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The structural basis of cephalosporin formation in a mononuclear ferrous enzyme

Karin Valegård¹, Anke C Terwisscha van Scheltinga², Alain Dubus³, Graziella Ranghino⁴, Linda M Öster², Janos Hajdu¹ & Inger Andersson²

Deacetoxycephalosporin-C synthase (DAOCS) is a mononuclear ferrous enzyme that transforms penicillins into cephalosporins by inserting a carbon atom into the penicillin nucleus. In the first half-reaction, dioxygen and 2-oxoglutarate produce a reactive iron-oxygen species, succinate and CO₂. The oxidizing iron species subsequently reacts with penicillin to give cephalosporin and water. Here we describe high-resolution structures for ferrous DAOCS in complex with penicillins, the cephalosporin product, the cosubstrate and the coproduct. Steady-state kinetic data, quantum-chemical calculations and the new structures indicate a reaction sequence in which a 'booby-trapped' oxidizing species is formed. This species is stabilized by the negative charge of succinate on the iron. The binding sites of succinate and penicillin overlap, and when penicillin replaces succinate, it removes the stabilizing charge, eliciting oxidative attack on itself. Requisite groups of penicillin are within 1 Å of the expected position of a ferryl oxygen in the enzyme–penicillin complex.

Mononuclear ferrous enzymes participate in a wider range of reactions than heme enzymes, but they are unstable and thus difficult to study. DAOCS is a 2-oxoglutarate-dependent mononuclear ferrous enzyme¹, and it was the first whose structure was determined². It catalyzes the oxidative expansion of the five-membered thiazolidine ring of the penicillin nucleus into the six-membered dihydrothiazine ring of the cephalosporins^{2,3} (see **Supplementary Fig. 1** online). More recently, crystal structures have been reported for other 2-oxoglutarate-dependent ferrous enzymes: clavaminic synthase (CAS)⁴, anthocyanidin synthase (ANS)⁵, taurine/α-ketoglutarate dioxygenase (TauD)⁶, proline 3-hydroxylase⁷ and the hypoxia-inducible factor asparaginyl hydroxylase (FIH)^{8–10}. In structural terms, isopenicillin N synthase (IPNS)¹¹ also belongs to this family, but IPNS does not use 2-oxoglutarate in catalysis. The overall three-dimensional structures of these enzymes are similar and have a β-barrel core with the ferrous iron ligated by two histidines and a carboxylate^{11,12}.

An understanding of catalysis by mononuclear ferrous enzymes would be significant in scientific, medicinal and industrial terms. The iron center in these enzymes provides a multifunctional and flexible platform for catalysis, but at a price. In contrast to heme enzymes, mononuclear ferrous enzymes easily suffer oxidative damage^{13–15} and can be inactivated by dioxygen (one of their substrates) in <1,000 turnovers¹³. Inactivation can be reversible or irreversible. When the ferrous iron is oxidized, the inactive ferric enzyme can be rescued by ascorbate in a redox reaction that is not part of the catalytic cycle. Irreversible inactivation involves radical side reactions, leading to the

hydroxylation of side chains^{14,15} and/or to the fragmentation of the polypeptide chain¹³. The control of the reactivity of the oxidizing species and the timing of the oxidative reaction steps seem to be important factors in catalysis, and play a central role in the survival of these enzymes during catalysis.

RESULTS

Binding of the cosubstrate and coproduct

We compared the high-resolution X-ray structures for the ternary DAOCS–Fe(II)–2-oxoglutarate complex² and for the DAOCS–Fe(II)–succinate complex (**Fig. 1a,b** and **Supplementary Fig. 2** online, in stereo). Crystallographic statistics are given in **Table 1**. As predicted², the distal carboxylate of succinate forms a salt bridge to Arg258, and the proximal carboxylate ligates the iron in a monodentate manner in a position that would be expected if it were produced by oxidative decarboxylation. The coordination geometry around the iron is octahedral. The succinate complex is likely to be more stable when the oxidation state of the iron is high. Although the soaking solution contained bicarbonate (see **Methods**), neither carbon dioxide nor bicarbonate is visible in the structure; instead, a new water molecule (Wat2) takes up the expected position of these weak ligands. The presence of Wat2 in the succinate complex (**Fig. 1b**) is significant because it permits isotope dilution through a mechanism based on a suggestion by Baldwin and Schofield¹⁶ (**Supplementary Fig. 3** online). The level of ¹⁸O isotope incorporation from labeled dioxygen into succinate is >95%, but the incorporation of the label into hydroxylated byprod-

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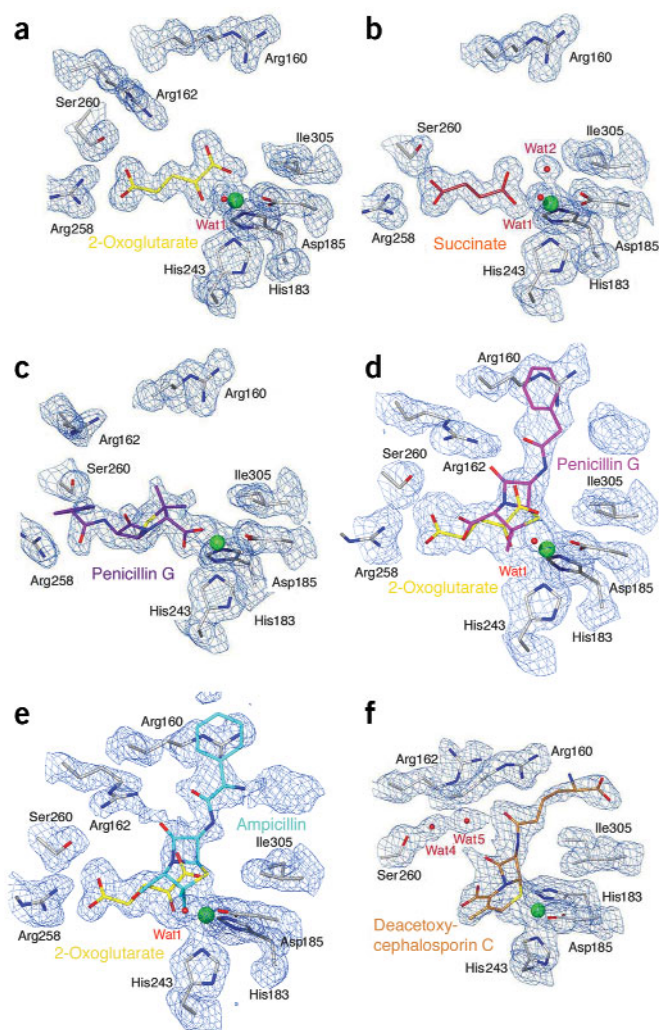


Figure 1 The active site region of DAOCS in complex with substrates and products. (a) The DAOCS–Fe(II)–2-oxoglutarate complex² at 1.5-Å resolution. (b) The DAOCS–Fe(II)–succinate complex at 1.5-Å resolution. (c) The DAOCS–Fe(II)–penicillin G complex at 1.6-Å resolution. (d) The DAOCS–Fe(II)–2-oxoglutarate–penicillin G complex at 1.7-Å resolution. (e) The DAOCS–Fe(II)–2-oxoglutarate–ampicillin complex at 1.5-Å resolution. (f) The DAOCS–Fe(II)–DAOC complex at 1.7-Å resolution. See text for details. The density next to the penicillin side chain in **d,e** corresponds to a minor alternative conformation of the side chain. Dioxxygen is expected to bind at the position of Wat1 in **a**. The oxygen of the ferryl iron would be formed at this site². The carbon atoms in 2-oxoglutarate are yellow, in succinate orange, in penicillin G magenta, in ampicillin cyan and in DAOC gold.

ligates the iron at the same site as succinate (Fig. 1b). This binding mode is believed to be unproductive, but might have a role in certain oxidative side reactions supported by the enzyme. The binding of the C3 carboxylate to the ferrous iron could activate the iron for dioxygen binding in a manner similar to 2-oxoglutarate (Fig. 1a). A model of oxygen binding in the DAOCS–Fe(II)–penicillin complex shows that the distal oxygen atom of a bound dioxygen molecule could approach both the C3 carbon of the thiazolidine ring and the carbon of the β -methyl group at a distance shorter than ~ 3 Å. The β -methyl group and the C3 carbon atom are the sites from which two hydrogens (protons and electrons) are removed in the ring expansion reaction. The enzyme does not sustain steady-state catalysis without 2-oxoglutarate¹⁶, but it may turn over once with the bound penicillin in this complex when exposed to dioxygen. Such a reaction could produce a ferryl species and a water molecule via the abstraction of two hydrogens, and could account for the formation of certain byproducts in catalysis (such as the 3- β -hydroxy or the 3-exomethylene cepham)¹⁶. This type of interaction may also be significant in the hydroxylation reaction catalyzed by the bifunctional DAOCS/DACS enzyme in eukaryotic cephalosporin producers¹⁶.

Productive penicillin binding

We also determined the structure of DAOCS soaked in a solution containing Fe(II), followed by a soak with 2-oxoglutarate and penicillin G (Fig. 1d). The electron density corresponds to a mixture of two different structures within the same crystal at more or less equal occupancies. One of these structures is the enzyme in complex with 2-oxoglutarate and water (Wat1) (Fig. 1a), whereas the other is the enzyme in complex with penicillin G. The iron in the penicillin complex seems to be pentacoordinated and ‘dry.’ The penicillin is in a considerably different orientation here than in Figure 1c. The order of substrate addition had no effect on the structural results. Similar results were obtained with ampicillin and 2-oxoglutarate (Fig. 1e), suggesting that this binding mode is not exclusive to penicillin G. Further tests in which 2-oxoglutarate was replaced by succinate gave identical penicillin orientations to those in Figure 1d,e (data not shown). These results suggest that succinate or 2-oxoglutarate primes the active site for productive penicillin binding.

In the complexes of Figure 1d,e, both penicillins bind via their sulfur atom to the iron *trans* to His243 at the position expected to be occupied by carbon dioxide after oxidative decarboxylation. The water in the electron density map corresponds to Wat1 of the 2-oxoglutarate complex, and has $\sim 50\%$ occupancy. If an oxo-ferryl group were present in the protein, its ferryl oxygen would be expected to occupy the position of this water². In the mixed structures of Figure 1d,e, the β -methyl group of the penicillin core takes a position ~ 1 Å away from the position previously occupied by Wat1 (Fig. 1a,b). This methyl group is inserted into the penicillin ring during catalysis. A ferryl oxygen at the site of Wat1 could trigger a redox reaction with the methyl group. The geometry of the iron–penicillin complex suggests produc-

ucts and into the product of a closely related enzyme, deacetyl cephalosporin C synthase, is only $\sim 50\%$. The structure in Figure 1b explains these findings (Supplementary Fig. 3 online).

Overlap of the binding sites

DAOCS has broad substrate specificity and can catalyze ring expansion in penicillins with various side chains^{16–18}. The physiological substrate of DAOCS is penicillin N (side chain: δ -(D- α -aminoadipoyl)), and the physiological product is deacetoxycephalosporin C (DAOC). In this study, we used penicillin G (side chain: phenylacetyl; product: phenylacetyl-7-aminodeacetoxycephalosporanic acid) and ampicillin (side chain: R-(2-amino)phenylacetyl; product: cephalixin). Penicillin G and ampicillin have relatively high overall stabilities; this was important for the crystallographic experiments. Unexpectedly, we found two different binding modes for each of these penicillins in the enzyme. Both penicillin sites (Fig. 1c–e) overlapped and clashed with the binding site of succinate or 2-oxoglutarate. This observation has mechanistic implications for catalysis by DAOCS, precluding simultaneous binding of the two types of substrates or products in the active site.

Unproductive penicillin binding

The DAOCS–Fe(II)–penicillin G complex (Fig. 1c) was prepared by soaking crystals of apo DAOCS in a solution of Fe(II) followed by a solution of penicillin G. The results show that penicillin binds at the succinate-binding site so that the C3 carboxylate on the thiazolidine ring

tive penicillin binding in the structures shown in Figure 1d,e. This is also supported by results on cephalosporin binding described below.

Formation of a functional active site

An explanation for the different penicillin-binding modes can be given by considering steric and electrostatic changes introduced into the active site when the cosubstrate or coproduct is bound to the iron. Under these conditions, the incoming penicillin encounters an active site with altered geometry and charge state, and will be guided and anchored differently than in the unliganded enzyme, expelling or replacing the cosubstrate or coproduct from the iron during this process. This also suggests that the active site is incomplete for productive catalysis without the cosubstrate or coproduct. The results show a dynamic equilibrium between the enzyme and these ligands in the crystal. In this equilibrium, 2-oxoglutarate or succinate seems to prime the active site for productive penicillin binding. The results also confirm the existence of a cosubstrate-substrate exchange in the crystal². We note that substrate exchange could have an additional role in isotope exchange reactions (see Supplementary Fig. 3 online) observed earlier¹⁶. Structural studies in a related enzyme, clavaminase synthase^{4,19}, suggest a reorganization of the cosubstrate or coproduct around the iron during catalysis in that enzyme.

Evidence for productive substrate binding

The structure of the natural cephalosporin product (DAOC) in a crystal soaked with Fe(II) first, followed by succinate and DAOC (Fig. 1f), shows that the binding mode of the cephalosporin product is similar to the binding mode of penicillin substrates in Figure 1d,e. The sulfur atom of the cephalosporin interacts with the iron, and as in Figure 1d,e, the position of the cephalosporin nucleus overlaps with the position of succinate, but succinate is not visible in the electron density map. Identical structures were obtained when the natural cephalosporin product alone was added without succinate to crystals of ferrous DAOCS (data not shown). In these structures, Arg160 and Arg162 line the binding pocket of the aminoacyl side chain, and the carbonyl oxygen atom of the β -lactam ring makes a water-mediated hydrogen bond to Ser260. The α -aminoacyl side chain of DAOC is in the same orientation as the penicillin side chains in Figure 1d,e. The identical orientation of the penicillin substrates and the cephalosporin product in Figure 1d–f suggests that this orientation is catalytically relevant. This is further supported by a comparison of penicillin binding in DAOCS and in isopenicillin N synthase (IPNS) (Fig. 2). These enzymes are closely related^{1,2}. A comparison of the active site of DAOCS in complex with (i) penicillin G (ii) ampicillin and (iii) DAOC, with the active site of IPNS¹¹ in complex with (iv) isopenicillin N²⁰ (Fig. 2) shows that the binding mode of these antibiotics is practically identical in these enzymes (Fig. 2).

Expected structure of the oxidizing iron species

The structural results show the site of the cosubstrate or coproduct overlaps with the site of penicillin or cephalosporin in the enzyme, and thus these compounds cannot bind simultaneously in the active site. This was unexpected in light of structural studies on CAS and TauD, which indicate simultaneous binding of cosubstrate and substrate^{4,6,19,21}. Spectroscopic studies of CAS and TauD indicate that only when both substrate and cosubstrate are present is the ferrous site converted into a five-coordinate species with enhanced reactivity toward dioxygen^{22–24}. Based on these results, a mechanism was proposed^{22–24} in which the binding of the main substrate triggers the rearrangement to a pentacoordinated species, and this serves as the conformational trigger for oxygen binding (and oxidation of the substrate). DAOCS seems to behave differently.

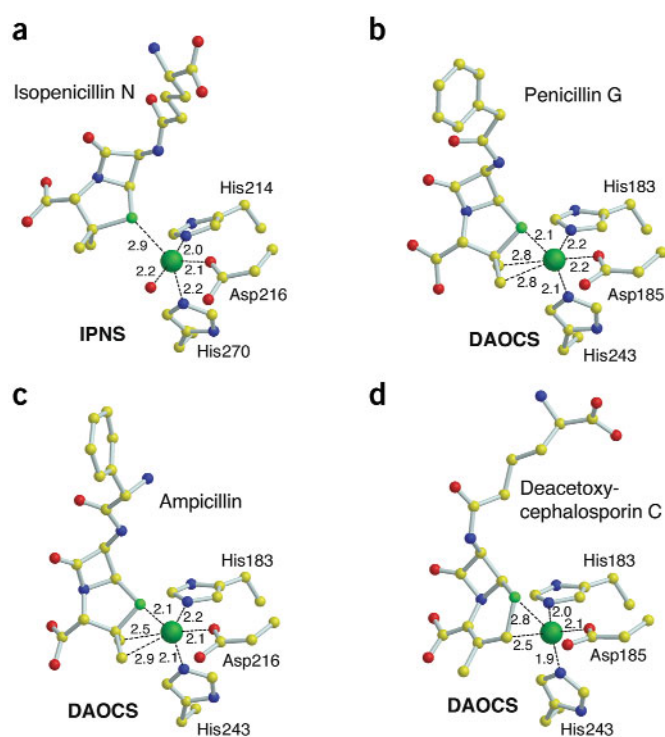


Figure 2 Penicillin binds in the same orientation to DAOCS as it does to isopenicillin N synthase (IPNS). (a) IPNS in complex with isopenicillin N. (b) DAOCS in complex with penicillin G. (c) DAOCS in complex with ampicillin (b and c are from the 2-oxoglutarate soaks in Fig. 1d,e). (d) DAOCS in complex with deacetoxycephalosporin C (DAOC). DAOCS and IPNS are structurally related². The iron centers and the His-Asp-His triad were aligned with O⁴¹.

Based on our structural data showing overlapping binding sites for the cosubstrate or coproduct with the binding site of penicillin in DAOCS, we propose that the oxidizing iron species of DAOCS has to be formed before the binding of penicillin. This also represents a storage problem for the reactive oxidizing species. In Figure 3 we assess the relative stabilities of different iron-oxygen adducts in DAOCS. The figure shows computed structures and their relative energies *in vacuo*. These structures were obtained from large basis set DFT (density functional theory) calculations combined with *ab initio* QMD (quantum molecular dynamics) and simulated annealing studies (see Methods). The errors at the level of theory concerned are at least an order of magnitude smaller than the differences in properties discussed. The key conclusion of these calculations is that the planar peroxo form is less reactive than the ferryl species and may thus be present in the active site for longer times. The iron is in oxidation state II in the peroxo intermediate and in oxidation state IV in the ferryl form. The results support the view² that the reactivity of the ferrous iron will be enhanced toward dioxygen by the 2-oxoacid cosubstrate (Fig. 1a), whereas the reactivity of the oxidizing intermediate would be reduced by the negative charge of the reaction product, succinate (Fig. 1b). The oxidizing iron species may thus be stored transiently in the peroxo form within the protein to await a reaction with the penicillin substrate. The mode of penicillin binding to DAOCS supports this proposal.

Steady-state kinetic studies

The structural data on DAOCS indicate a reaction sequence in which the binding of one substrate interferes with the binding of the other.

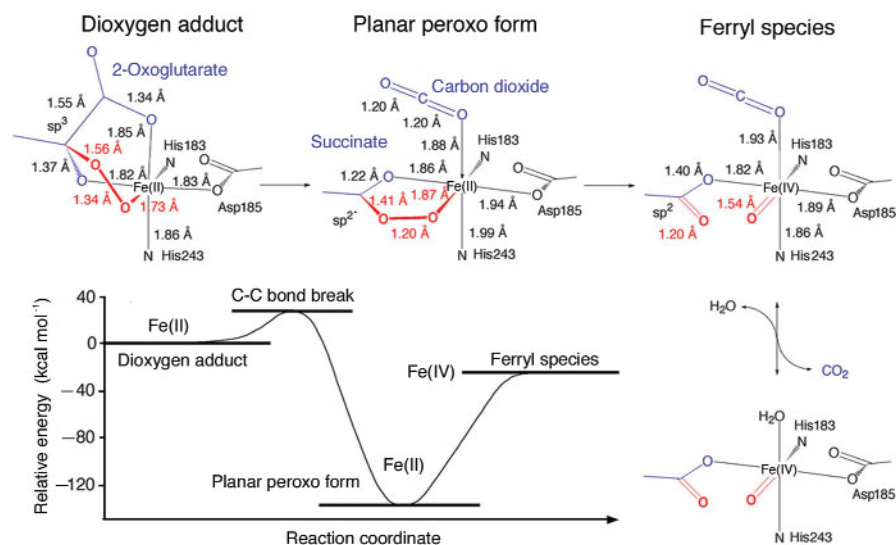


Figure 3 Quantum chemical models for the oxidizing species. Atoms of the cosubstrate and coproducts are blue. The two oxygen atoms of dioxygen are red. Inset, relative energies for the oxidizing species *in vacuo*. The planar peroxy form is more stable than the ferryl form (which is thus more reactive). The binding mode of CO₂ in the computed structure is similar to that observed by Lee *et al.*²⁶. This information was not fed into the model.

Interference of this type must affect the kinetic behavior of the enzyme in solution. The results of steady-state rate measurements of DAOCS (Fig. 4) in which the concentration of one substrate was varied (*x*-axis) while the concentration of the other remained constant confirm our expectations, and show a major decrease in the steady-state rates at fixed penicillin concentrations when the concentration of 2-oxoglutarate is increased. Although it is less pronounced, the same behavior can also be observed at fixed 2-oxoglutarate concentrations when the concentration of penicillin G is increased. This type of substrate inhibition and competition agrees with the structural results. Such kinetic data alone would not be sufficient to determine the nature of the inhibition mechanism, but in combination with structural data, they strongly support the mechanism we propose. This trend is expected when two ligands compete for the same binding pocket in the enzyme. Another line of evidence comes from the observation of uncoupled turnover in 2-oxoglutarate-dependent oxygenases. Many of these enzymes can convert 2-oxoglutarate to succinate and carbon dioxide without concomitant oxidation of their other or 'prime' substrate^{7,18,22–27}. The rate of uncoupled turnover increases with increasing concentrations of 2-oxoglutarate, supporting the mechanism proposed here. Indeed, in this model, excess 2-oxoglutarate would drive the reaction toward uncoupled turnover with a second molecule of 2-oxoglutarate reacting with the activated ferryl species during a coproduct-cosubstrate exchange. Recent results by Price *et al.*²⁴ suggest that the lifetime of a free oxoferryl species can be long enough to permit exchange reactions among substrate, cosubstrate and coproduct around a ferryl iron species.

DISCUSSION

Proposed mechanism for ring expansion

All of the complexes we have obtained so far show overlapping binding sites for substrate and cosubstrate, as well as for product and coproduct. The results indicate that these compounds cannot be simultane-

ously present in the active site and consequently suggest a definite sequence of events in catalysis. This has mechanistic implications and affects our views on the way the oxidizing iron species is created, stored and used by the enzyme.

DAOCS must keep a potentially very reactive oxidizing species in a tamed form but ready to attack the other substrate whenever it

appears. Based on structures in Figures 1 and 2, on results from solution kinetics (Fig. 4) and on quantum mechanical calculations (Fig. 3), we propose a mechanism in which a 'booby-trapped' oxidizing intermediate is created at the iron and then stored temporarily for use on the other substrate. The less reactive planar peroxy intermediate

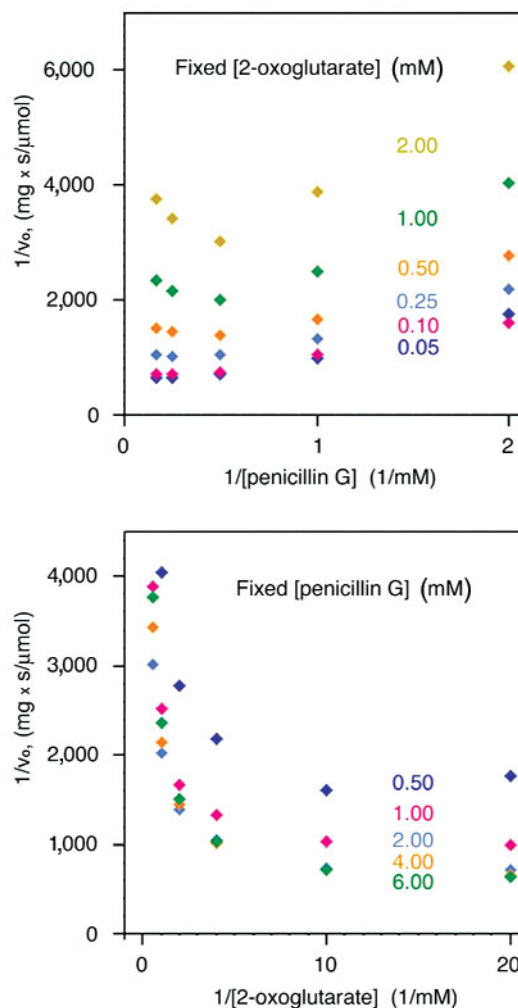


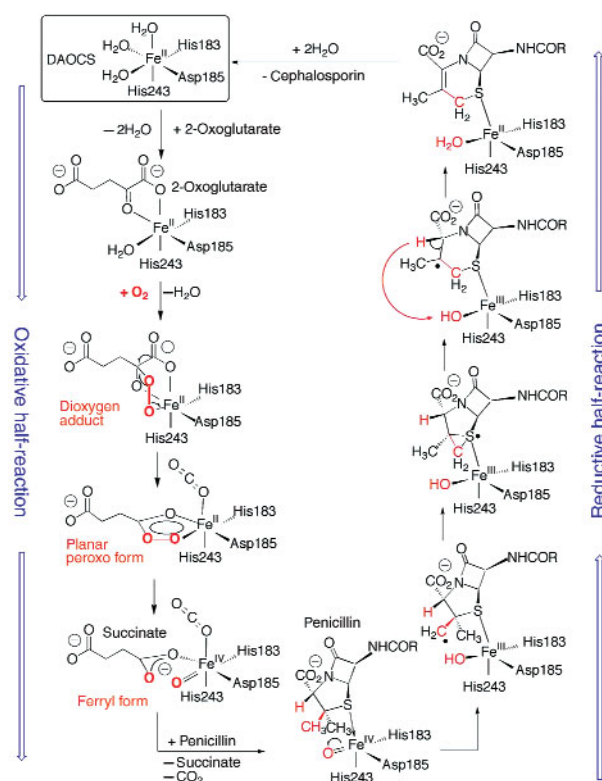
Figure 4 Substrate inhibition of DAOCS with increasing concentrations of penicillin G or 2-oxoglutarate. (a,b) Results from steady-state rate measurements as a function of penicillin G (a) or 2-oxoglutarate (b) concentrations. Similar results were obtained with ampicillin (data not shown). Measurements were taken by varying the concentration of one substrate (*x*-axis) while keeping the concentration of the other fixed (as indicated on each picture). See Methods for details.

Figure 5 A possible mechanism for the ring expansion catalyzed by DAOCS. The mechanism is based on the mode of penicillin and cephalosporin binding shown in **Figures 1d–f** and **2**. The presumed oxidation states of the iron are marked. In the oxidative half reaction, one of the oxygen atoms of dioxygen is incorporated into succinate while the other one remains on the iron. This oxygen can remove two electrons and two protons from the five-membered thiazolidine ring to form the six-membered dihydrothiazine ring of the cephalosporin product in the reductive half reaction. Note ligation of the penicillin sulfur to the iron (Fe–S distance: 2.1–2.0 Å in the various complexes) and that both α - and β -methyl groups are in van der Waals contact with the iron (iron–methyl distances: 2.1–2.5 Å).

(Fig. 3) could serve this purpose (probably in some equilibrium with the ferryl form). As the binding of the penicillin substrate destabilizes and replaces succinate from the active site, the oxidizing intermediate is activated and reacts with the incoming substrate. Activation is based on the withdrawal of the carboxylate from the first coordination sphere of the iron. The behavior of CAS and TauD in this respect may be different^{4,6,19,21,23}, although priming of the active site by 2-oxoglutarate might also have a role²³. In these cases, the oxidizing species seems to be created upon binding of the main substrate. However, the presence of an uncoupled turnover in these species^{22,23} suggests that this is probably not an exclusive mechanism and a mixture of various mechanisms may be present. In DAOCS, the large size and relative instability of the penicillin substrate may have shifted the mechanism so that other mechanistic features dominate.

We propose a mechanism (Fig. 5) based on the mode of penicillin binding in **Figure 1d,e**. This model has the following features. (i) Binding of 2-oxoglutarate activates the iron for oxygen binding². (ii) Oxidative decarboxylation of the cosubstrate results in formation of oxidizing intermediates plus succinate and carbon dioxide as byproducts. Carbon dioxide is a weak ligand for the iron and it is likely to leave easily, whereas succinate remains bound and stabilizes the oxidizing iron species (Fig. 1b). (iii) When penicillin expels succinate (Fig. 1d,e), it triggers oxidative attack on itself. (iv) Structures for the penicillin substrates in **Figure 1d,e** show that penicillin binds via its sulfur atom to the iron. This binding mode brings the requisite β -methyl group of the penicillin core within easy reacting distance of the expected position of the ferryl oxygen. Ring expansion, involving radical formation^{28–32} and transfer of two electrons and two protons to the oxygen, results in formation of water and the cephalosporin product (Fig. 1f). Electron transfer to the oxygen could be direct but could also proceed via the oxidized iron linking the sulfur and the ferryl oxygen. The binding of the sulfur to the iron allows electron transfer from the sulfur to the oxidized iron during catalysis, and may thus assist in the expansion of the thiazolidine ring. Penicillins can be expanded synthetically from their sulfoxides into the corresponding cephalosporins via a Pummerer-type reaction³³. The mechanism proposed here shows certain similarities to Pummerer reactions but without an oxygen directly attached to the sulfur atom. Such a reaction with an iron atom intervening between the sulfur and the oxygen could in principle be described as an indirect Pummerer reaction.

Kinetic data show that the first hydrogen atom to be removed comes from the β -methyl group¹⁶, which is nearest to the expected position of oxygen in the ferryl form. Transfer of the second hydrogen from the C3 carbon of the thiazolidine ring to the oxygen of the ferryl is not straightforward. The ferryl oxygen is probably too far away from this hydrogen (~3 Å) for direct transfer, and there are no bases visible in the protein in its vicinity. We speculate that electron transfer to the iron is followed by proton migration toward the iron. Transfer of an electron to the iron may proceed via the sulfur or via the two methyl



groups. This would be followed by the migration of a proton to the ferryl oxygen either from the C3 carbon or from the solvent. The disordered C-terminal arm of DAOCS may also provide a base for the abstraction of the C3 proton^{26,34}. In the present structure, however, the C-terminal arm is too far away for direct interaction with reacting groups of the substrate.

The biological context

Heme enzymes have a solid container for a highly charged iron in their porphyrin ring. Excess charge can be distributed in a cationic radical on the ring or elsewhere in the protein. Mononuclear ferrous enzymes lack such a solid iron container. The present studies on DAOCS suggest how oxygen activation and ferryl containment could be achieved in these enzymes. The results of these studies indicate a reaction sequence in which a 'booby-trapped' oxidizing species is created first; this could be stored and then activated to react with the second substrate later. Similar mechanisms may operate in other 2-oxoglutarate-dependent mononuclear ferrous enzymes. These enzymes couple a strongly exergonic redox reaction (the reduction of dioxygen) to drive an endergonic reaction uphill (Fig. 5). This can be achieved by transiently storing³⁵ a large fraction of the available high-grade energy from the drive reaction at the iron center that temporarily becomes highly oxidized in the enzyme. When formed in aqueous solution, such highly oxidized metals would subtract electrons from any electron source. This is usually a radical reaction with water (Fenton chemistry). In the enzyme, however, access to the metal is restricted as a result of steric and electrostatic shielding, ensuring that only a certain class of substrates can approach the metal center. As a result, many fewer reaction routes remain open for the system to return to the ground state than exist in solution. Selectivity is achieved mainly by exclusion³⁶, and the activated enzyme behaves as a reactant³⁵. These proteins are catalysts for the complete reaction cycle; they return to

Table 1 Data collection and refinement statistics

	DAOCS–Fe(II) –succinate	DAOCS–Fe(II)–2OG –ampicillin	DAOCS–Fe(II) –penicillin G	DAOCS–Fe(II) –2OG–penicillin G	DAOCS–Fe(II) –DAOC
Wavelength (Å)	0.80	1.076	0.886	1.092	0.965
Resolution (Å)	1.5	1.5	1.6	1.7	1.7
Space group	<i>R</i> 3	<i>R</i> 3	<i>R</i> 3	<i>R</i> 3	<i>R</i> 3
Unit cell dimensions (Å)					
<i>a</i> = <i>b</i>	106.8	106.7	106.5	106.6	106.6
<i>c</i>	70.1	71.6	72.1	71.6	74.0
Mosaicity (°)	1.3	0.5	0.6	1.0	0.45
Reflections					
Measured	108,054	299,664	207,167	216,411	192,826
Unique	45,627	45,862	39,909	30,873	31,415
Completeness (%) ^a	95.5 (94.2)	94.2 (99.9)	99.2 (100.0)	92.6 (99.0)	91.0 (99.5)
Twin fraction	0.25	0.38	0.01	0.01	0.35
<i>R</i> _{merge} (%) ^{a,b}	4.9 (28.0)	6.4 (31.2)	4.7 (13.4)	8.7 (23.1)	4.6 (18.6)
Residues in model	1–81, 98–166, 178–309	1–80, 91–166, 178–309	1–80, 97–164, 179–196, 201–248, 258–310	1–79, 97–167, 179–309	2–80, 98–164, 181–195, 204–246, 259–310
Occupancy of ligand					
Succinate	1	–	–	–	–
2-OG	–	0.5	–	0.5	–
Ampicillin	–	0.5	–	–	–
Penicillin G	–	–	1	0.5	–
DAOC	–	–	–	–	1
Solvent molecules	216	191	141	158	117
<i>R</i> _{cryst} ^c	0.174	0.190	0.196	0.188	0.204
<i>R</i> _{free} ^c	0.189	0.237	0.231	0.240	0.242

2OG, 2-oxoglutarate.

^aValues in parentheses are for the highest-resolution bin. ^b $R_{\text{sym}} = \sum_{hkl} \sum_j |I_j - \langle I \rangle| / \sum_{hkl} \sum_j I_j$, where *I* is the observed density and $\langle I \rangle$ is the mean density of reflection *hkl*. ^c $R = \sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl} |F_o|$, where *F*_o and *F*_c are the observed and calculated structure factor amplitudes, respectively.

their original state after a turnover, but instead of being selective rate accelerators (classical Pauling-type catalysts), they act mainly as energy converters³⁵. The loose substrate and product specificities on a versatile catalytic platform provide important advantages for such systems in evolutionary terms; running battles of intermicrobial warfare with antibiotics and anti-antibiotics are fought through such enzymes in biology.

METHODS

Crystallization and data collection. Recombinant DAOCS³⁷ from *Streptomyces clavuligerus* was purified and crystallized as described^{2,37}. The crystals belong to space group *R*3, and are merohedrally twinned^{2,38}. The twin fraction varies between crystals, and we screened many crystals to find ones with twin fractions <0.3. Complexes were prepared by soaking crystals of the apoenzyme² at 20 °C for 10–35 min at pH 7.0 in solutions containing: (i) first 20 mM Fe(NH₄)₂(SO₄)₂, then supplemented with 100 mM succinic acid and 100 mM NaHCO₃ for the succinate complex of Figure 1b; (ii) first 20 mM Fe(NH₄)₂(SO₄)₂, then supplemented with 50 mM penicillin G for the penicillin complex in Figure 1c; (iii) first 20 mM Fe(NH₄)₂(SO₄)₂, then supplemented with 45 mM 2-oxoglutarate and 65 mM ampicillin or 25 mM penicillin G for the penicillin complexes in Figure 1d,e or (iv) first 20 mM Fe(NH₄)₂(SO₄)₂, then supplemented with 80 mM succinate and 80 mM DAOC for the cephalosporin product complex (Fig. 1f). All solutions contained 1.75 M Fe(NH₄)₂(SO₄)₂ and 0.1 M HEPES and the complexes were prepared under strictly anaerobic conditions. Crystals were flash-frozen inside the anaerobic box (<http://www.belle-technology.com>). High-resolution X-ray data (Table 1) were collected at 100 K on beamlines BM14 (DAOCS–Fe(II)–succinate) and ID14:EH2(DAOCS–Fe(II)–penicillin G), European Synchrotron Radiation Facility (ESRF, Grenoble, France) and on station I711, Maxlab (DAOCS–Fe(II)–2OG–ampicillin, DAOCS–Fe(II)–2OG–penicillin G and DAOCS–Fe(II)–DAOC) (Lund University, Lund, Sweden).

Structure determination and analysis. Data were processed with the HKL package³⁹. Each dataset was assessed and corrected for merohedral twinning as described previously³⁸. Initial models were obtained by rigid-body refinement using the structure of the DAOCS–Fe(II) complex, and were then further refined using REFMAC5 (ref. 40) on the detwinned data. Electron density maps were interpreted using O⁴¹. The iron–ligand bond lengths were unrestrained throughout refinement. Restraints for ampicillin and DAOC were calculated from models obtained by energy minimization. Coordinates of IPNS–Fe(II)–IPN (1IPS), DAOCS–Fe(II)–ampicillin and DAOCS–Fe(II)–DAOC were superimposed centered at their iron ligands using O⁴¹. Figures were prepared using Molray⁴², MolScript, BobScript^{43,44} and Raster3D⁴⁵.

Steady-state kinetic studies. Kinetic assays were based on UV spectrophotometric measurements¹⁸ and were done at 30 °C in solutions containing 50 mM HEPES, pH 7.5, 50 mM ammonium sulfate, 1 mM Tris(carboxyethyl)phosphine (TCEP), 0.1 mM ascorbate. The assay mixture also contained penicillin and 2-oxoglutarate at concentrations shown in Figure 4. Before measurement, a small volume of concentrated ferrous ammonium sulfate solution was added (25 μM final concentration) and the mixture was preincubated at 30 °C for ~3 min. Reaction was started by adding a small volume of the concentrated enzyme (4–8 μM final concentration). Each measurement was repeated four times. Initial rates were proportional to the enzyme concentration. A blank assay was also recorded for each condition.

Quantum chemical calculations. Starting coordinates were obtained from PDB entries 1RXF and 1RXG, and were used to create a model of the active site, including the ferrous iron, its ligands and 2-oxopropionate as an analog for the cosubstrate, 2-oxoglutarate. The orientations of the primary protein ligands were constrained to their orientations in the high-resolution X-ray structures. Hydrogen atoms were added and the positions of all atoms were optimized. Energies were computed with the *ab initio* DFT program Fast Structure (<http://www.accelrys.com/ceirus2/faststructure.html>), using extended basis



sets and relaxed core. *Ab initio* quantum molecular dynamics calculations were made with the CASTEP (<http://www.tcm.phy.cam.ac.uk/castep/>) at 300 K with kinetic energy cutoff for plane waves at 600 eV, local spin density approximation, and a total spin of 0. The model was placed in a cubic cell with dimensions of 10 Å to prevent direct interactions between neighboring molecules. The output model of QMD was again energy-minimized and taken as starting point to carry out a Fast Structure simulated annealing with melting from 300 K to 700 K followed by quenching the system back to 300 K in 300 steps. This was followed by energy minimization.

Coordinates. Atomic coordinates and structure factors have been deposited in the Protein Data Bank (accession codes: DAOCS–Fe(II)–succinate, 1UO9; DAOCS–Fe(II)–penicillin G, 1UOF; DAOCS–Fe(II)–2-oxoglutarate–penicillin G, 1UOB; DAOCS–Fe(II)–2-oxoglutarate–ampicillin, 1UNB; DAOCS–Fe(II)–DAOC, 1UOG).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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