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Review

Towards new β-lactam antibiotics

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Abstract. Antibiotics have had a profound impact on human health and belong to one of the largest-selling classes of drugs worldwide. Introduced into industrial production only some half century ago, these miracle drugs have been the main contributors to the recent increase in human life expectancy. However, the accelerated emergence of bacteria that are resistant to multiple antibiotic types now appears as the most serious threat to continuing success in the treatment of infectious diseases. Recent advances in our knowledge of the structures and mechanisms of enzymes in the biosynthetic pathways of penicillins and cephalosporins, amongst the most important antibiotics in current use, have identified a common structural core together with common iron- and cosubstrate-binding motifs. The diversity in the catalytic specificities of these oxygenases using very similar structural platforms suggests that altering the substrate and product specificities of these enzymes should be possible in the laboratory. This opens up new avenues for industrial production and medical utilisation.

Key words. β-Lactam antibiotics; resistance; mononuclear ferrous oxygenases; new antibiotics.

Introduction

At the beginning of the twentieth century, infectious diseases such as pneumonia, tuberculosis and bacterial meningitis were among the leading causes of death in the world. A turning point came in the 1940s with the introduction of antibiotics such as penicillin into clinical use, resulting in a sharp decline in death caused by infections. Victory was declared against infectious diseases – as we now know, a little too soon. Perhaps as a result of this over-optimism, as recently as the 1980s, pharmaceutical companies began reducing development of new antibiotic drugs or began redirecting efforts away from antibiotics. However, the huge consumption of antibiotics following their discovery has lead to the concomitant emergence of bacterial resistance to antimicrobial agents. Although resistance has attracted increased interest in the past decade by virtue of its accelerated spread, the phenomenon is not new. For example, the main cause of bacterial resistance to β-lactams, the family of antibiotics that includes penicillin, is the presence of enzymes that hydrolyse the β-lactam moiety. The existence of such penicillinases or β-lactamases was predicted by scientists in the field even before penicillin was introduced into clinical use, and the enzymes appeared only a few years after the drug became generally available. However, the speed at which resistance develops could hardly have been foreseen, and it is now threatening to leave drug company chemists helplessly behind. Perhaps the most frightening aspect of resistance is the ability of bacteria to pass genes conferring resistance (often residing on plasmids) to other bacteria and to other species [1, 2]. Bacterial strains causing fatal hospital infections (methicillin-resistant *Staphylococcus aureus*, MRSA) are now immune to all but one existing antibiotic (vancomycin) and there are recent reports that

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this final barrier is now about to fall [3, 4]. The emergence of bacterial strains resistant to multiple drugs is threatening to put us back to pre-penicillin days and is now rightfully recognised as a medical disaster [see ref. 4 and references therein].

What to do? Apart from the obvious, such as more prudent use of antibiotics and a ban on the use of large quantities of antibiotics in agriculture [5, 6], there is also a need for cheaper and faster routes to new antibiotics and to those already in use. Most antibiotics in current use are derived from a surprisingly small number of basic compounds and very few new antibiotics are presently approved for clinical use [7]. The need to expand the repertoire and to produce combinations not yet encountered by bacteria is desperate. In addition, cheaper and more environmentally friendly routes to already existing compounds are required to pave the way for industrial production. The way ahead may depend on the class of drug concerned, but one idea is to study the micro-organisms that produce antibiotics and to use their machinery for novel production. This article reviews recent studies on one group of enzymes, the mononuclear non-haem ferrous oxygenases, involved in the biosynthesis of the most commonly used antibiotics, the β-lactam antibiotics. Further studies in this field are anticipated to lead to the development of new antibiotics, e.g. by exploiting and modifying the natural pathways for β-lactam biosynthesis.

β-Lactam antibiotics

β-Lactams are the most well known and most widely used of all naturally occurring antibiotics and are secreted by (among others) moulds of the genus *Penicillium*. The name penicillin was coined by Alexander Fleming, who in 1928 observed bacteriolysis in a nutrient broth at St Mary's Hospital in London [for accounts of the early history of penicillin, see articles by Abraham, refs 8–10]. Although the discovery was attributed to Fleming, there are indications that the same observation was made earlier by others (see e.g. Teiling [11]). However, several years passed until the clinical value of the substance was recognised and demonstrated. Due in particular to the work of scientists at Oxford, including Florey, Chain and Abraham, the drug was isolated and entered into clinical trials by 1941. It was used successfully to treat battle casualties at the end of World War II and was soon hailed as the true medical miracle of the century.

Penicillin is chemically characterised by the presence of a four-membered heterocyclic ring, the β-lactam ring, fused to a five-membered thiazolidine ring, as was demonstrated by Dorothy Hodgkin in her pioneering X-ray crystallographic work [12]. Penicillin exerts its antibiotic action on DD-transpeptidases (penicillin-binding proteins) which are membrane-bound enzymes involved in the biosynthesis of the bacterial cell wall [13]. β-Lactam drugs act as pseudosubstrates and acylate the active sites of these enzymes, thereby inhibiting their action. Following the discovery of penicillin, a range of naturally occurring antibiotics were discovered, often by screening for the production of β-lactam compounds by micro-organisms isolated from e.g. soil samples and waste water. Cephalosporins, another class of compounds containing the β-lactam ring fused to a six-membered dihydrothiazine ring, were discovered in organisms isolated from a sewer in Sardinia [14], subsequently isolated and further analysed [15]. Due to their effectiveness and lack of toxicity, β-lactam compounds are amongst the most widely used and successful antibiotics in history.

Resistance

Bacteria have evolved several strategies to avoid the lethal action of antibiotics directed against them, processes eventually leading to resistance [6]. Destruction is one alternative to escape the drugs, others are efflux systems to pump the drug out of the cell, or camouflaging the target so it is not recognised by the drug. Abraham and Chain [16] were able to detect resistance to penicillin in some strains of *Escherichia coli* as early as 1940. The bacteria were able to synthesise β-lactamases which hydrolyse the chemical warhead of the antibiotic, the β-lactam ring. This is the most common mode of resistance to β-lactam antibiotics and several hundred β-lactamases have now been identified that threaten the continued use of all β-lactam antibiotics. In fact, one of the reasons for the success of cephalosporin antibiotics is their somewhat lower susceptibility to certain β-lactamases. Based primarily on sequence, β-lactamases have been classified into four groups: A, B, C and D [13]. Of these, the class B β-lactamases are zinc-dependent enzymes and the remaining three are serine hydrolases [17]. There is ample structural and mechanistic information on the class A, B and C enzymes in particular [reviewed in ref. 13], whereas a three-dimensional structure of the class D enzymes has been reported only recently [18]. At present, the most clinically important are the class A and C enzymes [19], both active-site serine enzymes. An alternative classification of β-lactamases based on substrate profiles and inhibition characteristics puts the emphasis on clinical aspects [reviewed in ref. 20].

There are basically two ways that have been employed to avoid resistance due to β-lactamases. One is to tinker with the structure of the antibiotic, to produce one that is not recognized by the β-lactamase, until a new mutation allows the modified enzyme to efficiently recognise and hydrolyse the new compound [21, 22]. The other is to neutralise the action of the β-lactamase. Clavulanic acid,

a naturally occurring β-lactam, has little antibacterial activity in itself but is a suicide substrate for the lactamase and acts as a 'guardian angel' to coadministered β-lactam antibiotics [6, 23]. The drawback is that clavulanate effectively inhibits only class A β-lactamases. There is a clear need for broad-spectrum inhibitors, but such compounds are currently too expensive. Thus, new and inexpensive routes must be found to new antibiotics and inhibitors that could enable pharmaceutical companies to develop a whole range of variations on the theme in a cost-effective manner. There is a particular demand for drugs whose activity is highly selective, and thereby less likely to contribute to the general rising level of antibiotic resistance of unrelated pathogens. To achieve this, insight into the synthetic machinery of the microbes is required.

Biosynthesis

The biosynthetic routes to penicillin and cephalosporin antibiotics are outlined in figure 1. Biosynthesis of penicillins starts with the condensation of three L-amino acids with configurational inversion of one of the centres to form the unusual linear tripeptide α-aminoadipoyl-L-cysteinyl-D-valine (ACV). This is non-ribosomal biosynthesis catalysed by a single multifunctional enzyme, ACV synthase. In the next step, the enzyme isopenicillin N synthase (IPNS) catalyses in one single step the bicyclisation of ACV to form isopenicillin N, the precursor of all penicillin-type antibiotics [24, 25].

In those micro-organisms that produce cephalosporins, there are additional steps to consider (fig. 1) [24]. First, an epimerisation reaction produces penicillin N (Pen N). The next reaction is the expansion of the thiazolodine ring of Pen N to form the six-membered dihydrothiazine ring characteristic of all cephalosporin antibiotics. In the eukaryote *Cephalosporium acremonium*, the ring expansion to deacetoxycephalosporin C (DAOC) and a subsequent hydroxylation reaction to form deacetylcephalosporin C (DAC) is catalysed by a bifunctional enzyme deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) [26]. In prokaryotic cephalosporin producers, e.g. *Streptomyces clavuligerus*, the ring expansion and hydroxylation reactions are catalysed by separate enzymes [27], DAOCS and DACS (fig. 1), although the substrate specificity of the recombinant DAOCS enzyme is apparently not absolute [28]. The ring expansion reaction depends on Fe(II), uses oxygen as a cosubstrate and couples the two-electron oxidation of its prime penicillin substrate to oxidative conversion of a 2-oxoacid cosubstrate, most commonly 2-oxoglutarate (2-OG), to succinate and CO_2. The reaction with 2-OG and dioxygen produces a reactive iron intermediate which attacks the penicillin substrate. This key intermediate is generally believed to be a high-valent iron (ferryl) intermediate of formal oxidation state $\geq 4+$, or a peroxo intermediate. Such a ferryl state has never been captured experimentally in these enzymes although there is circumstantial evidence from model complexes [29, 30].

The reaction catalysed by IPNS, which uses the full four-electron oxidising power of dioxygen, is unique and has no precedent in synthetic organic chemistry. In contrast, attempts to chemically synthesise the rather unstable penicillin molecule were unsuccessful for a long time and not until 1957 was the total synthesis accomplished [31, 32]. However, compared to the single-step biosynthetic route, the multi-step chemical synthesis is not economically viable. Therefore, to date, all medicinally used penicillin and cephalosporin antibiotics are produced from natural fermented products or by semisynthesis starting from a fermentation intermediate and involving complex and expensive modifications of the fermented products.

The discovery of methods to produce the nucleus of the penicillin molecule, 6-aminopenicillanic acid (6-APA), and that of the cephalosporin nucleus, 7-aminocephalosporanic acid (7-ACA) [33], enabled the production on a large scale of semisynthetic penicillins and cephalosporins [34, 35]. However, the complex modifications necessary to convert the fermented intermediates to the final antibiotic product render production both costly and environmentally unfriendly. Considering the extraordinary efforts and costs involved in bringing a new drug onto the market, the need for more direct routes to new β-lactam antibiotics is obvious.

A common structural theme

IPNS and DAOCS are members of an extended family of mononuclear ferrous oxygenases that use Fe(II) in catalysis. Due to their sensitivity and the relative difficulty expressing and purifying large quantities of these enzymes, information on members of this family was hard to obtain and until relatively recently no three-dimensional structures were known. This is in contrast to the haem-containing enzymes, for which a wealth of information on structures and mechanisms of action has been gathered [36]. The structure of IPNS from *Aspergillus nidulans* was determined in 1995 and was the first from this family of enzymes [37, 38]. The structure (fig. 2a) consists of a core of β-strands forming a distorted jelly-roll β-barrel surrounded by flanking α-helices. The active site is buried within the jelly-roll motif with ligands to the iron provided by two histidines, one aspartate, two water molecules and a glutamine residue [37]. The latter residue, Gln330, is the penultimate residue in *A. nidulans* and is displaced on substrate binding. The use of anaerobic crystallisation techniques has allowed the crystallisation of a complex, IPNS-Fe(II)-ACV [39] into which a dioxy-

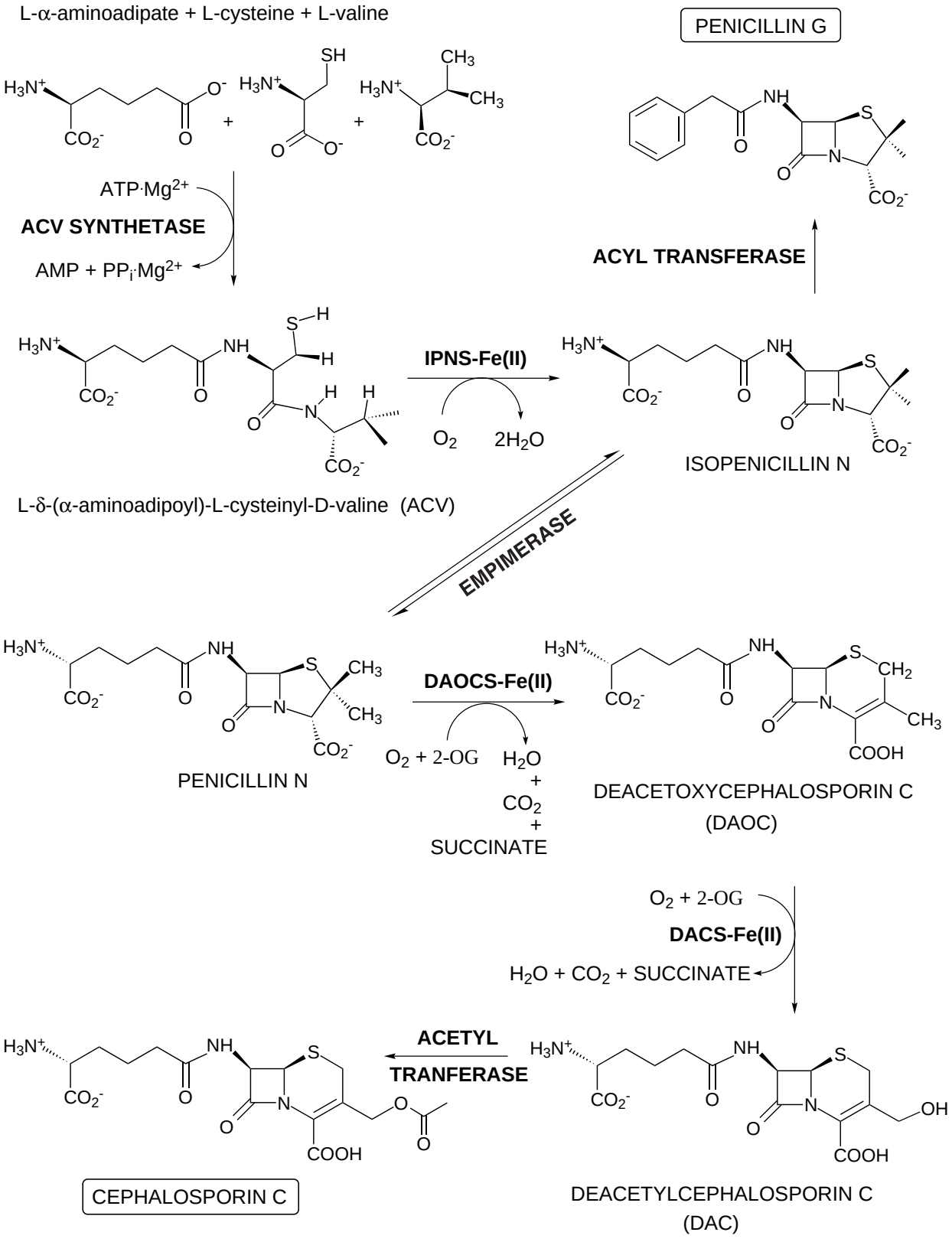

Figure 1. Biosynthetic routes to penicillin and cephalosporin antibiotics.

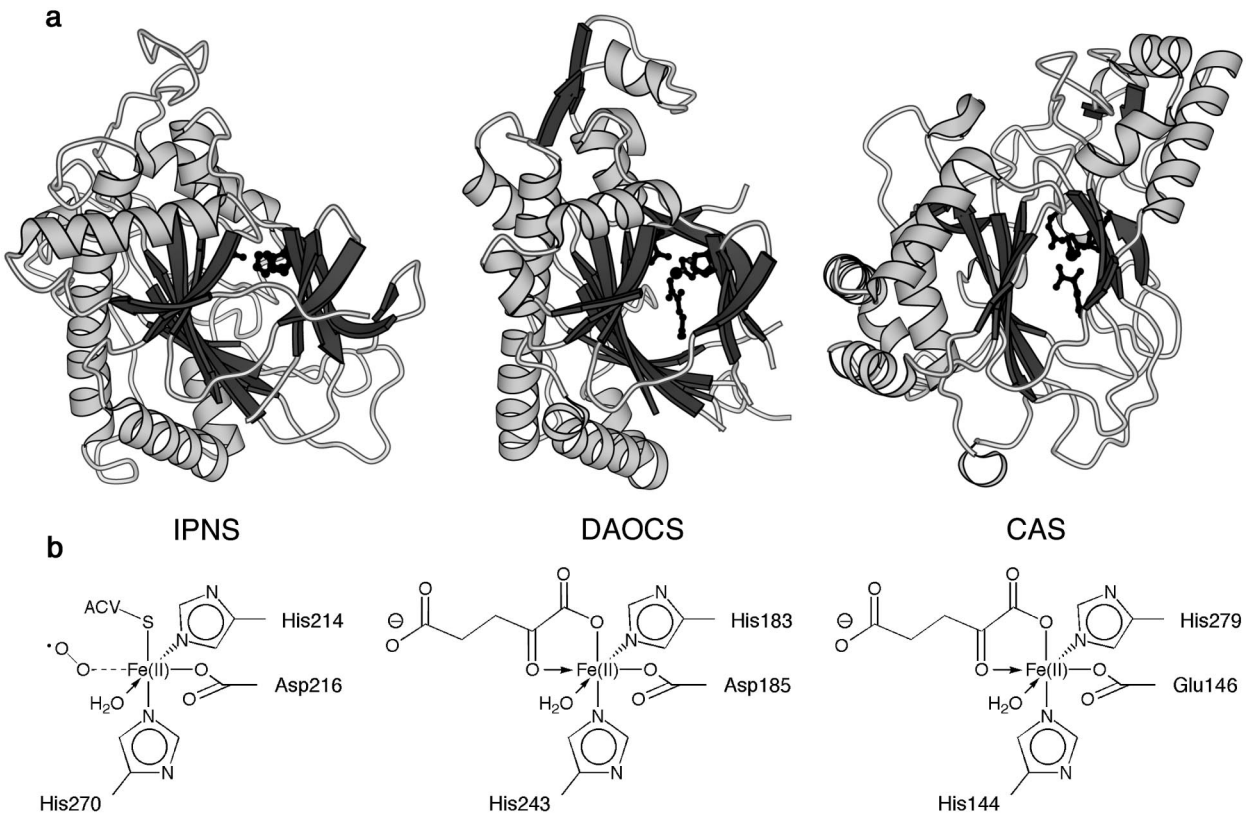

Figure 2. Structures of three enzymes involved in the biosynthesis of β-lactam compounds: IPNS, DAOCS, and CAS. (*a*) Overall fold. Coordinates from the Protein Data Bank [1IPS.pdb (IPNS), 1RXG.pdb (DAOCS), 1DS1.pdb (CAS)] were superimposed with their respective active-site β barrel motifs using O [77]. Prepared using MOLSCRIPT [78]. (*b*) Coordination of the active-site iron in the respective enzymes, showing the conserved (co)substrate-binding mode.

gen analogue, nitric oxide (NO), was diffused to yield the quaternary complex IPNS-Fe(II)-ACV-NO [40]. The structure shows that ACV displaces one water molecule and Gln330 around the iron (fig. 2b), thus allowing the C-terminal helix to extend and cover the active site. ACV coordinates with its thiol to the iron, and thereby changes the metal coordination geometry from octahedral to square pyramidal [25, 40]. In the quaternary complex, NO binds to the vacant site opposite Asp216 in a non-linear manner [40]. It is striking that no residues capable of acid-base catalysis are found in the vicinity of the active site, implying that the iron-dioxygen-derived species is totally responsible for this unique catalysis, which includes removal of four hydrogens from ACV. Burzlaff et al. [41] were able to follow the cyclisation reaction of the ternary IPNS-Fe(II)-ACV crystals by exposure to high pressures of oxygen. The structure of the resulting IPNS-Fe(II)-IPN shows a rotation of the substrate during catalysis. Use of a modified substrate in place of ACV further allowed the observation of a monocyclic β-lactam species bound in the active site with the presence of a metal-bound sulphoxide indicative of the formation of an Fe(IV)-oxo species during catalysis.

The 2-OG-dependent oxygenases form a large and diverse subgroup of the mononuclear ferrous oxygenases and include enzymes from microbes, mammals, plants and viruses [42]. The first three-dimensional structure from this group, that of the ring-expanding enzyme DAOCS from *S. clavuligerus,* was determined in 1998 [43].

The overall three-dimensional structure of DAOCS (fig. 2a) is remarkably similar to that of IPNS considering the relatively low level of protein sequence identity of 19% between the two enzymes. Structural differences are mainly in loop regions, peripheral helices, and in the carboxy-terminal arm. At the level of individual residues, despite a similar structural framework, the character of the active sites in IPNS and DAOCS is strikingly different, reflecting differences in the chemistry of the substrates. By using anaerobic techniques, crystals of the apoenzyme were soaked with solutions containing Fe(II) or Fe(II) and 2-OG [43]. In the binary complex, the iron is ligated by two histidines, an aspartate and three water molecules in an almost perfect octahedral geometry. The presence of the metal ion causes minimal structural changes in the active-site region. In the ternary DAOCS-

Fe(II)-2-OG complex (fig. 2b), the cosubstrate binds to the iron in a bidentate manner, replacing two solvent molecules. The 1-carboxylate group of 2-OG binds trans to His243, and the 2-carbonyl group binds trans to Asp185. 2-OG makes a number of contacts with residues that are conserved within the 2-OG-dependent oxygenases. The stereochemistry of cosubstrate binding leaves one site around the iron (the site opposite His183) accessible for binding. This pocket is lined by hydrophobic residues. This arrangement suggests a mechanism for cosubstrate oxidation and ferryl formation in DAOCS [43, 44]. There is presently no structural information on how the 'prime' substrate binds in the active site.

Recently, the X-ray structure of another 2-OG-dependent enzyme, clavaminate synthase (CAS), a key catalyst in the biosynthetic pathway leading to clavulanic acid was reported [45]. This pathway, which starts from the precursors L-arginine and a C3 carbohydrate, constitutes a completely different mechanism for β-lactam biosynthesis [23, 46, 47] compared to the previously mentioned penicillin and cephalosporin pathway. CAS catalyses three distinct non-succesive oxidation reactions in the clavulanic acid pathway, including a hydroxylation, an oxidative cyclisation, and a desaturation reaction (fig. 3).

The crystal structure of CAS was determined in complex with Fe(II), 2-OG and the substrates N-α-acetyl-L-arginine (NAA) and proclavaminic acid [45]. Whereas the three-dimensional structural similarity of IPNS and DAOCS could be predicted on the basis of a certain (low) protein sequence similarity, protein sequence alignment revealed little similarity of CAS to IPNS/DAOCS [48]. Nevertheless, the fold of the core β-barrel is remarkably similar to those of IPNS and DAOCS (fig. 2a), as is the iron ligation in the active site (fig. 2b). In the CAS-Fe(II) binary complex, the iron is ligated by two histidines, one glutamate and three water molecules. In the CAS-Fe(II)-2-OG ternary complex, the cosubstrate binds in a bidentate manner to the iron, replacing two water molecules (fig. 2b) [45]. This is analogous to the binding of 2-OG in the DAOCS-Fe(II)-2-OG structure [43]. However, in CAS, the 2-oxo group of 2-OG is ligated trans to Glu146, and the 1-carboxylate binds trans to His144 with a water molecule binding opposite to His279, swapping the positions of the metal-ligating water molecule and the 2-oxo acid groups relative to the arrangement of these ligands in DAOCS. In the substrate structures, both the β-lactam of proclavaminic acid and NAA are located in the active site in a similar manner. The substrate-binding pocket is large, reflecting the need to accommodate three different substrates and to catalyse three different oxidative reactions [45]. The structure with proclavaminate indicates the possibility of a switch from octahedral hexa-coordination to penta-coordination during catalysis as suggested by spectroscopic studies [49, 50].

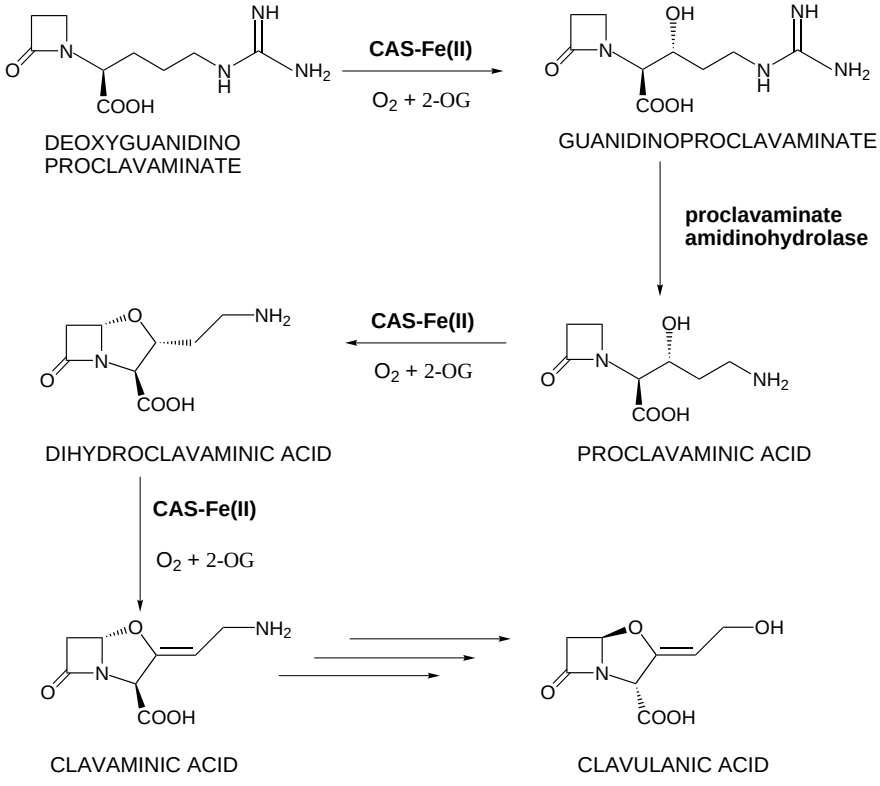

Figure 3. Biosynthetic pathway leading to clavulanic acid and other clavams.

Differences and similarities reflect catalytic diversity on a common structural scaffold

The β-lactam biosynthetic enzymes for which three-dimensional structures are now available, IPNS, DAOCS and CAS, belong to an extended family of non-haem ferrous oxygenases. In addition to their pivotal role in the β-lactam biosynthetic pathways, enzymes in this diverse family assist in a broad range of reactions in the biosynthesis of e.g. collagen [51], L-carnitine [52], plant hormones and other plant metabolites [53–55]. These enzymes catalyse diverse reactions such as oxidative cyclisations, desaturations and hydroxylations, involving a wide range of substrates and products differing both in size and charge. This indicates the extent of variation in the nature of the active sites provided by these enzymes on a common structural core, the jelly-roll β-barrel.

The presence of the jelly-roll motif in CAS [45] was totally unpredicted since there was little overall similarity in protein sequence to the DAOCS/IPNS subfamily. Clavulanic acid, a potent β-lactamase inhibitor, bears strong three-dimensional structural similarity to isopenicillin N (the product of IPNS) although their biochemical activity and synthesis differ fundamentally. That the structural similarity is extended to the biosynthetic machinery by the fact that CAS, the major biosynthetic enzyme of the pathway leading to clavulanic acid, uses the same structural scaffold as IPNS and DAOCS brings an intriguing twist to the story.

The non-haem ferrous oxygenases catalyse some of the most 'difficult' reactions in nature, such as the oxidation of an unactivated aliphatic C-H bond. The thermodynamic cost of such a reaction is paid by the reduction of a molecule of dioxygen. In the non-haem ferrous oxygenases, an active oxidising species, most likely a ferryl Fe(IV)=O state, is generated in the reaction of dioxygen with Fe(II). The role of the enzyme in catalysis is to control the reactivity of the highly reactive intermediate, a process that has been referred to as negative catalysis [56]. The position of the O_2 binding site is different in IPNS, DAOCS and CAS (fig. 2b). This feature, which at first seems puzzling, could allow the reactivity of the oxygen intermediate to be modulated by the electronic state of the liganding sphere and increase the range of reactions that can be catalysed.

The key to the versatility offered by these enzymes is the use of a mononuclear iron in catalysis – as opposed to a haem iron (fig. 4). Haem enzymes feature an iron porphyrin cofactor that is ligated to an amino acid residue on the protein. Such a ligand environment leaves only one site available for binding of an exogenous ligand – substrate or cofactor. In contrast, mononuclear non-haem iron enzymes offer a more flexible metal coordination environment, which allows for the binding of up to three ligands at the same time. From the knowledge gathered from

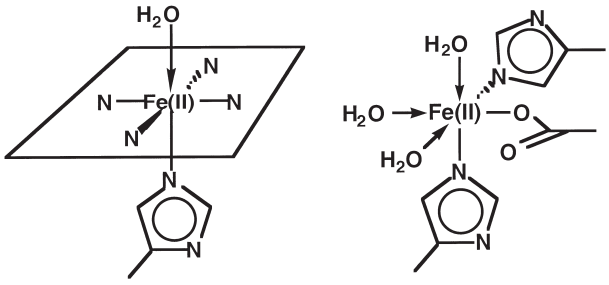

Figure 4. Differences in coordination chemistry around the iron in haem enzymes and non-haem ferrous oxygenases.

available structural evidence, some common features of the active sites of the mononuclear non-haem ferrous enzymes can be deduced. The iron is ligated by two histidines and one carboxylate amino acid occupying one face of an octahedron. This so called 2-His-1-carboxylate facial triad [57, 58] leaves three remaining sites on the opposite side of the iron for binding additional ligands, endogenous or exogenous, such as substrates or cofactors, and has been suggested to be a common structural motif for the iron active site in these enzymes.

New β-lactam antibiotics – the outlook

Structural studies on non-haem ferrous oxygenases have provided new insights into the mechanism of these versatile biocatalysts and provide a platform for protein engineering. The diversity of the catalytic selectivities of the non-haem ferrous enzymes, coupled with the discovery that they utilise very similar structural platforms, suggests that altering their substrate and product selectivities will be possible in the laboratory. By employing combinatorial approaches, rules for engineering substrate and product specificities using the common jelly-roll structure as a scaffold could be established. This may enable the clean synthesis of existing and new antibiotics and β-lactamase inhibitors. Figure 5 depicts the core structures of some of the targets. In particular, it could facilitate the production of new (unsaturated) bicyclic templates and synthetically inaccessible β-lactam nuclei, but it could also offer new, cheaper and cleaner routes to existing β-lactam templates.

Several directions may be envisaged. Fine-tuning of the synthetic machinery is anticipated to enable the introduction of alternative side-chains. The redesign of IPNS to accept modified tripeptides as substrates could offer new routes to penems and other bicyclic β-lactam nuclei. The natural substrate of DAOCS, Penicillin N, has a polar side-chain. One approach could therefore be to engineer the selectivity of DAOCS to accept unnatural substrates with different (hydrophobic) side-chains. Products with hydrophobic side-chains have the advantages

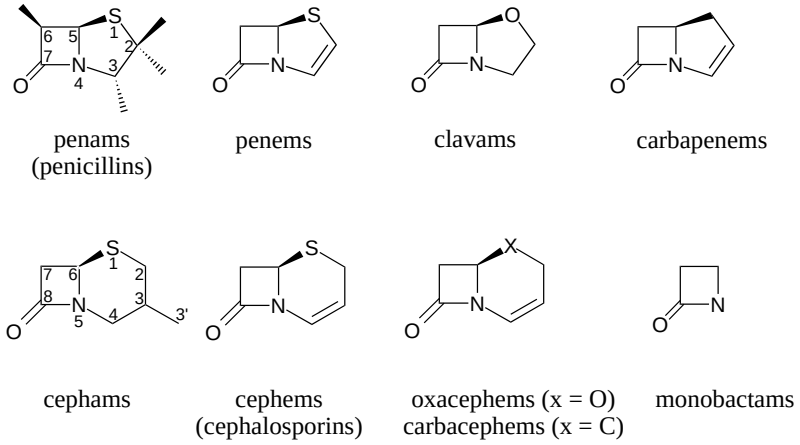

Figure 5. Core structures of major classes of β-lactam compounds.

of being readily extractable compared to the corresponding compounds with polar side-chains. Such conversions have been demonstrated with the wild-type enzyme under certain conditions [59–62], but a redesign of DAOCS could allow for a more efficient conversion. Such a transformation would allow the direct fermentation of penicillins and cephalosporins of choice without the need for synthetic modifications, with a simultaneous cut in production costs and toxic by-products as an added benefit. Another possibility would be to alter the selectivity of DAOCS to provide new biosynthetic routes to intermediates for the production of commercial cephems. Introduction of a methoxyl (-OCH_3) group as a substituent at carbon 7 of the cephem nucleus converts cephalosporins into the β-lactamase-resistant cephamycins [63, 64; reviewed in ref. 65]. Cephamycin C is a naturally modified cephalosporin antibiotic produced by e.g. *Streptomyces* or *Nocardia* spp., but one could envisage the redesign of enzymes in the later stages of cephem biosynthesis to make similar substitutions on other β-lactam templates. Note that penams or cephems with alternative side-chains could also be obtained using alternative or modified (trans) acylases [59, 66–67].

The examples above aim to beat resistance by avoiding recognition by the β-lactamase. Another approach would be to disarm the β-lactamase by inhibiting it. Clavulanic acid, a naturally occurring inhibitor, inactivates β-lactamases by forming a slowly hydrolysing covalent acyl enzyme intermediate, and is used in combination with a β-lactam antibiotic. The commercial drug Augmentin is a combination of clavulanate and amoxicillin [6, 23]. The 'problem' with clavulanate is that it has little or no activity towards cephalosporinases (class C β-lactamases). One way around this problem could thus be to reengineer clavams in such a way that they would be recognised by cephalosporinases. CAS, the trifunctional enzyme in the biosynthetic pathway of clavulanic acid, offers itself as the obvious target for development of new broad-spectrum β-lactamase inhibitors and hybrid β-lactam antibiotics/β-lactamase inhibitors. Such broad-spectrum β-lactam compounds with penem, oxapenem or carbapenem cores have been produced by traditional synthetic procedures [e.g. 35, 68–73], but their commercial large-scale production is at present problematic. Insight into the machinery of the β-lactam biosynthetic enzymes now offers a unique opportunity for experiments aimed to develop new biosynthetic routes to these targets. By combining information on the three-dimensional structure of the active sites of different biosynthetic enzymes, new classes of compounds that are active against the resistant bacteria could be developed. Different approaches include: (i) targeted random mutagenesis, (ii) site-directed mutagenesis and (iii) directed evolution, e.g. domain shuffling involving several genes [74–76].

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