Crystal structure of a clavaminate synthase–Fe(II)–2-oxoglutarate–substrate–NO complex: evidence for metal centred rearrangements

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Abstract Clavaminate synthase (CAS), a 2-oxoglutarate (2OG) dependent dioxygenase, catalyses three steps in the biosynthesis of clavulanic acid. Crystals of CAS complexed with Fe(II), 2OG and deoxyguanidinoproclavaminate were exposed to nitric oxide (NO) acting as a dioxygen analogue. Prior to exposure with NO, the active site Fe(II) is octahedrally coordinated by a water molecule, the 2-oxo and 1-carboxylate groups of 2OG, and the side-chains of an aspartyl and two histidinyl residues. NO binds to the position previously occupied by the 2OG 1-carboxylate concomitant with rearrangement of the latter to the position previously occupied by the displaced water.

Key words: Clavaminate synthase; Clavulanic acid; Dioxygenase; Non-haem; 2-Oxoglutarate; Oxygenase

1. Introduction

Non-haem oxidising enzymes containing mono- and di-nuclear iron centres are established as an important super-family of oxidising enzymes [1]. Like their haem counterparts they catalyse the oxidation of inactivated carbon–hydrogen bonds and ferryl species have been proposed as intermediates. The largest family of non-haem iron oxygenases utilise a single ferrous iron cofactor and 2-oxoglutarate (2OG) as a cosubstrate [2,3]. These oxygenases catalyse a variety of reactions, including hydroxylations, desaturations and oxidative ring closures, in the biosynthesis of signalling molecules, carnitine, antibiotics, and many modified amino acids and peptides. They also catalyse the post-translational hydroxylation of proline residues during collagen biosynthesis and in the hypoxia inducible factor (HIF)-mediated pathway for dioxygen sensing [4–6].

In all but two known reactions catalysed by 2OG dependent and related oxygenases, the two electron oxidation of the 'prime' substrate is coupled to the conversion of 2OG into succinate and CO_2 (Fig. 1a). There is stoichiometric incorporation of one of the oxygen atoms of the dioxygen molecule into succinate and incorporation of the other into the alcohol of the product. Significant levels of incorporation of oxygen from water into the hydroxyl of the product have been observed in the case of bacterial enzymes [7–9], but not in the case of human prolyl-4-hydroxylase [10].

Crystal structures for four members of the 2OG dependent oxygenase family have been reported: deacetoxycephalosporin C synthase (DAOCS) [11,12], anthocyanidin synthase [13], proline-3-hydroxylase [14] and clavaminate synthase (CAS) [15]. Together with that of isopenicillin N synthase [16,17], which is structurally related to the 2OG oxygenases but does not utilise 2OG, these structures reveal a 'jelly roll' core and imply divergent evolution with respect to overall structures within the sub-family of 2OG and related oxygenases [2].

Structures for both CAS [15] and ANS [13,20] in the presence of their prime substrate have been obtained. CAS catalyses three steps during the biosynthesis of the clinically used β-lactamase inhibitor clavulanic acid and other clavams. These steps encompass the hydroxylation of deoxyguanidinoproclavaminate, oxidative ring closure of proclavaminic acid to give dihydroclavaminic acid, and desaturation of the latter to give clavaminic acid (Fig. 1b) [18,19]. The diversity in the type of oxidative reaction catalysed by CAS makes it a useful case study for structural investigations aimed at understanding the general principles of how the substrate and product selectivities of 2OG oxygenases are achieved.

Crystal structures for apo-CAS (PDB accession code: 1DSO), and the CAS–Fe(II)–2OG (1DS1), CAS–Fe(II)–2OG–proclavaminate (1DRT) and CAS–Fe(II)–2OG–(L)-*N*-α-acetylarginine (1DRY) complexes have been reported [15]. In the latter case (L)-*N*-α-acetylarginine substituted for the less readily available natural substrate, deoxyguanidinoproclavaminate. These structures reveal the iron ligands of CAS were provided by the side-chains of His-144, Glu-146 and His-279 and that CAS is unusual amongst the 2OG oxygenases in that it uses a glutamate rather than an aspartate-derived carboxylate iron ligand (Fig. 1c). In the CAS–Fe–2OG structure, the 2OG cosubstrate was bound in a bidentate fashion to the metal, as observed in the DAOCS–Fe(II)–2OG structure. The sixth coordination position *trans* to His-279 in the 2OG complexes was occupied by a water molecule, although its occupancy level was lowered in the case of

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CAS structures in which a prime substrate (proclavaminate or (L)-*N*-α-acetylarginine) was bound. Solution studies [21,22] imply binding of the prime substrate causes displacement of the ligating water and as a result allows binding of dioxygen.

A conundrum is presented by the observation that the relative ligation positions of the 1-carboxylate of 2OG and ligating water were transposed in the structures of CAS complexed with Fe(II) and 2OG (with or without prime substrate) compared to those of both DAOCS (without prime substrate) [11,12] and ANS (with or without prime substrate) [13] (Fig. 1c). For CAS, dioxygen binding and ferryl (Fe(IV) = O ↔ Fe(III)–O•) reaction to/from the position of the ligating water (*trans* to His-279) seemed reasonable since this position projects towards the binding site for the prime substrates. In contrast, structural and modelling studies suggest dioxygen binding in the alternative water ligation position, as observed in DAOCS and ANS, would lead to a ferryl intermediate incorrectly orientated for substrate oxidation. Thus, it was proposed that the ANS and DAOCS structures did not reflect the correct geometry for dioxygen binding or that a rearrangement occurs during catalysis. Here we report a crystallographic study with CAS involving the use of nitric oxide (NO) as a dioxygen analogue revealing an unusual rearrangement that offers the possibility of reconciling previous observations.

2. Materials and methods

2.1. Crystallisation and data collection

Recombinant CAS1 isozyme from *S. clavuligerus* was prepared as reported [15]. Anaerobic crystals of CAS complexed to Fe(II), 2OG and its natural 'hydroxylation' substrate deoxyguanidinoproclavaminate (Fig. 1) for the CAS-catalysed hydroxylation reaction were prepared in a similar manner to that reported for the analogous complex formed with the substrate analogue (L)-*N*-α-acetylarginine [15]. Thus, using the hanging drop vapour diffusion method, crystals were grown by mixing 2 μl of a 20 mg/ml CAS1 solution with 2 μl of the reservoir buffer (100 mM Tris, pH 7.5/2.0 M ammonium sulphate/10% glycerol) [15], and equilibration against the reservoir solution at 20°C. The crystals belong to space group $P2_12_12_1$ and contain one CAS1 molecule per asymmetric unit. The complex of CAS1 with substrate and cofactors was obtained by overnight soaking of crystals in the reservoir solution supplemented with deoxyguanidinoproclavaminate substrate at 50 mM, 2OG at 5 mM and Fe(II) at 5 mM. The soaked crystals were then treated with NO under anaerobic conditions by injecting NO gas (4 ml) under the cover-slip and rapidly resealing the well. After 2 h equilibration crystals were frozen using liquid nitrogen in an anaerobic glove box. Data collection was carried out at < 100 K using Cu $K\alpha$ radiation from a Rigaku RU200H rotating anode generator and a 34.5 cm MAR image plate (wavelength of 1.54 Å). Indexing and integration of data images were carried out using DENZO and data merged with SCALEPACK [23].

2.2. Structure determination

The orientation and position of the CAS1 molecule in the unit cell of the structure were determined using rigid-body refinement using the

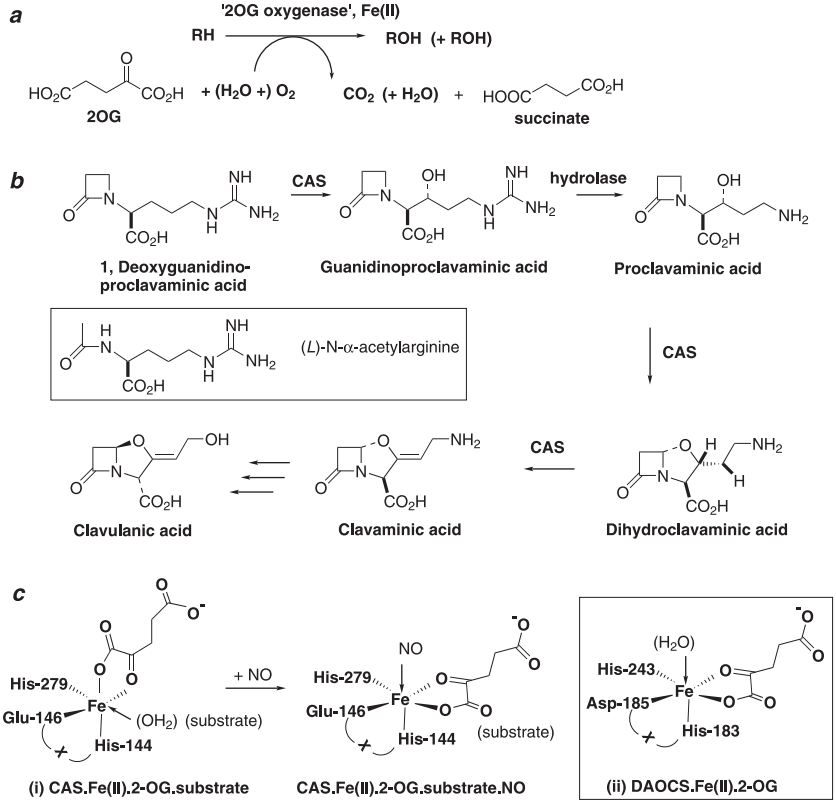

Fig. 1. a: Stoichiometry of a 'typical' hydroxylation reaction as catalysed by a 2OG dependent dioxygenase. Incorporation of oxygen from dioxygen into the alcohol group can be less than stoichiometric with 'exchange' with oxygen from water sometimes occurring (see text). b: The trifunctional role of CAS in the biosynthesis of clavulanic acid. Each CAS-catalysed step is coupled to the conversion of 2OG and dioxygen to succinate and CO_2. The first CAS-catalysed step (hydroxylation) is separated from the latter two (oxidative cyclisation/desaturation) by the action of proclavaminate amidinohydrolase. c: Comparison of the coordination chemistry of (i) CAS and (ii) DAOCS. Binding of NO (a dioxygen analogue) to the iron of CAS results in rearrangement such that the position of the 1-carboxylate of the 2OG relative to the other ligands is the same as observed in the DAOCS–Fe(II)–2OG complex. Note the latter structure was obtained in the absence of the 'prime' penicillin N substrate. For CAS the approximate binding position of substrate relative to ligands is indicated (substrate). ANS has a similar coordination chemistry to that of DAOCS (with the analogous coordinating residues being His-232, Asp-234, His-288), but structures have been obtained in the presence of 'prime substrate'.

Table 1
Data collection and refinement statistics

Data collection	CAS–Fe(II)–2OG–NO–deoxyguanidinoproclavaminate
λ (Å)	1.5418
Unit cell (Å)	64.27, 69.52, 70.19
Resolution range (Å)	30.0–1.54
Observations	301 822
Unique reflections	46 381
Completeness (%)	97.7
Average $I/(\sigma)I$	29.1
R^{a}_{merge}	0.054
Outer resolution shell	Resolution range (Å)
1.60–1.54	
Unique reflections	4 038
Completeness (%)	86
Average $I/(\sigma)I$	3.8
R^{a}_{merge}	0.298
Refinement	
Resolution range (Å)	30.0–1.54
Number of reflections (working/test)	44 008/2 325
$R_{working}/R^{b}_{free}$	0.1813/0.2074
Number of atoms[c]	2 434/74/387RMS bond length deviation (Å)[d]
0.010	
RMS bond angle deviation (°)	1.60

[a] $R_{merge} = \Sigma_h \Sigma_j |I_{j,h} - \langle I_h \rangle| / \Sigma_h \Sigma_j \langle I_h \rangle$, where $I_{j,h}$ are intensities of symmetry redundant reflections and $\langle I_h \rangle$ = mean intensity of reflections.
[b] R-factor $= \Sigma_h ||F_o|_h - |F_c|_h| / \Sigma_h |F_o|_h$ where the sum is over the unique reflections, h. For each data set 5% of reflections were randomly selected and not used in refinement. R_{free} is the R-factor calculated with these reflections, $R_{working}$ is the R-factor calculated from 95% reflections used in refinement.
[c] Protein atoms/substrate, cofactors, glycerol, and sulphate atoms/water atoms.
[d] Root mean square deviation from ideality.

structure of CAS–Fe–2OG (1DS1) as an initial model. The structure was refined with CNS [24] using positional, simulated annealing and individual B-factor refinement with bulk solvent correction and anisotropic B-factor scaling. Models were rebuilt, and water molecules, sulphate ions and glycerol molecules were added using the program O [25] after each round of refinement. The iron–ligand bond lengths were not restrained throughout refinements. In the final model, segment Gly-207 to Ala-214 could not be traced and is presumably disordered. The final model corresponding to an $R_{working}/R_{free}$ of 18.1%/20.75% includes 2434 defined protein atoms and 387 solvent molecules. Crystal data and refinement statistics are given in Table 1. The PDB accession code for the structure is 1GVG.

3. Results and discussion

Anaerobic crystals of CAS complexed to Fe(II), 2OG and deoxyguanidinoproclavaminate (Fig. 1) were prepared as for the analogous complex formed with substrate analogue (L)-*N*-α-acetylarginine [15]. The crystals were then exposed to NO. In most regards, including the orientation of prime substrate binding, the structure obtained after exposure to NO was similar to that obtained in its absence. A clear exception in the region of the iron atom is discussed below (Fig. 2). The 2OG 5-carboxylate is bound in a pocket and is in position to form interactions with the side-chains of Arg-293 and Thr-172. The β-lactam ring of deoxyguanidinoproclavaminate is located in a pocket defined in part by the side-chains of Leu-114, Met-147, Tyr-149, Pro-154, Asp-202, Phe-205 and Tyr-299. Like that of (L)-*N*-α-acetylarginine, the guanidino side-chain of the substrate is in position to form electrostatic and hydrogen bonding interactions with the side-chains of Asp-233 and Asp-202, respectively. The carboxylate of the β-lactam substrate is also in position to bind to the side-chain of Arg-297, via a water molecule with the side-chain of Arg-115 (which is too distant to form a direct interaction), and with the backbone amide NH of Ser-134.

Binding of NO occurs with displacement of water from the position *trans* to His-279 (Fig. 2). In the absence of NO the occupancy level of this water was lower in the presence of prime substrates consistent with solution studies suggesting that dioxygen binding is partly 'triggered' by that of prime substrate [15,21,22]. The electron density map further clearly indicated that NO binding occurs in the position *trans* to His-144 and that the 1-carboxylate of 2OG previously observed in this position has rearranged, via rotation by ca. 40–50° about the C-2 C-3 bond of 2OG, to the position vacated by the ligating water (*trans* to His-279, Fig. 2). The Fe–N distance for NO is 1.79 Å and an Fe–N–O angle of 146° reflects non-linear binding (note that it cannot be certain that NO ligates via its nitrogen rather than its oxygen atom). B-factors for the nitrogen and oxygen atoms of the bound NO are 18.1 Å^2 and 20.1 Å^2, respectively. Refined distances for the other Fe ligands are all within 0.1 Å of those observed in the absence of NO for the CAS–Fe(II)–2OG–(L)-*N*-α-acetylarginine structure. The distance of the assigned oxygen atom of NO to C-2 of 2OG is 2.85 Å and the angle N–O–C, where C is the C-2 carbonyl carbon of 2OG, is ca. 80°. The latter is non-optimal for nucleophilic attack onto the 2OG carbonyl ketone, but it is reasonable to assume that during catalysis dioxygen may adopt a slightly different conformation from that of NO. Transfer of electron density from Fe to dioxygen would promote the nucleophilicity of the latter (or subsequently formed superoxide/peroxide) and enhance the electrophilicity of the carbonyl of the 2OG.

Thus, the coordination position of the 2OG 1-carboxylate in the crystal structures of CAS–Fe(II)–2OG–substrate complexes (i.e. *trans* to His-144, using the CAS nomenclature) differs from that in the CAS–Fe(II)–2OG–substrate–NO, ANS–Fe(II)–2OG–(±substrate), and DAOCS–Fe(II)–2OG structures (i.e. *trans* to His-279). Assuming a common mechanism for 2OG oxygenases two possibilities, both consistent with kinetic studies [26], present themselves for the site of dioxygen binding. Firstly, that the ANS–Fe(II)–2OG–(±substrate), and DAOCS–Fe(II)–2OG structures in which the 2OG 1-carboxylate is *trans* to His-279 do not represent correct coordination states for dioxygen binding, i.e. rearrangement of the 1-carboxylate to the position occurs *trans* to His-144, occurs prior to/concomitant with dioxygen binding. In support of this proposal it may be argued that dioxygen binding and ferryl (Fe(IV) = O ↔ Fe(III)–O$^{\bullet}$) formation *trans* to His-279 are consistent with CAS structures in the absence of NO and lead to a simpler mechanism. Further, it may be argued that NO is an imperfect analogue of dioxygen.

Alternatively and consistent with the CAS–Fe(II)–2OG–substrate–NO structure and reported DAOCS and ANS structures, all the enzymes may bind dioxygen in the position *trans* to His-144 (using the CAS numbering system). The 'problem' with this proposal is that if ferryl formation occurs in this position, it will be incorrectly orientated to react with substrate. In this case the following process is proposed: (i) dioxygen binding to Fe(II) *trans* to His-144, (ii) oxidative decarboxylation to give a ferryl intermediate or equivalent

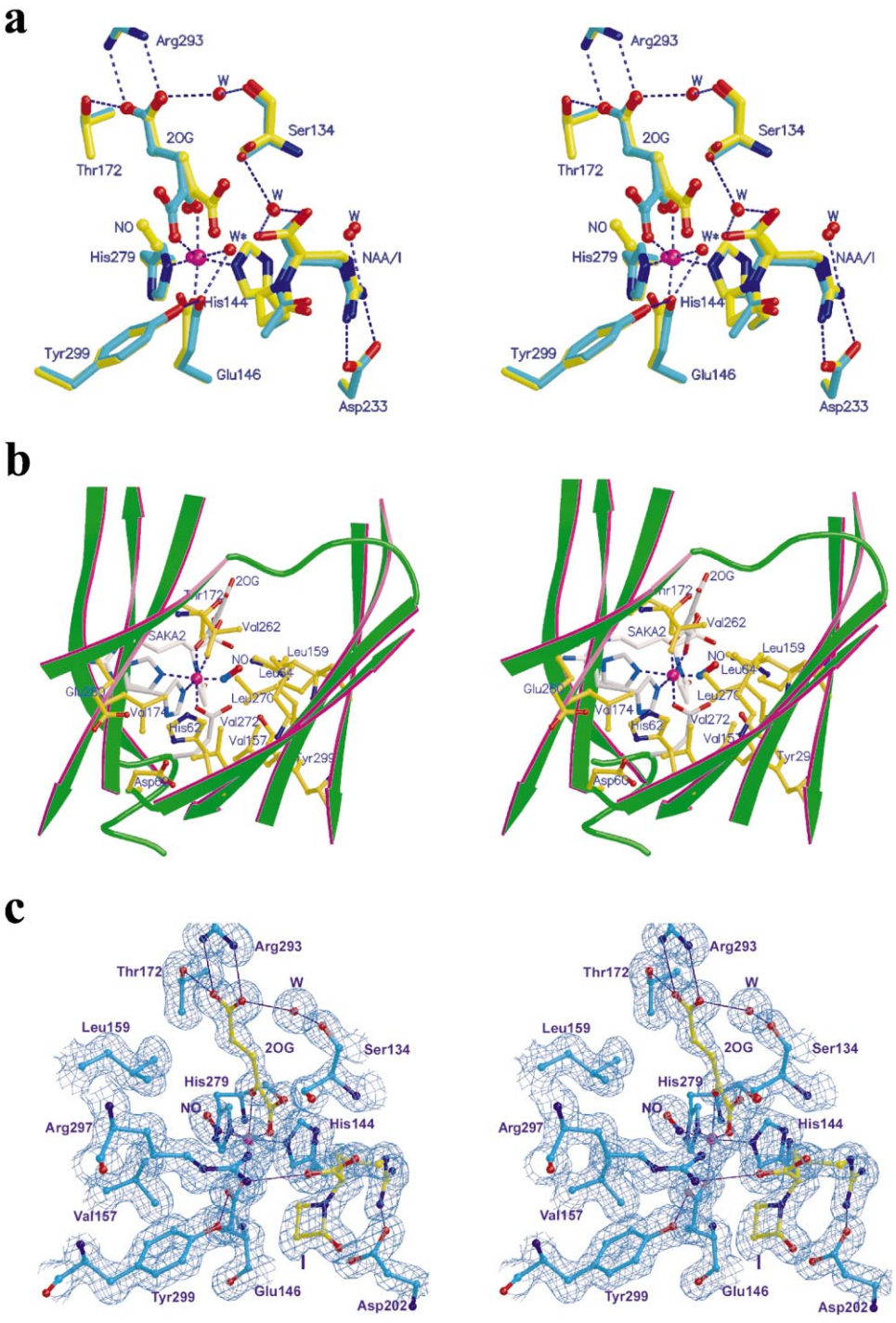

Fig. 2. a: Stereoview of the superimposed structures of CAS–Fe(II)–2OG–deoxyguanidinoproclavaminate (1)–NO and that of CAS–Fe(II)–2OG–L-*N*-α-acetylarginine (NAA). Carbons of the former structure are in yellow while those of the latter are in blue. NO is coloured yellow, iron is in magenta. Dotted lines denote electrostatic or ligating interactions between selected atoms including iron ligands (His-144, Glu-146 and His-279). b: Stereoview of the putative 'tunnel' through the centre of the jelly roll motif through which the NO (and dioxygen) may enter the active site and bind to iron. Note the view is from the opposite side of the active site to the view in a. Selected residues are in gold, the iron is in magenta and carbons for the iron ligands and substrate are in light grey. c: Stereoview of the CAS–Fe(II)–2OG–deoxyguanidinoproclavaminate (1)–NO structure with the $2F_o-F_c$ electron density map contoured at 1.5σ.

in the same position and CO_2 in the position *trans* to Glu-146, (iii) release of CO_2, (iv) intermediate rearrangement ('flip') of the ferryl to the position *trans* to His-279, (v) substrate oxidation via a radical rebound or analogous process and sequential release of product then succinate (Fig. 3). Catalysis proceeding wholly or partly via a mechanism involving a ferryl flip process is attractive since it unites the structural observations, and the presence of a penta-coordinate ferryl species as an intermediate rationalises how exchange of oxygen derived from dioxygen with that derived from water may occur. This mechanism also suggests that dioxygen may approach the iron from the opposing face of the active site to

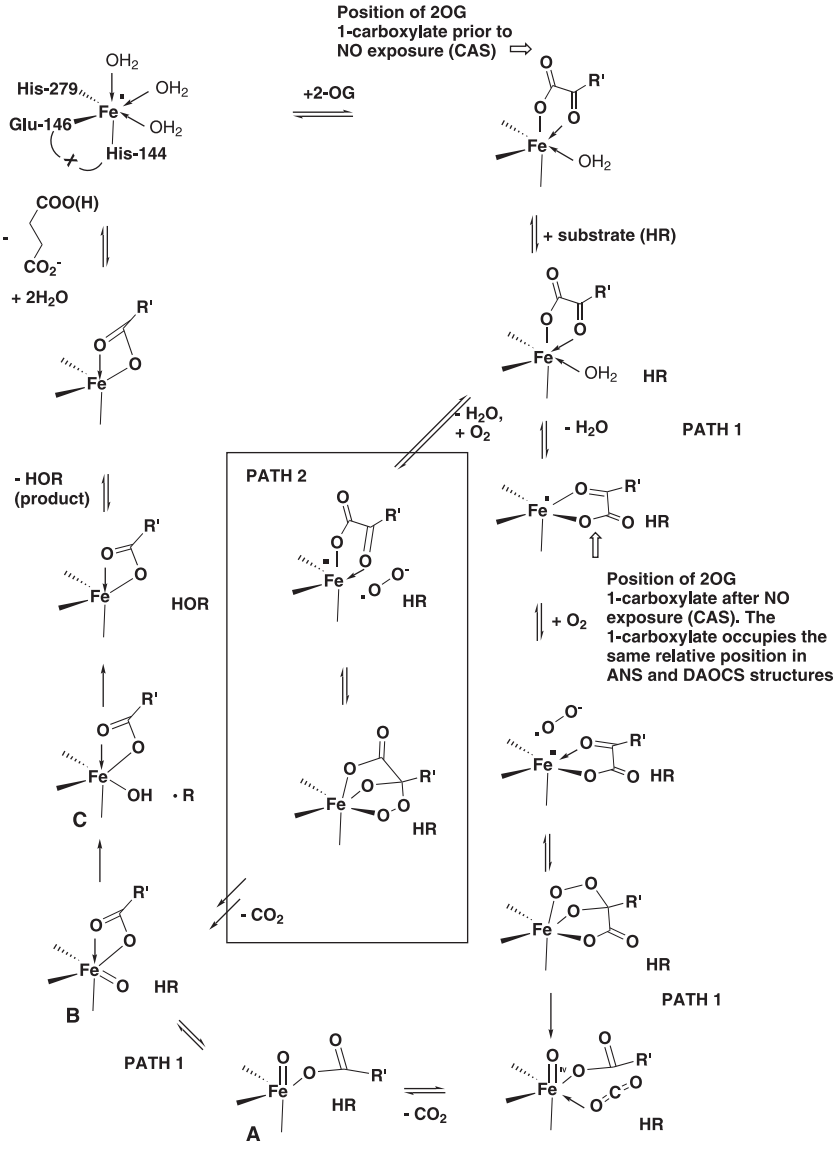

Fig. 3. Proposed mechanistic for the catalytic cycle of 2OG dependent oxygenases involving rearrangement of a ferryl intermediate (path 1). R–H = substrate. $R' = CH_2CH_2CO_2^-$. Exchange of oxygen from dioxygen for that from water may occur at penta-coordinate ferryl intermediates A or B or at a subsequent intermediate C. Catalysis may also proceed (partially) via dioxygen binding and reaction from the ligation position *trans* to His-279 (path 2, see text).

which 2OG and the prime substrate bind. A putative tunnel through the centre of the jelly roll motif lined by predominantly hydrophobic residues (including the side-chains of Leu-64, Val-157, Leu-159, Val-174, Val-262, Val-272 and Leu-270 in CAS) provides a possible mode of entry for dioxygen. The presence of a dioxygen binding tunnel may be related to the dioxygen sensing properties assigned to the HIF prolyl hydroxylase [6]. Dioxygen binding and ferryl formation *trans* to His-144 will also help to isolate the reactive oxidising intermediates both from the environment and, until the 'correct' point in the catalytic cycle, the substrate.

It is recognised that there may not be a unique mechanistic pathway either amongst the family of 2OG oxygenases, i.e. catalysis may proceed via dioxygen binding *trans* to both His-144 and His-279. Further studies are required to test the proposals presented here. However, the results clearly reveal the potential for Fe–O rearrangements during catalysis by 2OG oxygenases, as such a process has been unequivocally observed for the 2OG 1-carboxylate in the crystalline state with complexes of CAS.

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