G [3], and adipoyl-6-APA [13].

The conversion of acetyl-6-APA was unexpected but easily rationalised. The expansion of penicillins with sidechains ranging from butyryl to decanoyl-6-APA has been reported with cell-free extracts of *S. clavuligerus* NP1 [33] using a bioassay method. These substrates probably mimic the natural penicillin N substrate with their aliphatic carbon chain bound by the residues which normally bind the methylene groups of the α-aminoadipoyl sidechain. Residue Leu-158, in close proximity to this area, is a good candidate for providing part of the requisite hydrophobic interactions. The absence of conversion observed with penicillanate and 6-APA is even more interesting because while the former is devoid of any sidechain, the latter lacks the acetyl group which suffices to make acetyl-6-APA a substrate. Moreover, the amino group of 6-APA presents a very atypical pKa value of 4.9 [34] and thus would not possess a positive charge under the pH conditions (7.5) used in the experiment. Thus, the failure of 6-APA to be expanded appears to be due to the lack of the amide group connecting the sidechain. Consequently, the acetyl group appears to be the minimal sidechain requirement for a penicillin to bind and be expanded by DAOCS. In connection with this proposal, residue Asn-304 has been suggested to form an electrostatic interaction with the amide bond connecting the penicillin nucleus with the sidechain [3]. The conversion of acetyl-6-APA to acetyl-7-ADCA is of potential industrial interest as a method of production of the 7-ADCA nucleus, perhaps in conjunction with the use of an amidase.

It is difficult to conclusively rationalise the precise sidechain selectivity of DAOCS on a structural basis in the absence of crystal structures of the enzyme complexed with penicillin substrate, as well as complementary studies on the enzyme in solution. Previous studies have suggested [3] that the monomeric form of DAOCS is the active form. In the crystal structures, the C terminus is close to the putative binding pocket(s) for the penicillin sidechain, but in solution the conformation of these residues is likely to be somewhat different [8]. The catalytic expansion of penicillins with aromatic sidechain (penicillin G and ampicillin) by DAOCS suggests that the phenyl rings may be bound by generalised hydrophobic or π-stacking interactions.

Roy et al. [35] have shown direct production of cephalexin (a clinically used cephem) from ampicillin using *Streptomyces* DRS-1. The results presented in this paper have implications for the fermentation of novel antibiotics or those presently made by chemical synthesis, using recombinant host strains [2] with modified enzymes engineered to accept desirable substrates. However, efficient conversion of hydrophobic penicillins is likely to be quite difficult, requiring multiple changes to the binding site of the enzyme whilst at the same time maintaining catalytic competency, including efficient coupling of 2-oxoglutarate oxidation to substrate oxidation.

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