Penicillins and cephalosporins are among the most widely used therapeutic agents. These antibiotics are produced from fermentation-derived materials as their chemical synthesis is not commercially viable. Unconventional steps in their biosynthesis are catalysed by Fe(II)-dependent oxidases/oxigenases; isopenicillin N synthase (IPNS) creates in one step the bicyclic nucleus of penicillins, and deacetoxycephalosporin C synthase (DAOCS) catalyses the expansion of the penicillin nucleus into the nucleus of cephalosporins. Both enzymes use dioxygen-derived ferryl intermediates in catalysis but, in contrast to IPNS, the ferryl form of DAOCS is produced by the oxidative splitting of a co-substrate, 2-oxoglutarate ($\alpha$-ketoglutarate). This route of controlled ferryl formation and reaction is common to many mono- and dioxo-quinonoid enzymes. Here we report the first crystal structure of a 2-oxoacid-dependent oxygenase. High-resolution structures for apo-DAOCS, the enzyme complexed with Fe(II), and with Fe(II) and 2-oxoglutarate, were obtained from merohedrally twinned crystals. Using a model based on these structures, we propose a mechanism for ferryl formation.

In 1945, from sea water near a sewage outflow on Sardinia, Giuseppe Brotsu, a bacteriologist and local politician, isolated a new species of **Cephalosporium** (named *C. acremonium*), which secreted material with strong antibacterial activity. It was later established that this fungus produced several different antibiotics. The compound in the mixture arbitrarily named cephalosporin C (ref. 5) became the first known member of a new family of antibiotics that are now called cephalosporins. Chemical and X-ray studies established that cephalosporin C contained a $\beta$-lactam ring which, in contrast to penicillins, was resistant to hydrolysis by known penicilinase ($\beta$-lactamases). Bacterial resistance to penicillins was new at the time, and the need to combat resistant strains had just begun to emerge.

The biosynthetic pathways of penicillins and cephalosporins share the first two steps (Fig. 1): the condensation of three L-amino acids to give L-$\beta$-(6-$\alpha$-amino-3-oxopropyl)-L-cysteinyl-D-valine (ACV), and the four-electron oxidation of this tripeptide by molecular oxygen catalysed by IPNS to give the first formed penicillin, isopenicillin N (refs 9, 10). The committed step in cephalosporin biosynthesis is the expansion of the five-membered thiazolidine ring of the penicillin nucleus into the six-membered thiazolidine ring of cephalosporin.
dihydrothiazine ring of the cephalosporin nucleus. The reaction requires dioxygen, and is catalysed by a 2-oxo-glutarate-dependent ferrous enzyme. In prokaryotic cephalosporin producers, this enzyme is DAOCS, whereas in eukaryotic cephalosporin producers the reaction is catalysed by a bifunctional enzyme, deacetoxy/deacetylcephalosporin C synthase. The prokaryotic and eukaryotic enzymes are closely related by sequence.

Here we report three high-resolution crystal structures for prokaryotic DAOCS from Streptomyces clavuligerus: the structure of the iron-free apoenzyme, the enzyme complexed with Fe(ii), and the structure of the complex with Fe(ii) and 2-oxo-glutarate. The results imply a mechanism by which the enzyme-Fe(ii) complex reacts with 2-oxo-glutarate and dioxygen to give the reactive ferryl species, a process common to many non-haem oxygenases. Other notable 2-oxoacid-dependent ferrous enzymes are prolyl hydroxylase, which is involved in collagen biosynthesis; gibberellin 3β-hydroxylase, a mutation of which influences stem length in plants and was one of the traits studied by Mendel; 1-aminocyclopentane-1-carboxylate oxidase (the ethylene-forming enzyme); and clavaminic acid synthase, which is involved in the biosynthesis of the β-lactamase inhibitor, clavulanic acid. Within the family of 2-oxoacid-dependent enzymes, DAOCS belongs to a subfamily of the members of which show sequence similarity with IPNS, an enzyme that does not use a 2-oxoacid in catalysis.

The iron-free form of DAOCS crystallizes in space group R3 as a crystallographic trimer. The main chain of the protein folds into a conserved jelly roll with flanking helices. Three surface loops are disordered (Table 1). An intersubunit contact within the crystal

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**Figure 2** Comparison of the structures of deacetoxycephalosporin C synthase (DAOCS) and isopenicillin N synthase (IPNS). DAOCS (a) is a 2-oxoacid-dependent enzyme, whereas IPNS (b) is not. The two sequences show only 19% identity. Major departures from the IPNS structure can be found in the amino-terminal loop, in the region between residues 272 and 292 where two strands and a helix are inserted, in the active site and its environment, and at the C-terminal arm (highlighted in red in DAOCS).
lographic trimer is maintained by residues 308–311 (the carboxy-terminal arm, highlighted in Fig. 2a) in the apoenzyme. This arm penetrates the active site of the neighbouring subunit in a cyclic fashion, so that Lys 310 from one subunit lines the active-site pocket of the neighbouring subunit. Shortening the polypeptide chain from the C terminus by six residues diminishes activity. Together with the intricate network of interactions between symmetry-related subunits, this suggests that the trimeric structure of the apoenzyme found in the crystal might have some functional significance, for example in the protection of the C-terminal arm (rich in lysines and arginines) against proteases in the apo- or resting forms of DAOCS. However, DAOCS dissociates into monomers in the presence of Fe(II) and 2-oxoglutarate.

Figure 3 shows the geometry of the active site of DAOCS. Soaking crystals of the apoenzyme in a solution of Fe(II) results in iron binding (Fig. 3b). This causes only small structural changes; however, in agreement with the observation that the monomer is the catalytically competent form, the protruding C-terminal arm of the oxoglutarate complex. Structures for molecule in given in red.

Figure 3 Structure of the active site in DAOCS. The site is shown in a, the apoenzyme; b, in the enzyme reconstituted with Fe(ii); and c, in the Fe(ii)-2-oxoglutarate complex. Structures for b and c were obtained under anaerobic conditions. The ferrous iron is ligated by Asp 185, His 183, His 243 and three solvent molecules (labelled 1, 2 and 3) in b. Solvent molecules 1 and 2 are replaced by 2-oxoglutarate in c. The co-substrate binds in a bidentate manner, with the 2-oxo-group trans to Asp 185. The position where nitric oxide, a dioxygen analogue, binds in IPNS (ref. 2). The stereochemistry of the co-substrate binding is similar to that proposed from chemical and mutagenesis studies for prolyl 4-hydroxylase7. The only accessible site around the iron in the 2-oxoglutarate complex of DAOCS is located in a hydrophobic pocket lined by residues Ile 192, Val 262, Phe 225, Phe 264 and the methyl group of Thr 190. This site is occupied by a solvent molecule in the structure (Fig. 3c), and we propose that this site is the binding site for dioxygen in DAOCS and related 2-oxoacid-dependent ferrous enzymes. The bound 2-oxoglutarate is flanked by the side chains of Met 180 and Val 262; the sulphur atom of Met 180 is in van der Waals contact with the C2 carbon atom of 2-oxoglutarate, whereas Val 262 maintains contact with the C3 carbon atom from the opposite side. The 5-carboxylate of the co-substrate forms a salt bridge with Arg 258 and is hydrogen bonded to Ser 260 (Fig. 3c). These residues are conserved within the DAOCS subfamily of enzymes.

Despite the conserved structural motifs in IPNS and DAOCS (Fig. 2), there is a significant difference in their use of dioxygen (Fig. 1). Whereas IPNS uses the full four-electron reduction of
molecular oxygen to water to create the strained ring structure of the penicillin core, DAOCS, like other 2-oxoglutarate-dependent ferrous enzymes, performs only a two-electron oxidation of its substrate (penicillin N in this case) for each molecule of dioxygen consumed. The ferryl form of IPNS is created by splitting dioxygen in a reaction coupled to the oxidative decarboxylation of the co-substrate, 2-oxoglutarate (Fig. 4). After these initial steps, both enzymes use their ferryl state to complete the remaining parts of their reactions. An important role of the protein in these reactions is to provide a shelter to reactive intermediates, and a possible function of the C-terminal arm of DAOCS and IPNS is to isolate reactive intermediates during catalysis.

The high reactivity of iron–dioxoiron and iron–oxyintermediates can give rise to unwanted side reactions and may lead to the destruction of such enzymes. The control of the reactivity of intermediates is therefore an important aspect of catalysis by these enzymes. Our structural results give indications of how control may be exercised in 2-oxoacid-dependent enzymes. In the ferrous form of the enzyme (Figs 3b and 4), the iron is ligated by three protein ligands and three solvent molecules. Binding of the co-substrate introduces a negative charge into the coordination sphere of the iron, thereby activating it for productive dioxygen binding. The presence of the co-substrate thus gives the signal for the start of the reaction. Oxidative decarboxylation of 2-oxoglutarate maintains the negative charge around the iron, but the position of the carboxylate ligand shifts from opposite His 243 to opposite Asp 185 (Fig. 4, last step) as 2-oxoglutarate is converted to succinate. An electron-donating carboxylate ligand has a stabilizing effect on the ferryl species, whereas the shift in the position of the carboxylate during the reaction may have a role in directing the ferryl in later steps. The other reaction product, carbon dioxide, could either leave the iron or react with water in situ to give bicarbonate (or carbamate, through a reaction with an amine). This reaction could introduce a further negative charge into the environment of the ferryl. We note that some 2-oxoacid-dependent ferrous enzymes are activated by carbon dioxide. The scheme outlined above could ensure that the reactivity of the ferrous iron is first enhanced towards dioxygen by the negative charge of the co-substrate, while the reactivity of the ferryl intermediate is tuned down by the charge of the products. The ferryl species may thus be stored safely within the protein to await a reaction with the ‘main’ substrate (penicillin N in this case). Controlled changes in the coordination sphere of the ferryl after substrate binding could modify its reactivity and ensure selective oxidation. A detailed understanding of how these processes occur may assist in the design of non-protein-based oxidative catalysts using a ferryl intermediate.

Many members of the family of mononuclear ferrous enzymes catalyse reactions that are synthetically impossible at present. Our results, together with sequence data, show that members of the DAOCS subfamily have similar three-dimensional structures (Fig. 2). The diversity in catalytic specificities of these enzymes on very similar structural platforms suggests that altering substrate and product specificities should be possible in the laboratory. This could be used for the synthesis of new antibiotics to combat the increasing threat of resistant microorganisms.

Methods

Crystallization and X-ray data collection. The DNA fragment that contained the DAOCS gene from S. clavuligerus was subcloned into the PET11a vector and overproduced in Escherichia coli (M.D.L. et al., in preparation). The purified protein was crystallized from 1.75 M ammonium sulphate, 5 mM 2-oxoglutarate and 0.1 M HEPES, pH 7.0–7.5. Crystals belong to space group P212121 with unit-cell dimensions of a = b = 106.4 Å, c = 71.2 Å, α = β = 90°, γ = 120° (in the hexagonal setting), and one monomer in the asymmetric unit. They diffract X-rays beyond a resolution of 1.3 Å. All crystals were merohedral twins, with twinning ratios between 0.06 and 0.45. Data were collected at 90 K on station BM4, ESRF, Grenoble, and on beamline I-711, MAX-Lab, Lund, Sweden (Table 1).

Structure determination. The structure of the enzyme in its apoform was determined by multiple isomorphous replacement (Table 1) and by a space group general dêtwinning strategy that was based on an extension and modification of ref. 20. Details of this procedure will be published elsewhere (A.C.T.v.S. et al., in preparation). Data were processed and scaled with HKL21 and CCP4 suites.22 After detwinning, an initial phase set was calculated that could extend to a resolution of 1.3 Å (Table 1). The procedure included solvent flipping by SOLOMON23 and the subsequent construction of a free-model of DAOCS as described for the WARP procedure.24 This model was refined by ARB25 and REFMAC26. At the end of this procedure, Rmetal (defined as $\Sigma|F_{o} - F_{c}| / |F_{o}| \times 100$) dropped from 50.7% for the initial free-model to 18.5% for the final free-model. Subsequent automatic chain tracing (A.P. and V.S. Lamzin, unpublished data) allowed 262 residues out of the 311 residues of the DAOCS molecule to be built in a single step. Electron-density maps were inspected and further interpreted with the program O27, and the new models were refined with XPLOR28 and SHELX97 (ref. 29). Quality checks with programmes from the Uppsala Software Factory (http://alpha2.bmc.uu.se/ufsd/) showed excellent geometry and a good agreement between models and data.

Structure determination of DAOCS complexes with Fe(n) and Fe(n)-2-oxoglutarate. Crystals of the apoenzyme were soaked at 15 °C for 30–60 min in solutions containing 50 mM Fe(n) or 20 mM Fe(n) and 100 mM 2-oxoglutarate in an artificial mother liquor solution. Anaerobically (0.1 p.p.m. oxygen) was maintained throughout, and crystals were flash frozen inside the anaerobic box. Initial models were obtained by rigid-body refinement using the structure of the apoenzyme (except for residues 307–311), and were then further refined with REFMAC26 and SHELX97 (ref. 29) (Table 1).

Figures. Figures were drawn with a modified version of MOLSCRIPT29.
Millennial-scale climate instability during the early Pleistocene epoch


Figures 2 (below) and 3 (right) of this Letter contained some typographical errors. The corrected figures are reproduced here.