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Crystallographic studies on the binding of selectively deuterated LLD- and LLL-substrate epimers by isopenicillin N synthase

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ABSTRACT

Isopenicillin N synthase (IPNS) is a non-heme iron(II) oxidase which catalyses the biosynthesis of isopenicillin N (IPN) from the tripeptide δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (LLD-ACV). Herein we report crystallographic studies to investigate the binding of a truncated LLL-substrate in the active site of IPNS. Two epimeric tripeptides have been prepared by solution phase peptide synthesis and crystallised with the enzyme. δ-L-α-Aminoadipoyl-L-cysteinyl-D-2-amino-3,3-dideuteriobutyrate (LLD-ACd_2Ab) has the same configuration as the natural substrate LLD-ACV at each of its three stereocentres; its epimer δ-L-α-aminoadipoyl-L-cysteinyl-L-2-amino-3,3-dideuteriobutyrate (LLL-ACd_2Ab) has the opposite configuration at its third amino acid. LLL-ACV has previously been shown to inhibit IPNS turnover of its substrate LLD-ACV; the all-protiated tripeptide δ-L-α-aminoadipoyl-L-cysteinyl-D-2-aminobutyrate (LLD-ACAb) is a substrate for IPNS, being turned over to a mixture of penam and cepham products. Comparisons between the crystal structures of the IPNS:Fe(II):LLD-ACd_2Ab and IPNS:Fe(II):LLL-ACd_2Ab complexes offer a possible rationale for the previously observed inhibitory effects of LLL-ACV on IPNS activity.

1. Introduction

Isopenicillin N synthase (IPNS) is a non-heme iron(II) oxidase (NHIO) central to the biosynthesis of penicillin and cephalosporin antibiotics [1]. IPNS catalyses the oxidative bicyclisation of the linear tripeptide δ-L-α-aminoadipoyl-L-cysteinyl-D-valine (LLD-ACV, **1**) to isopenicillin N (IPN, **2**) (Scheme 1A). This unusual transformation takes place in a single step via a putative iron(IV)-oxo intermediate [2,3], with one molecule of oxygen fully reduced to two molecules of water in the process [1]. It is broadly accepted that non-heme iron oxidases generate highly reactive iron(IV)-oxo (ferryl) species in their reaction cycles [4,5].

IPNS has been studied extensively over a number of years [1,6,7], on account of the unique transformation that it catalyses and the importance of this reaction to the industrial preparation of β-lactam antibiotics for the clinic. The IPNS mechanism has been investigated primarily using solution-phase incubation experiments [6], solution-phase spectroscopy [7], and protein crystallography [8–14]. Incubation experiments in solution and *in crystallo* involve synthesizing analogues of ACV and incubating them with IPNS, then deducing mechanistic information by analysing the incubation products formed using either NMR for solution-phase experiments or crystallography for solid-phase experiments.

Reaction of the truncated analogue δ-(L-α-aminoadipoyl)-L-cysteinyl-D-α-aminobutyrate (LLD-ACAb **3**) with IPNS gave three products: the epimeric C2-methyl penams α-methyl **4** and β-methyl **5**, plus cepham **6** (Scheme 1B) in a 1:7:3 product ratio (α-methyl penam:β-methyl penam:cepham) [15,16]. LLD-ACAb analogues in which the α-aminobutyrate residue is stereospecifically deuterated at its β-carbon have been incubated with IPNS, and intriguingly both isomers give the same β-methyl penam product **7** (Scheme 1B) [17]. In contrast IPN formation from ACV is highly stereoselective with respect to thiazolidine closure [18], presumably because the bulk of the isopropyl side-chain restricts its ability to rotate within the IPNS active site [2,19,20]. β-Methyl penam and cepham natural products similar to **5** and **6** but bearing a D-α-aminoadipoyl side-chain have been isolated from fermentations of *Streptomyces ACC* 13285 [21], and are presumably formed by IPNS-mediated cyclization of ACAb followed by side-chain epimerisation [1,21].

ACAb **3** lacks one methyl group relative to ACV **1** – the α-aminobutryate residue presents an ethyl group in place of the isopropyl group of valine – and it has been postulated that the penam:

Abbreviations: AC6FV, δ-α-aminoadipoyl-cysteinyl-3,3,3,3′,3′,3′-hexafluorovaline; ACA, δ-α-aminoadipoyl-cysteinyl-alanine; ACd_2Ab, δ-α-aminoadipoyl-cysteinyl-2-amino-3,3-dideuteriobutyrate; ACG, δ-α-aminoadipoyl-cysteinyl-glycine; ACOmC, δ-α-aminoadipoyl-cysteine (1-carboxy-2-thiomethyl)ethyl ester; ACV, δ-α-aminoadipoyl-cysteinyl-valine; IPN, isopenicillin N; IPNS, isopenicillin N synthase; NHIO, non-heme iron(II) oxidase.

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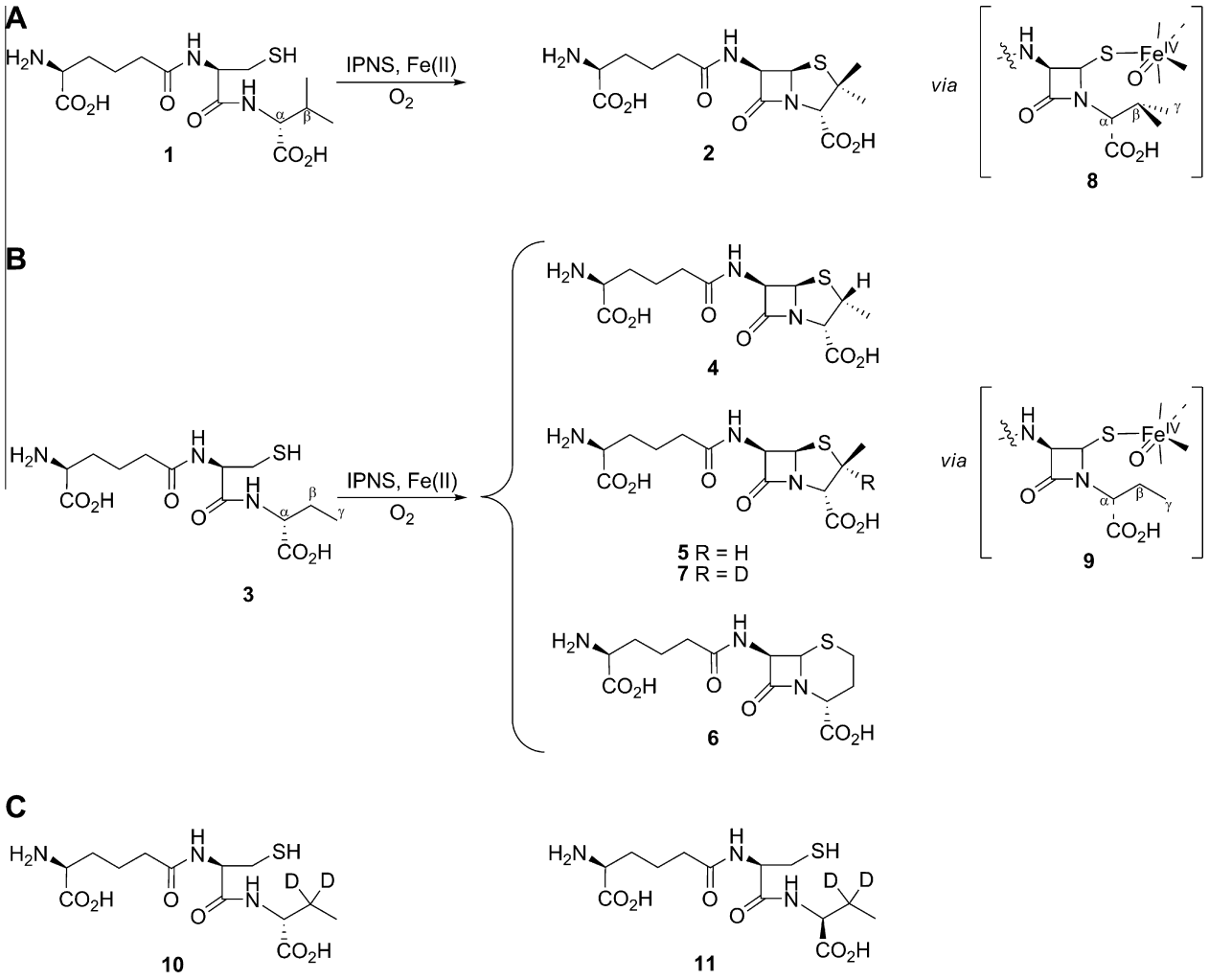


Scheme 1. (A) The reaction of IPNS with its natural substrate LLD-ACV **1** to give IPN **2**; (B) IPNS-mediated turnover of LLD-ACAb **3** gives a mixture of two epimeric penams **4** and **5** plus the cepham **6**; (C) the selectively deuterated ACAb analogues used in this study LLD-ACd_2Ab **10** and LLL-ACd_2Ab **11**.

cepham product ratio observed with ACAb is controlled by the relative energy barriers to reaction of the iron(IV)-oxo intermediate with primary and secondary C—H bonds [22,23]. Compare the structures of the putative intermediates **8** and **9** formed in the reaction of IPNS with ACV **1** and ACAb **3** respectively (Scheme 1): the iron(II)-oxo moiety must distinguish between primary and tertiary C—H bonds in the first case, but between primary and secondary in the second. Crystallographic experiments and modelling studies based on the crystal structures have provided a structural rationale for the diminished product selectivity shown by IPNS in reaction with ACAb **3** and deuterated isotopomers [24].

The selectively di-deuterated substrate analogue δ-L-α-aminoadipoyl-L-cysteinyl-D-2-amino-3,3-dideuteriobutyrate (LLD-ACd_2Ab **10**) was conceived of as a mechanistic trap to probe the reaction of IPNS with ACAb **3** and isotopomers: by replacing both β-hydrogen atoms with deuterium it was envisaged that the resulting kinetic isotope effect would slow reaction at the β-position of ACAb, increasing reaction at the γ-carbon to probe cepham formation from these substrates. However attempts to trap intermediates in the reaction cycle of LLD-ACd_2Ab **10** with IPNS *in crystallo* have not been successful, returning only complex mixtures of products at the enzyme active site. Thus attention has turned instead to the stereochemistry of the third amino acid in this truncated analogue, and we report herein crystal structures of IPNS in complex with the epimeric, truncated substrate analogues LLD-ACd_2Ab **10** and δ-L-α-aminoadipoyl-L-cysteinyl-L-2-amino-3,3-dideuteriobutyrate (LLL-ACd_2Ab **11**).

2. Materials and methods

2.1. Synthesis of LLD-ACd_2Ab **10** and LLL-ACd_2Ab **11**

Commercially available 1,1-dideutereio ethanol **12** was treated with sodium hydride and *p*-toluenesulfonyl chloride to afford the corresponding *O*-*p*-toluenesulfonate, which was used to alkylate diethyl acetamidomalonate forming **13**. Acid-mediated deprotection and decarboxylation gave the free amino acid, which was protected as its benzhydryl ester **14** by reaction with *p*-toluenesulfonic acid and diphenyldiazomethane [25]. Coupling of **14** with known dipeptide **15** [20] was achieved using EDCI and HOBt in anhydrous dichloromethane to give the fully protected tripeptide **16** as a mixture of diastereoisomers [26]. Compound **16** was deprotected by heating in refluxing TFA for 30 min, in the presence of anisole as a cation scavenger [27]. The resultant crude mixture of the LLD- and LLL-tripeptides was purified by reversed phase HPLC (10 mM NH_4HCO_3, Hypersil 5 μ C18 column, 250 × 10 mm internal diameter, λ = 254 nm; 4 mL min^{-1}) to afford pure LLL-ACd_2Ab **11** (R_t = 11.3 min) and LLD-ACd_2Ab **10** (R_t = 11.8 min) in a 1:1 ratio. Stereochemical assignment of the two isomers was deduced by

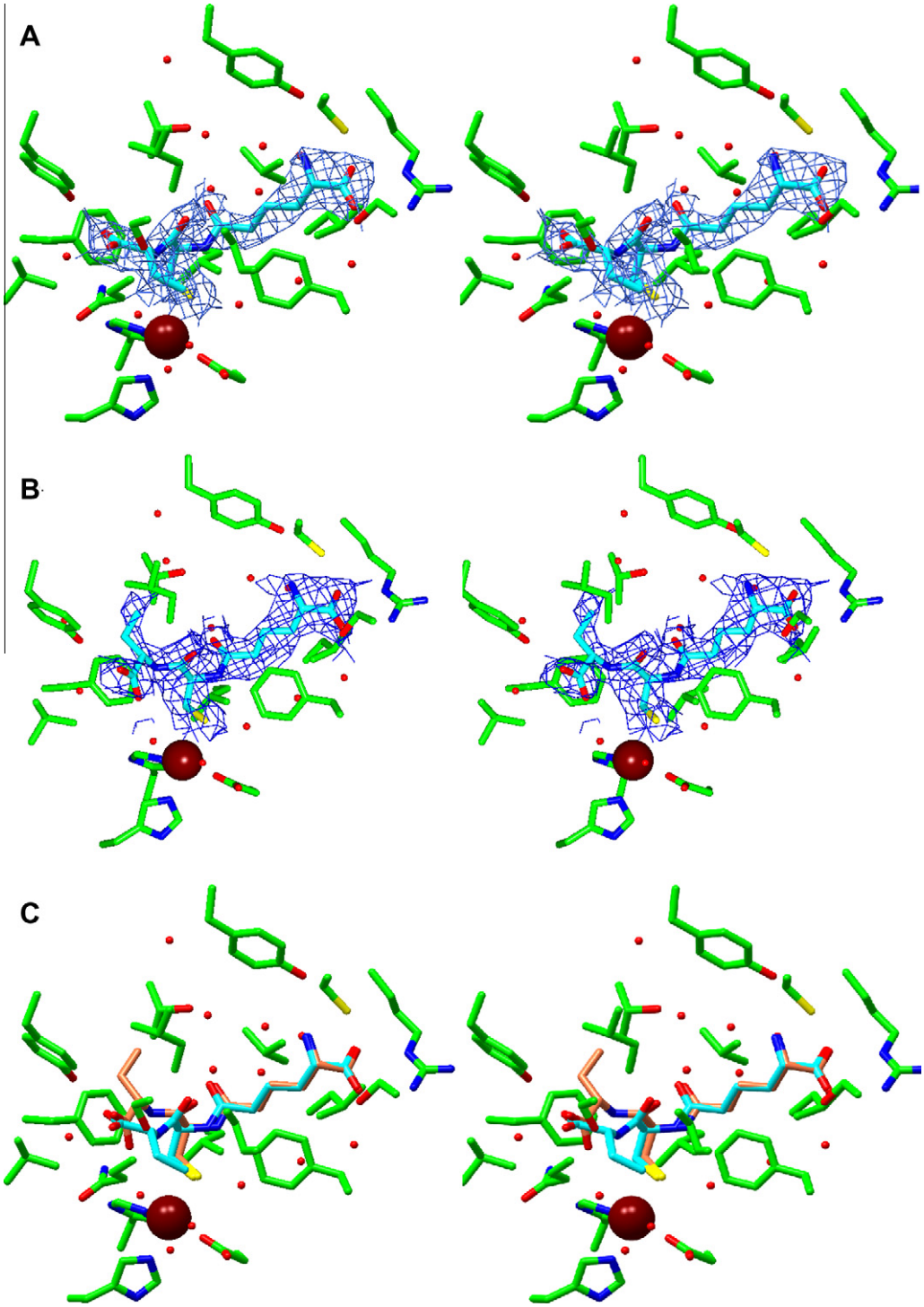


Fig. 1. (A) The active site of anaerobic IPNS:Fe(II):LLD-ACd_2Ab complex; (B) the active site of anaerobic IPNS:Fe(II):LLL-ACd_2Ab complex; (C) overlay showing the relationship between the active site regions of the LLL-ACd_2Ab and LLD-ACd_2Ab complexes. A $2mF_o–DF_c$ map electron density map is shown in blue at 1σ in (A) and (B); the red sphere is iron (II).

analogy with related tripeptides, and confirmed by the X-ray crystal structures of their respective complexes with IPNS.

2.2. Crystallization experiments

Crystals of the IPNS:Fe(II):LLD-ACd_2Ab and IPNS:Fe(II):LLL-ACd_2Ab complexes were grown under anaerobic conditions as reported previously with minor modification [28,29]. Crystals suitable for structure determination were selected using a light microscope, then removed from the anaerobic environment, exchanged into a cryoprotectant solution (1:1 mixture of well buffer: saturated lithium sulfate in 40% v/v glycerol solution), and flash-frozen in liquid nitrogen [28,29].

2.3. Data collection and structure determination

Data were collected at the Synchrotron Radiation Source (SRS), Daresbury, UK at 100 K, with temperature control achieved

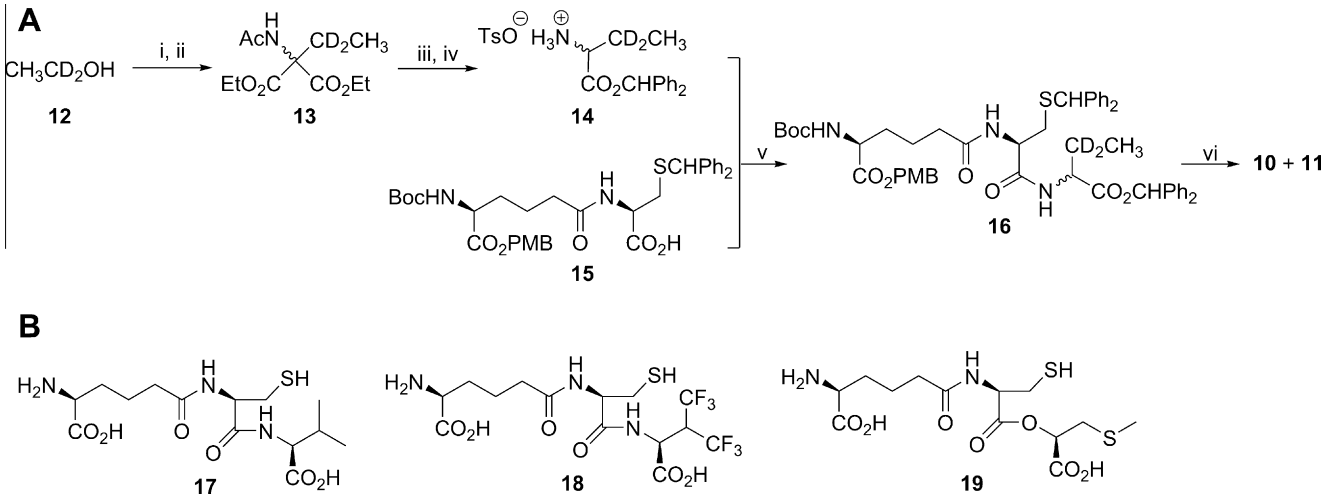
through the use of an Oxford Cryosystems Cryostream. Data processing was carried out using MOSFLM [30] and programs from the CCP4 suite [31]. Refinement was undertaken with REFMAC5 [32], and the program O was used for model building [33]. Initial phases were generated by molecular replacement, using co-ordinates for the protein from the IPNS:Fe(II):ACV structure published previously [19], and manual rebuilding of protein side-chains was performed as necessary. Crystallographic coordinates and structure factors have been deposited in the Worldwide Protein data Bank, accession numbers 2vbp for LLL-complex, 2wo7 for LLD. The colour (Fig. 1) was prepared using the program CCP4mg [34].

3. Results and discussion

It has been reported previously that LLL-ACV **17** – epimeric to the natural substrate LLD-ACV in the configuration of its third residue (valine) – is not turned over by IPNS, but inhibits turnover of the natural substrate [35]. It was proposed at the time and in subsequent work with the LLL-configured substrates δ-L-α-aminoadipoyl-L-cysteinyl-L-3,3,3,3′,3′,3′-hexafluorovaline (LLL-AC6FV **18**) [36] and δ-L-α-aminoadipoyl-L-cysteine (1-(*R*)-carboxy-2-thiomethyl)ethyl ester (LLL-ACOmC **19**) [37] that LLL-substrates cannot be turned over by IPNS because they do not fit into the active site properly.

The novel substrate analogues LLD-ACd_2Ab **10** and LLL-ACd_2Ab **11** were prepared using solution phase peptide synthesis (Scheme 2) and crystallised with IPNS. Plate-shaped crystals of IPNS:Fe(II):LLD-ACd_2Ab **10** and IPNS:Fe(II):LLL-ACd_2Ab **11** were obtained using standard conditions [28], and the structures of the IPNS:Fe(II):LLD-ACd_2Ab and IPNS:Fe(II):LLL-ACd_2Ab complexes were solved to 2.50 Å and 1.50 Å resolution respectively (Fig. 1, Table 1).

The crystal structure of IPNS:Fe(II):LLD-ACd_2Ab (Fig. 1A) mirrors the IPNS:Fe(II):LLD-ACAb structure published previously, as is to be expected [24]. The substrate is bound in the active site by



Scheme 2. (A) Synthesis of tripeptides **10** and **11**. Reagents and conditions: (i) TsCl, NaH, rt, 68%; (ii) diethyl acetamidomalonate, Na, EtOH, 42%; (iii) HBr, 49%; (iv) TsOH, H_2O, then Ph_2CN_2, MeCN, 50%; (v) EDCL, HOBt, Et_3N, 48%; (vi) TFA, anisole, reversed phase HPLC, 20%; (B) 'all L' ACV analogues previously studied: LLL-ACV **17** [35], LLL-AC6FV **18**, [36] and LLL-ACOmC **19** [37].

Table 1
X-ray data collection and crystallographic statistics for IPNS:Fe(II):LLL-ACd_2Ab and IPNS:Fe(II): LLD-ACd_2Ab.

	IPNS:Fe(II):LLL-ACd_2Ab		IPNS:Fe(II):LLD-ACd_2Ab	
X-ray source	SRS, Daresbury, UK		SRS, Daresbury, UK	
Wavelength (Å)	1.488		1.488	
PDB acquisition code	2vbp		2wo7	
Resolution (Å)	1.50		2.50	
Space group	$P2_12_12_1$		$P2_12_12_1$	
Unit cell dimensions (a Å, b Å, c Å)	40.51, 74.34, 100.70		41.29, 74.76, 101.13	
Resolution shell (Å)	29.05–1.50	1.58–1.50	41.50–2.50	2.64–2.50
Total number of reflections	288 319	30 679	50 629	6815
Number of unique reflections	44 669	5345	11 384	1635
Completeness (%)	90.8	76.1	98.8	98.7
Average $I/\sigma(I)$	21.9	7.8	6.0	2.4
R_{merge} (%)[a]	5.3	20.9	20.8	58.9
R_{meas} (%)[b]	5.8	23.1	23.4	66.8
R_{pim} (%)[c]	2.2	9.6	10.5	30.7
R_{cryst} (%)[d]	22.3		18.9	
R_{free} (%)[e]	26.8		25.7	
RMS deviation[f]	0.021; 1.9		0.013; 1.432	
Average *B* factors (Å^2)[g]	12.1; 14.8; 20.1; 27.1		17.1; 17.9; 18.0; 13.4	
Number of water molecules	305		304	

[a] $R_{merge} = \sum_j \sum_h |I_{h,j} - \langle I_h \rangle| \sum_j \sum_h \langle I_h \rangle \times 100$.
[b] $R_{meas} = \sum_{hkl} \sqrt{N/(N-1)} \sum_i |I_i(hkl) - \overline{I(hkl)}| / \sum_{hkl} \sum_I I_i(hkl) \times 100$ [38,39].
[c] $R_{pim} = \sum_{hkl} \sqrt{1/(N-1)} \sum_i |I_i(hkl) - \overline{I(hkl)}| / \sum_{hkl} \sum_i I_i(hkl) \times 100$ [39].
[d] $R_{cryst} = \sum ||F_{obs}| - |F_{calc}|| / \sum |F_{obs}| \times 100$.
[e] R_{free} = based on 5% of the total reflections.
[f] RMS deviation from ideality for bonds (followed by the value for angles).
[g] Average B factors in order: main chain; side-chain; substrate and iron; solvent and sulfate.

coordination of the L-cysteinyl thiol to iron and a salt bridge between the L-α-aminoadipoyl carboxylate and Arg87. The dideutero-ethyl side-chain of the third residue (d_2Ab) is oriented directly towards iron, into the putative oxygen binding site opposite Asp216. However the metal is hexacoordinate, with two water molecules coordinated to it, versus the single aquo ligand in the IPNS:Fe(II):LLD-ACV complex [19]: the smaller size of the aminobutyrate side-chain allows a water molecule to bind opposite Asp216, where the valinyl isopropyl group excludes water from this site when ACV **1** binds. It is important to note that LLD-ACAb and isotopomers are turned over by IPNS [15–17], so the water molecule bound opposite Asp216 does not tightly lock this site nor exclude oxygen binding and turnover.

The IPNS:Fe(II):LLL-ACd_2Ab structure (Fig. 1B) is similar to the LLD-ACd_2Ab complex around its L-α-aminoadipoyl and L-cysteinyl residues (the L-α-aminoadipoyl side-chain is tethered by the salt bridge to Arg87 and the L-cysteinyl sulfur to iron), but substantially different in the region of the third resiδue (d_2Ab). The L-d_2Ab side-chain points away from iron, into a region of space near Tyr189 that is occupied by water molecules in the IPNS:Fe(II):LLD-ACd_2Ab and IPNS:Fe(II):LLD-ACV structures. The L-d_2Ab carboxylate is oriented towards the metal, and hydrogen bonds to a water ligand in the iron binding site opposite Asp216. The dideutero-ethyl side-chain of L-d_2Ab is comfortably accommodated in the region above Tyr189 that has previously been shown to accept the hexafluoro-isopropyl side-chain of **18** and the methylsulfide of **19** [36,37] even without the capacity for new hydrogen bonding or other interactions to compensate for the loss of hydrogen bonded water molecules usually present in this region.

Smaller tripeptides such as δ-L-α-aminoadipoyl-L-cysteinyl-glycine (LL-ACG) and δ-L-α-aminoadipoyl-L-cysteinyl-D-alanine (LLD-ACA) demonstrate a similar conformation when bound in the IPNS active site, with the carboxylate of their third residue oriented towards iron [40]. The smaller, less hydrophobic nature of these side-chains allows extra water molecules into the region opposite Asp216, and the glycinyl carboxylate of ACG is also tied by hydrogen bonding to a water ligand at the iron centre.

4. Conclusion

It has been shown previously that IPNS can tolerate great variation in the third residue of its substrate ACV **1**, reacting with a wide range of D-configured amino acids in the valinyl binding pocket [6]. Numerous analogues bearing a diverse range of side-chains in place of the valinyl isopropyl group are turned over by IPNS, including substrates that incorporate smaller side-chains (e.g. LL-ACG, LLD-ACA and LLD-ACAb discussed above), more sterically demanding alkyl chains (LLD-AC-isoleucine, LLD-AC-*allo*-isoleucine, LLD-AC-α-aminopentanoate), cyclopropyl groups (LLD-AC-cyclopropylalanine, LLD-AC-methylcyclopropylglycine), π-bonds (LLD-AC-vinylglycine, LLD-AC-dehydrovaline, LLD-AC-allylgylcine) and heteroatoms (LLD-AC-*O*-methyl-*allo*-threonine, LLD-AC-*S*-methylcysteine) [6].

However LLL-configured analogues are not substrates for the enzyme, and can inhibit turnover [35]. The crystal structures of IPNS with LLD-ACd_2Ab **10** and LLL-ACd_2Ab **11** reported here demonstrate that for LLL-configured analogues like **11**, the side-chain of the third residue is oriented away from the active site metal, directed instead into a region of the protein above Tyr189. As a result the iron is unprotected: the binding site opposite Asp216 is open and coordinates an extra water ligand. This water molecule is held by hydrogen bonding to the terminal carboxylate of the tripeptide substrate, and the hydrogen bonding network appears robust enough to prevent oxygen from binding. Thus turnover is blocked before it can begin, and LLL-configured analogues inhibit turnover of LLD-substrates.

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