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Isopenicillin N Synthase: An Enzyme at Work

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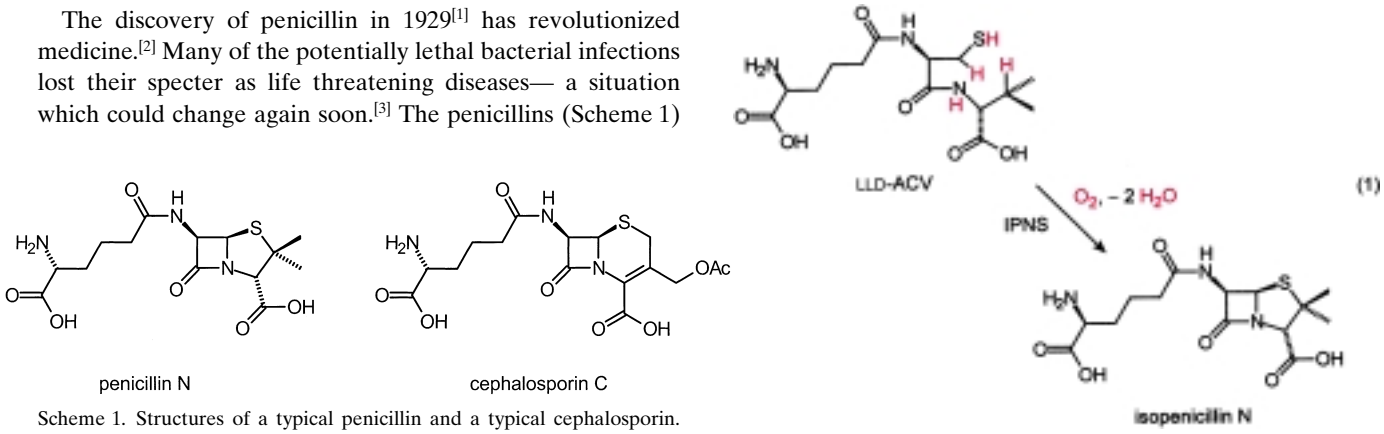
The discovery of penicillin in 1929[1] has revolutionized medicine.[2] Many of the potentially lethal bacterial infections lost their specter as life threatening diseases— a situation which could change again soon.[3] The penicillins (Scheme 1)



Scheme 1. Structures of a typical penicillin and a typical cephalosporin. Ac = acetyl.

were the first antibiotics, and for a long time the term "penicillin" was used by the general public as a synonym for "antibiotic".

Further milestones were set with the determination of the structure of penicillin[4] and the first total synthesis of a naturally occurring penicillin.[5] The laboratory synthesis of penicillin and its derivatives turned out to be quite a challenge because of its bicyclic structure, which makes the β-lactam ring particularly labile.[6] It is not surprising then that the question, "how does nature do it?" attracted the attention of the scientific community.

Most of the early information came from fairly indirect evidence.[7] The immediate precursor, the linear tripeptide L-α-aminoadipoyl-L-cysteinyl-D-valine (LLD-ACV), is first assembled from its component amino acids by the action of ACV synthase, which also mediates the necessary epimerization of valine. The key step, the stoichiometrically simple oxidative cyclization [Eq. (1)], is brought about by a single, non-heme iron-containing enzyme dubbed isopenicillin N synthase (IPNS). Further enzymes are then responsible for the epimerization of isopenicillin N to penicillin N, the derivatization to other penicillins, and the ring expansion that eventually leads to the various cephalosporins.

Despite considerable effort enzyme-free intermediates of this process have never been found. This indicates that both rings are formed within the same enzyme–substrate complex. Studies with modified substrates served to define the properties of the active site. A surprising range of variations of both the aminoadipoyl and the valinyl termini of ACV are tolerated, which helped the gathering of mechanistic information. Thus, if D-valine was replaced by other amino acids containing allyl or cyclopropylmethyl groups as "radical clocks", the appearance of the typical rearrangement products indicated that an (perhaps conformationally restrained) isopropyl radical intermediate is presumably involved in the formation of the thiazolidine ring.[8] The L-aminoadipoyl terminus may be replaced by a range of nonpolar substituents of similar size that are not necessarily terminated by a carboxylic group. This is good evidence that the corresponding part of the binding region of IPNS is nonpolar but contains a hydrogen-bonding site at the end of the pocket.[7] The central cysteine unit, however, is essentially inviolate. This is

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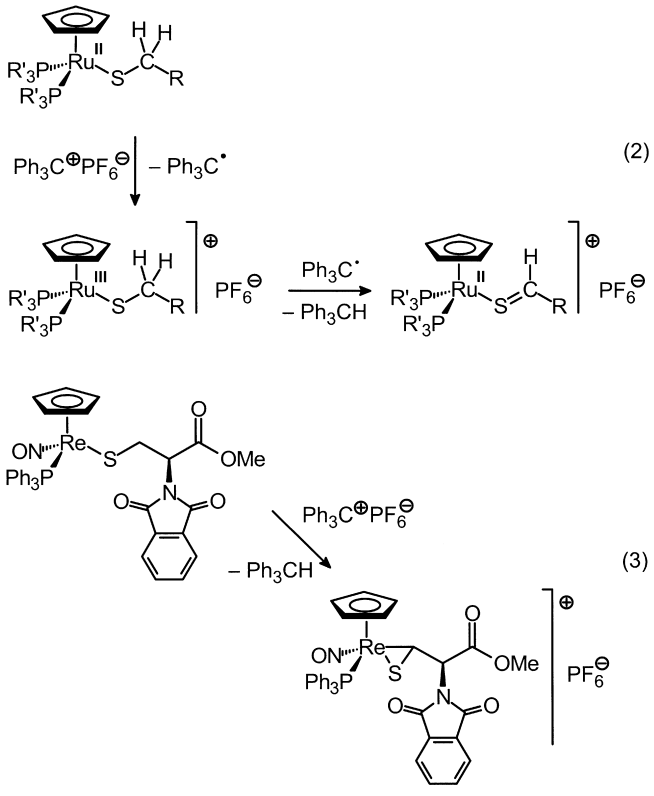
understandable in view of the constraints imposed by the formation of the strained β-lactam ring.

Further insight into the details of the mechanism came from a series of ingeniously planned deuteration experiments. For example, a large kinetic isotope effect was found when IPNS was fed a mixture of ACV doubly labeled in the 3-position of cysteine, and unlabeled ACV. On the other hand, IPNS does not discriminate between unlabeled ACV and ACV labeled in the 3-position of valine. This was taken as compelling evidence that the formation of the β-lactam ring is rate-limiting and precedes the closure of the thiazolidine ring.[9] Furthermore, through the use of ACV containing stereospecifically deuterated cysteine it was demonstrated that the hydrogen abstraction and ring-closure sequence occurs with retention of configuration, perhaps via an enzyme-bound thioaldehyde.[7a]

Transition metal complexes of thioaldehydes are well known since 1977.[10] A synthesis of metal-bound thioaldehydes by hydrogen abstraction from thiolate complexes was reported in 1992[11] [Eq. (2)] and recently extended to a range of aliphatic thioaldehydes, and includes an example of a side-on coordinated thioaldehyde derived from cysteine [Eq. (3)].[12]



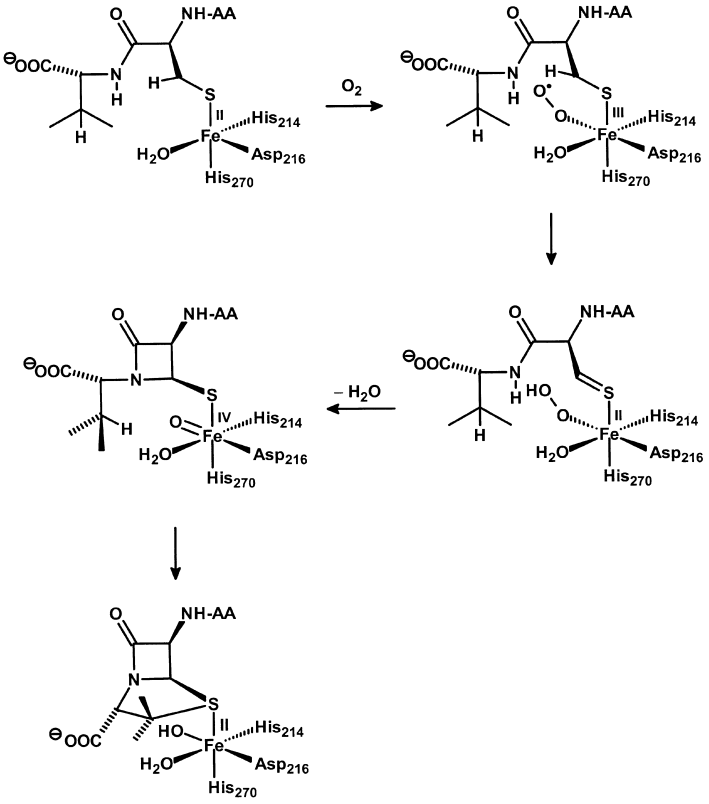
(2)

(3)

These reactions, which even show a comparably high kinetic isotope effect,[11] may be taken as organometallic analogues of the hydride abstraction step of the penicillin biosynthesis. Furthermore, the addition of nucleophiles to metal-bound thioaldehydes is well documented,[13] lending further support to the proposed mechanism.

A major breakthrough in this field came again from the group of J. E. Baldwin with the successful determination of the structure of IPNS.[14] The crystals were grown under anaerobic conditions so that the enzyme lacked the oxygen necessary for the reaction. By using this trick it was even possible to study the intact enzyme–substrate complex. Furthermore, after treatment with nitric oxide the corresponding nitrosyl complex was obtained which can serve as an unreactive structural model of the O_2-addition intermediate. In both cases the carboxylate groups at the ends of ACV are anchored through hydrogen bridges to the surrounding protein. This forces the substrate into an extended conformation which facilitates the closure of the β-lactam ring.

Based on this wealth of information a detailed mechanism of the action of IPNS could be formulated (Scheme 2). After binding the substrate ACV through the deprotonated thiol



Scheme 2. Mechanism of the biosynthesis of isopenicillin N. AA = aminoadipoyl.

group of the cysteine, the iron center takes up an oxygen molecule and changes oxidation state. Intramolecular transfer of a hydrogen atom from C-3 of the cysteine unit takes the iron back to the +II state and produces a thioaldehyde as well as a hydroperoxy ligand. The latter serves to deprotonate the amide function, which then closes the β-lactam ring by nucleophilic attack at the thioaldehyde carbon atom. With this the hydrogen atom at C-3 of valine comes in closer proximity to the highly electrophilic iron(IV) oxo ligand. A second hydrogen transfer follows, presumably producing an isopropyl radical which attacks the thiolate sulfur atom and thus closes the thiazolidine ring.

This already conclusive work has now been topped, again by the group around J. E. Baldwin, with a series of experiments which make IPNS now one of the best understood

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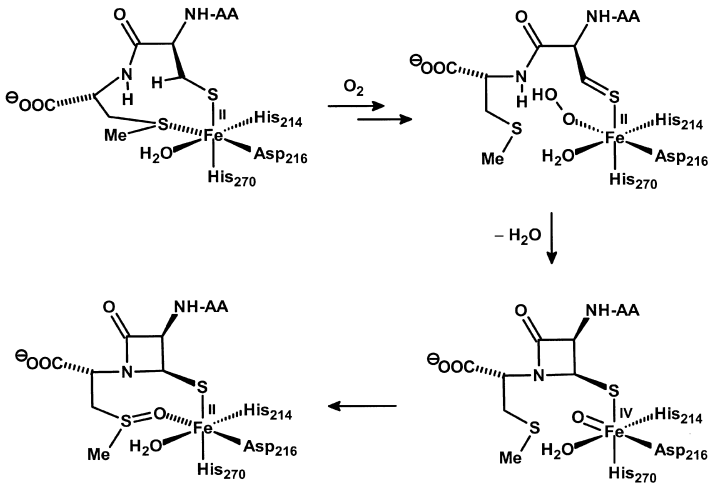
enzymes.[15] A crystal of the IPNS-ACV complex has been incubated with oxygen at 4 MPa for 320 min and the structure of the complex determined. It turned out that the substrate ACV had been transformed to isopenicillin N (IPN) which remained bound to the active site (some residual electron density indicated that under these conditions ca. 30 % of the substrate remain unchanged). In a separate set of experiments IPNS was complexed with a modified substrate (ACmC) in which valine had been replaced by (*S*)-methylcysteine. The structure of the active site of IPNS-ACmC is very similar to that of IPNS-ACV, except that the (*S*)-methyl group now occupies the sixth iron coordination site. Treatment of this crystal with oxygen (2 MPa, 10 min) gave a monocyclic β-lactam product complexed to the active site through the cysteinyl sulfur atom and a methylsulfenyl group, which may have originated from an interception reaction at the proposed Fe^{IV} oxo intermediate (Scheme 3).



Scheme 3. IPNS-mediated oxidative cyclization of ACmC.

This product is a good model of the monocyclic β-lactam intermediate of the IPN synthesis, whose transient existence had been inferred previously (see above). An overlay of the four structures is shown in Figure 1. Note in particular the extension of the Fe–S bond of the final product which anticipates the release of IPN from the active site.

With the oxidized ACmC taken as a model of the β-lactam intermediate, this series of structures provides a kind of animated cartoon of the details of the function of an enzyme. The rapid development of crystallographic instrumentation and methods may soon make investigations such as this a routine part of the elucidation of enzymatic reactions.

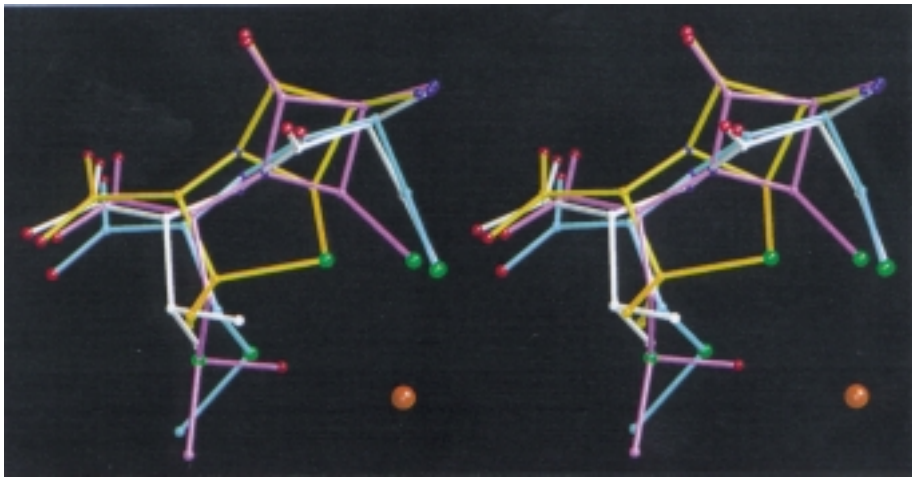


Figure 1. Stereoview of overlaid structures of the active site of IPNS complexed with ACV (white), ACmC (blue), oxidized ACmC (pink), and IPN (yellow). Reprinted with permission from *Nature* (*Nature* **1999**, *401*, 721 – 724), Copyright **1999**, Macmillan Magazines Ltd.

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