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Contrasting Fates for 6-α-Methylpenicillin N upon Oxidation by Deacetoxycephalosporin C Synthase (DAOCS) and Deacetoxy/deacetylcephalosporin C Synthase (DAOC/DACS)

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Abstract—6-α-Methylpenicillin N was synthesised via known routes from 6-aminopenicillanic acid, and tested as a substrate for recombinant DAOCS and DAOC/DACS. Incubation with DAOCS resulted in conversion of 2-oxoglutarate without oxidation of the penicillin substrate ('uncoupled turnover'). Incubation with DAOC/DACS resulted in oxidation to the cephem aldehyde. This is the first example of substrate-induced 'uncoupled turnover', which has been proposed to be an editing mechanism for these enzymes.

Introduction

Iron(II) and 2-oxoglutarate-dependent oxygenases play a key role in the biosynthesis of cephem antibiotics. In *Streptomyces clavuligerus*, two sequence-related enzymes, deacetoxycephalosporin C synthase (DAOCS) and deacetylcephalosporin C synthase (DACS), are responsible for the ring-expansion and hydroxylation of penicillin N to give the key intermediate deacetylcephalosporin C (DAC). This is then converted to cephamycin C via a series of reactions. In *Cephalosporium acremonium* a single bifunctional enzyme, deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS), which has primary sequence homology to the two monofunctional *S. clavuligerus* enzymes, catalyses both the ring-expansion and hydroxylation reactions. Cephalosporin C is formed by acetylation of DAC.[1,2]

Stereochemical studies on the ring-expansion reaction catalysed by DAOC/DACS are consistent with a radical mechanism.[3,4] Radical mechanisms have also been proposed for the hydroxylation reactions catalysed by the related enzymes γ-butyrobetaine hydroxylase[5] and clavaminate synthase.[6] Even though the crystal structure of DAOCS has been determined,[7,8] many of the details of its reaction mechanism remain obscure. However, recent crystallographic studies have suggested a radical mechanism operates throughout the ring-expansion reaction, with a ferric hydroxide complex abstracting a hydrogen atom to give DAOC in the final step.[9]

The ring-expansion and hydroxylation reactions catalysed by DAOCS, DACS and DAOC/DACS all potentially follow radical mechanisms. However, in order to determine how 2-oxoglutarate conversion is coupled to oxidation of the penam or cephem substrates, and which of the two reactions is performed by DAOC/DACS, crystallographic information about substrate and product complexes of both these enzymes is required in conjunction with functional and kinetic studies. Obtaining crystals of the enzyme–penicillin N complexes is difficult on account of the inherent instability of these compounds under the required aqueous conditions. In order to facilitate crystallisation of this complex, synthesis of the stabilised substrate 6-α-methylpenicillin N was undertaken, and this analogue has now been evaluated as a substrate for both DAOCS and DAOC/DACS. The results reveal a fundamental difference in reactivity between the two enzymes, in what appears to be the first example of a substrate being 'proof-read'[8] during the DAOCS-catalysed ring-expansion reaction.

Synthesis of 6-α-Methylpenicillin N

6-α-Methylpenicillin N (**1**) was prepared from 6-aminopenicillanic acid (6-APA, **2**) and D-α-aminoadipic acid

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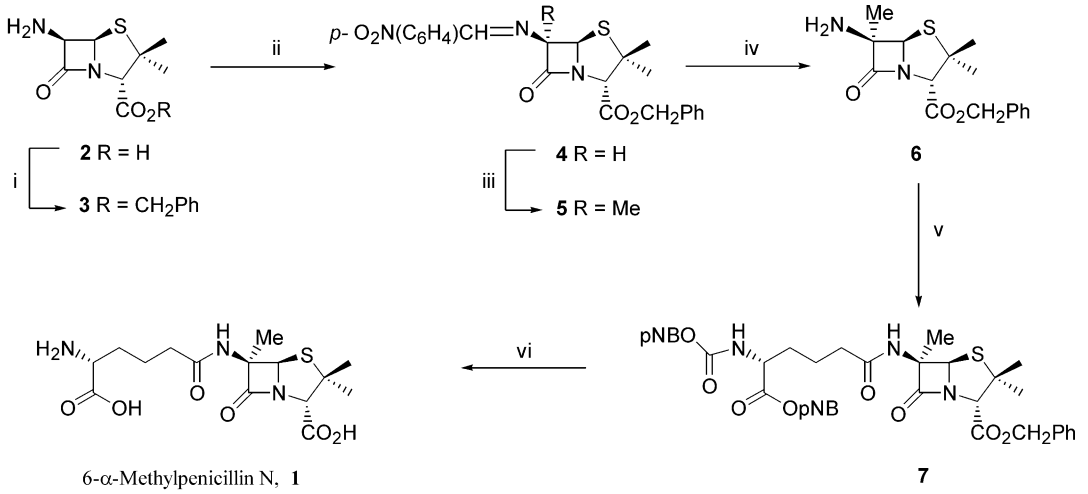
in six steps in 55.5% overall yield, using methods modified from those of Firestone[10,11] (Scheme 1). 6-APA (**2**) was protected as the benzyl ester (**3**), and converted into the *p*-nitrobenzylidine imine (**4**). Methylation to **5** followed by imine hydrolysis afforded the free amine (**6**), for coupling with diprotected D-α-aminoadipic acid. HBTU [*O*-(1H-benzotriazol-1-yl)-*N*,*N*′,*N*′-tetramethyluronium hexa-fluorophosphate]-mediated coupling of the two portions to **7**,[12] followed by hydrogenolytic deprotection[13] and HPLC purification gave 6-α-methylpenicillin N (**1**).[14]

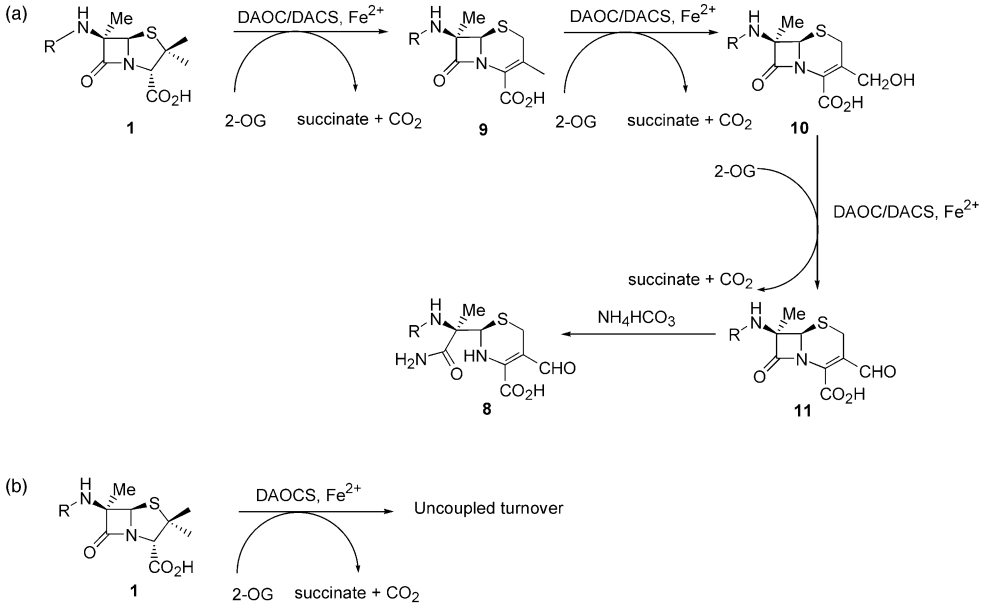
Incubation of 6-α-Methylpenicillin N with Enzymes

All incubations were carried out using highly purified recombinant enzymes.[15] When 6-α-methylpenicillin N (**1**) was incubated with DAOC/DACS, the product isolated was the ring-opened aldehyde (**8**) (Scheme 2).[14] This product parallels that observed with penicillin N,[16] and is probably produced via **9**, **10**, and **11**. The latter apparently undergoes facile non-enzymatic ring opening in the presence of NH_4HCO_3 buffer. A small amount of 6-α-methylpenicillin N (**1**) was recovered from the crude reaction mixture. No evidence for the presence of **9** or **10** was obtained, suggesting that the ring-expansion reaction was rate limiting.

In contrast, ^{1}H NMR analyses showed no evidence for the conversion of 6-α-methylpenicillin N (**1**) to 6-α-methyl-DAOC (**9**) by DAOCS (Scheme 2). 2-Oxoglutarate utilisation assays[17] with both DAOCS and DAOC/DACS showed that the presence of 6-α-methylpenicillin N (**1**) stimulated co-substrate conversion markedly. Parallel incubation studies suggest that the oxidation of



Scheme 1. Synthesis of 6-α-methylpenicillin N (**1**). Reagents and conditions: (i) Et_3N, $PhCH_2Br$, acetone, 0 °C, then rt, 2 days, 53%; (ii) *p*-nitrobenzaldehyde, $MgSO_4$, CH_2Cl_2, rt, 16 h, 94%; (iii) PhLi, THF, −78 °C, then MeI, −78 °C to rt, 0.5 h, 95%; (iv) *p*-TsOH, Girard's Reagent P, CH_2Cl_2/EtOH, 35 °C, 18 h, 55%; (v) *N*-(*p*-nitrobenzyloxycarbonyl)-D-α-aminoadipic acid 1-*p*-nitrobenzyl ester, Et_3N, HBTU, CH_3CN, rt, 16 h, 52%; (vi) H_2, Pd/C, $NaHCO_3$, THF/H_2O, rt, 24 h, then HPLC purification [ODS (250×10 mm), gradient elution with 0–25% (v/v) acetonitrile in 10 mM aq NH_4HCO_3, 4 mL/min, λ = 254 nm, 5 AUFS], 41%.



Scheme 2. Conversion of 6-α-methylpenicillin N (**1**) with (a) DAOC/DACS and (b) DAOCS. 2-OG, 2-oxoglutarate; R, D-δ-(α-aminoadipoyl).

6-α-methylpenicillin N (**1**) by DAOCS is significantly uncoupled from 2-oxoglutarate conversion, with a coupling ratio of >1:29. In contrast, the oxidation of both penicillin N and penicillin G by DAOCS is tightly coupled to 2-oxoglutarate conversion.[9,18] Thus, a large change in catalytic efficiency has been brought about by an apparently minor modification to the structure of the natural substrate.

Discussion and Conclusions

While DAOCS and DAOC/DACS have similar biological functions, similar amino acid sequences and by extrapolation similar structures, they apparently exhibit significantly different substrate specificities. DAOC/DACS reacts with a much broader range of substrates than DAOCS, a fact most graphically illustrated by the bifunctionality of the former enzyme and the contrasting monofunctionality of the latter. Further structural information is required before the precise molecular basis for this difference in selectivity can be fully understood. In particular, a structure for DAOC/DACS and structures of enzyme–substrate complexes for both enzymes are needed. However, in the absence of these structures, the results reported here provide further insight into the different selectivities of the two enzymes.

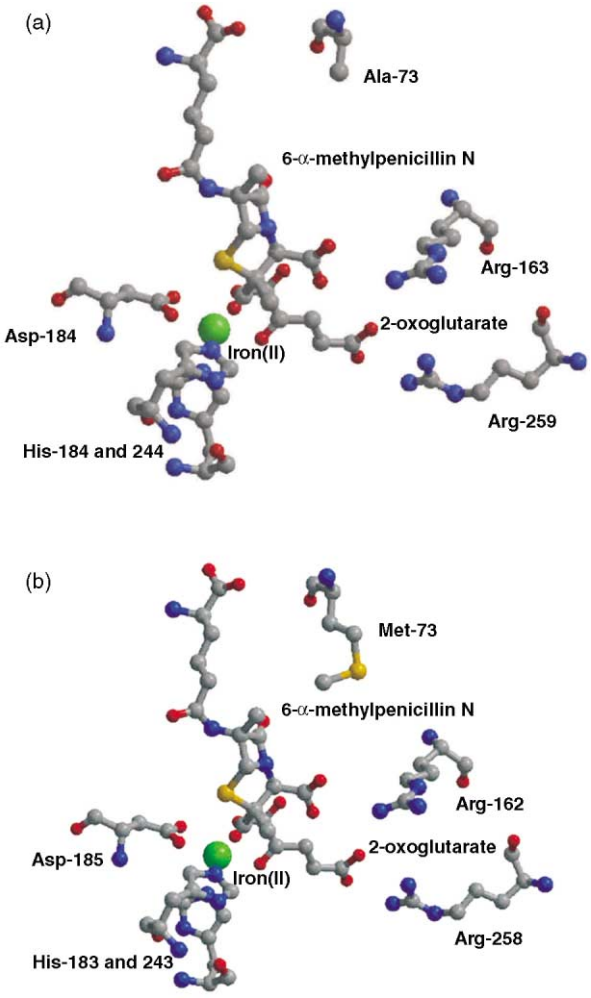


Figure 1. Binding of 6-α-methylpenicillin N (**1**) in the active site of: (a) DAOCS; (b) DAOC/DACS. Arg-160 (DAOCS) or Arg-161 (DAOC/DACS) have been removed for clarity, but are situated directly behind Met/Ala-73. Figures represent models (ref 21) and were produced using Bobscript.[26,27]

The differing reactivity of DAOCS and DAOC/DACS with 6-α-methylpenicillin N (**1**) reflects a subtle difference in the active site geometries of the two proteins. This difference in geometry is necessitated by the requirement to accommodate a concave bicyclic penicillin nucleus versus a 'flattened' unsaturated cephem nucleus. DAOC/DACS accepts the analogue (**1**) as a substrate in its active site and performs the ring expansion and hydroxylation reactions on it, leading ultimately to the aldehyde product (**8**). In contrast, the uncoupled turnover observed with DAOCS indicates that 6-α-methylpenicillin N (**1**) binds in the active site well enough to stimulate 2-oxoglutarate conversion, but not sufficiently tightly to be converted itself. It then apparently dissociates without any other reaction occurring. A similar difference in reactivity between the two enzymes has been previously reported with a range of substrate analogues[19] (although it is difficult to be certain whether this is truly a difference in reactivity or more a reflection of the limitations of the various assays used[20]). However, the structural difference between the 6-methyl analogue (**1**) and the natural substrate penicillin N is comparatively small, and thus **1** could be expected to react in an analogous manner with both enzymes.

To date no X-ray crystallographic structure of DAOC/DACS has been solved, but the enzyme shows high levels of primary sequence homology with DAOCS. In order to understand the molecular basis for the different fates of 6-α-methylpenicillin N (**1**) with these two enzymes, a model of the DAOC/DACS structure was constructed[21] and compared with the crystal structure of DAOCS. This model suggests that the majority of differences occur at the surface of the protein. Comparison of the sequences for the regions responsible for binding iron(II), 2-oxoglutarate (and substrate) suggests that the active sites of the two enzymes are extremely similar (Fig. 1).

Recent results[22] support the hypothesis that binding of the bicyclic penicillin nucleus in DAOCS is mediated by Arg-162 and Arg-160, as had previously been proposed.[7,8] Assuming this hypothesis is correct, then the only substitution within close proximity of the substrate 6-methyl group occurs at residue 73: a methionine in DAOCS versus alanine in DAOC/DACS (Fig. 1). It is plausible that the 6-α-methyl group results in unfavourable steric interactions between the side chain of Met-73 and the penicillin substrate in the active site of DAOCS. In DAOC/DACS, however, the small alanine side chain occupies the same position and thus unfavourable steric interactions will be diminished.

However, DAOCS possesses four disordered loops within the crystal structure[7] (residues 83–90, 166–177, 196–200 and 249–257), and all but residues 196–200 are within close proximity of the penicillin binding site. It is probable that the three other disordered loops will

become ordered on penicillin binding, thus isolating the active site during catalysis. Further experiments are required in order to determine whether these residues are important for efficient coupling of 2-oxoglutarate conversion to penicillin oxidation.

The factors that control the coupling of 2-oxoglutarate conversion to penicillin oxidation by DAOCS are subtle and hitherto poorly defined. However, it is known that modifications to either the C-terminus[9] or Arg-258[23] (the residue that binds the 5-carboxylate group of 2-oxoglutarate) both lead to substantial uncoupling of these reactions. Low levels of uncoupled 2-oxoglutarate conversion have been proposed to be a mechanism of 'proof-reading' in order to deselect incorrect substrates, or correct substrates incorrectly bound.[8]

More information, and in particular structural information, is required before the factors behind the coupling and uncoupling of these processes can be fully elucidated. However, the differing fates of 6-α-methylpenicillin N (**1**) upon oxidation with DAOCS and DAOC/DACS brings some further insight to this interesting puzzle.

Acknowledgements

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14. ^{1}H NMR (200 MHz, D_2O) δ 1.35 (3H, s, Me), 1.43 (3H, s, Me), 1.57 (3H, s, Me), 1.46–1.88 (4H, m, $CHCH_2CH_2CH_2CONH$), 2.23 (2H, t, *J* 7.0 Hz, CH_2CH_2CONH), 3.57 (1H, m, HO_2CCHNH_2), 4.11 (1H, s, H-3), 5.27 (1H, s, H-5). IR (KBr disc) 1764 cm^{-1}, strong (β-lactam C=O).
15. DAOCS and DAOC/DACS were produced using *Escherichia coli* BL21 (DE3) with the pET24a-derived plasmids pHL1 and pKC200 (constructed by Dr. K. S. Hewitson, University of Oxford), respectively. Purification to ca. 95% purity was achieved using anion-exchange and gel filtration chromatographies.

6-α-Methylpenicillin N (**1**) (1.5 mg) was incubated with DAOCS (6.47 mg, 116 IU/mg using penicillin G as substrate[20]) as previously reported[8] for 90 min. ^{1}H NMR analysis (500 MHz) of the quenched reaction suggested <2% conversion to cephem products.

6-α-Methylpenicillin N (**1**) (1.5 mg) was incubated[8] with DAOC/DACS (2.86 mg, 135 IU/mg with 6-α-methylpenicillin N as substrate[20]). ^{1}H NMR analyses (500 MHz) of the quenched reaction mixture suggested >95% conversion to (**8**). HPLC purification [ODS (250×4.6 mm), isocratic elution with 25 mM NH_4HCO_3 and 1% (v/v) acetonitrile, 1 mL/min, λ = 254 nm, 2 AUFS] resulted in isolation of (**8**) (107 μg) with a retention volume of 4.4 mL: ^{1}H NMR (500 MHz, D_2O) δ 1.55–1.65 (5H, m, Me and CH_2CH_2CONH), 1.70–1.85 (2H, m, $CH_2CH_2CH_2CONH$), 2.35 (2H, m, CH_2CONH), 3.28 (0.6H, s, residual MeOH), 3.40 (1H, d, *J* 17 Hz, one of SCH_2), 3.50 (1H, m, H_2NCH), 3.55 (1H, d, *J* 17 Hz, one of SCH_2), 4.97 (1H, s, $NHCHSCH_2$), 9.05 (1H, s, CHO); *m*/*z* (−ve ESI MS) 401.33 ([M−H]$^-$, 100%; calcd 401.4). A small amount of **1** was recovered as a broad peak with a retention volume of 6.6–7.0 mL: *m*/*z* (−ve ESI-MS) 372.3 [M−H]$^-$, (100%).
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21. The structure of DAOC/DACS was modelled from the DAOCS: iron(II):2-oxoglutarate complex (1rxg.pdb) with the programme O,[24] followed by model refinement within CNS.[25] Residues 310–332 of DAOC/DACS could not be adequately modelled, but are predicted to form at least one further helix. Models for 6-α-methylpenicillin N and dioxygen were produced using Quanta (Molecular Simulations), docked into the protein structures and refined.
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