

Incorporation of oxygen into the succinate co-product of iron(II) and 2-oxoglutarate dependent oxygenases from bacteria, plants and humans

Richard W.D. Welford^{a,1}, Joanna.M. Kirkpatrick^{b,1}, Luke A. McNeill^{b,1}, Munish Puri^c,
Neil J. Oldham^b, Christopher J. Schofield^{b,*}

^a UC Berkeley, Department of Chemistry, Berkeley, CA 94720, USA

^b Chemistry Research Laboratory, Department of Chemistry and Oxford Centre for Molecular Sciences, Mansfield Road, Oxford OX1 3TA, UK

^c Biochemical Engineering Research and Process Development Centre, Institute of Microbial Technology, Chandigarh, India

Received 20 July 2005; revised 17 August 2005; accepted 17 August 2005

Available online 30 August 2005

Edited by Stuart Ferguson

Abstract The ferrous iron and 2-oxoglutarate (2OG) dependent oxygenases catalyse two electron oxidation reactions by coupling the oxidation of substrate to the oxidative decarboxylation of 2OG, giving succinate and carbon dioxide coproducts. The evidence available on the level of incorporation of one atom from dioxygen into succinate is inconclusive. Here, we demonstrate that five members of the 2OG oxygenase family, AlkB from *Escherichia coli*, anthocyanidin synthase and flavonol synthase from *Arabidopsis thaliana*, and prolyl hydroxylase domain enzyme 2 and factor inhibiting hypoxia-inducible factor-1 from *Homo sapiens* all incorporate a single oxygen atom, almost exclusively derived from dioxygen, into the succinate co-product. © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Hypoxia-inducible factor; Hydroxylase; Hypoxia; Oxygenase; 2-Oxoglutarate; Prolyl hydroxylase; Succinate

1. Introduction

Iron(II) and 2-oxoglutarate (2OG) dependent oxygenases form a ubiquitous family of oxidative enzymes that catalyse a diverse range of reactions, most commonly hydroxylations, but other types of reaction including desaturations, epimerisations and rearrangements have also been observed [1–3]. The 2OG oxygenases are found in organisms ranging from bacteria to mammals and play a wide variety of biological roles [4]. All require ferrous iron, dioxygen, and 2OG for full activity; in some cases L-ascorbic acid also is required or beneficial for activity, at least in vitro [5]. During each reaction cycle the 2OG co-substrate is oxidatively decarboxylated to give a succinate co-product and a ferryl species that is proposed to effect two-electron oxidation of the substrate (Fig. 1) [6,7]. The incorporation of oxygen from dioxygen into the alcohol product during hydroxylation reactions by this class of enzymes is well documented [8]. Further, incubations of prokaryotic 2OG oxygenases under an $^{18}\text{O}_2$ atmosphere have demonstrated that,

during hydroxylation reactions, a less than stoichiometric incorporation of oxygen into the hydroxyl group of the product can occur in some cases (e.g., hydroxylation of some substrates catalysed by clavaminic acid synthase and deacetoxy/deacetyl cephalosporin C synthase) [9,10]. This is thought to be due to solvent exchange of one of the reactive iron–oxygen intermediates, although the identity of the particular intermediate(s) that undergo exchange has not been defined. Results with some eukaryotic 2OG dependent hydroxylases, such as thymine 7-hydroxylase (T7H) [11], mammalian type I prolyl 4-hydroxylase [12], enzymes of flavonoid biosynthesis [13] and human prolyl and asparaginyl hydroxylases involved in hypoxic sensing, PHD1 [14] and FIH [15], show that >90% incorporation of oxygen from dioxygen occurs on hydroxylation of their substrates (in the case of some reactions catalysed by the flavonoid oxygenases exchange may occur after initial hydroxylation by a non-oxidative process [13]). This contrasts with results for eukaryotic lysyl hydroxylase where only approximately 10% of ^{18}O was reported to be incorporated into the peptide product [16] (data reviewed in [8]). In the case of taurine dioxygenase and 2,4-dichlorophenoxyacetate oxygenase where inactivating self-hydroxylation of the protein occurs, it was found that no oxygen from dioxygen was incorporated into the modified protein when the reaction was carried out under $^{18}\text{O}_2$ gas. This was proposed to be the result of a relatively long-lived intermediate, which exchanges with or reacts with water before carrying out the self-hydroxylation reaction [17,18].

There is less available data on the incorporation, or otherwise, of the other atom of dioxygen into the succinate co-product. The enzymes thymine-7-hydroxylase and γ -butyrobetaine hydroxylase have been reported to incorporate >95% and 68% ^{18}O from $^{18}\text{O}_2$ into succinate, respectively [11,19]. These data were obtained by mass spectrometric analysis of the succinate co-product derivatised with bis(trimethylsilyl)trifluoroacetamide to give the bis-trimethylsilyl ester. In the case of the enzyme deacetoxy/deacetyl cephalosporin C synthase, greater than 90% ^{18}O incorporation into succinate from $^{18}\text{O}_2$ was observed in experiments analysing the shift in ^{13}C resonance of succinate by NMR spectroscopy [20].

A related reaction to the 2OG dependent dioxygenases is carried out by 4-hydroxyphenylpyruvate dioxygenase, which incorporates both atoms of oxygen from the dioxygen cosubstrate into its product, homogentisate (although exchange

*Corresponding author. Fax: +44 1865 275625.

E-mail address: christopher.schofield@chem.ox.ac.uk (C.J. Schofield).

¹ The contributions of these authors should be considered equal.

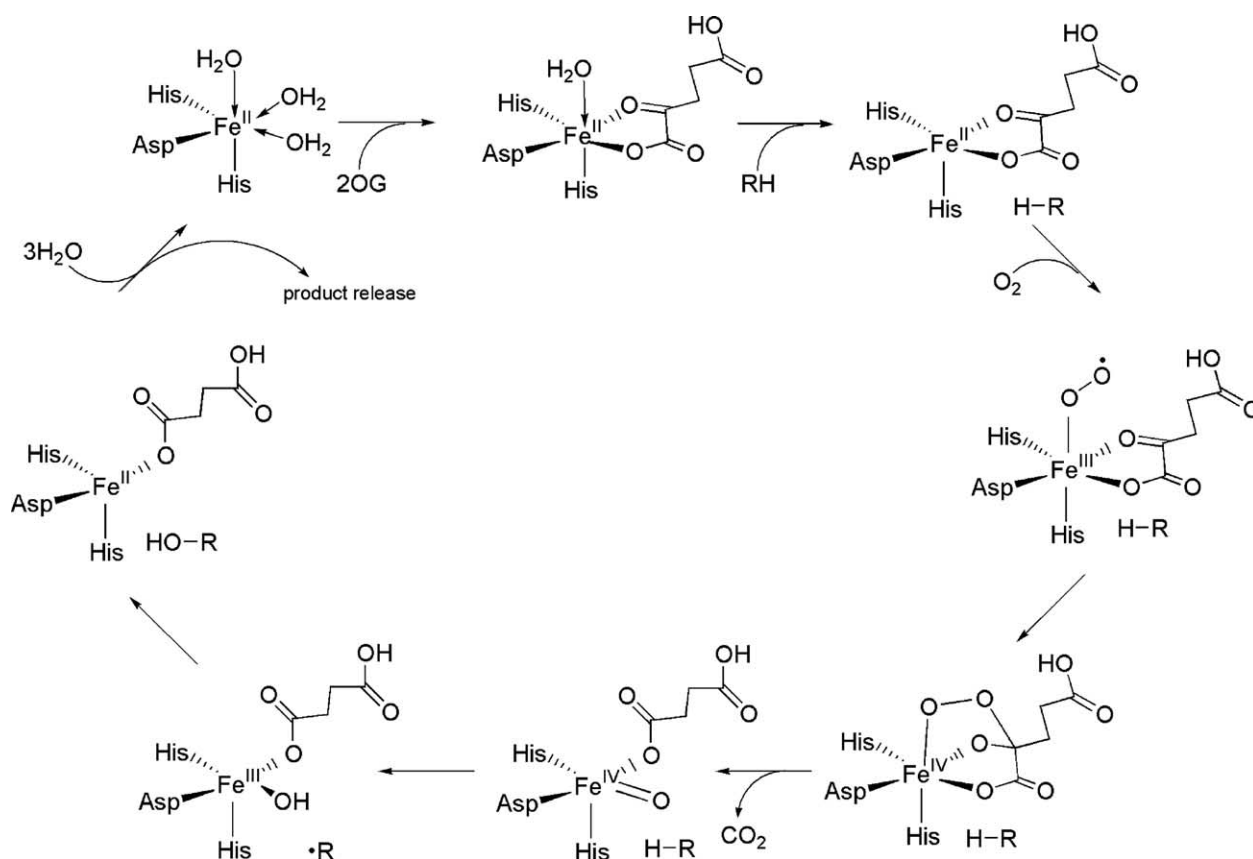


Fig. 1. Outline reaction cycle for a 2OG dependent oxygenase (adapted from [3]). R-H = prime substrate, R-OH = prime product. The resting enzyme binds iron(II) using the 2His-1-carboxylate motif common to the 2OG dependent dioxygenase family. 2OG and dioxygen occupy the final three coordination sites on the iron(II) and form a cyclic intermediate which collapses with the loss of CO₂ to give the reactive ferryl intermediate that carries out the oxidation reaction and the succinate product with one atom from the dioxygen. There are two possibilities for the oxidation, either a stepwise hydroxylation or direct insertion of the oxygen atom into the C-H bond. The choice of pathway followed in these enzymes is unknown, as is the exact timing of CO₂ dissociation and occupation of the vacant sites by water.

occurs of the phenolic oxygen occurs after its introduction) [21].

Here, we report the results of experiments analysing the extent of incorporation of an ¹⁸O label from ¹⁸O₂ and H₂¹⁸O into succinate with various iron(II) and 2OG dependent oxygenases. We selected enzymes from bacteria, plants and humans to provide a representative range of members of the ubiquitous superfamily of 2OG oxygenases. These were the *Escherichia coli* DNA repair enzyme AlkB [22,23], two *Arabidopsis thaliana* enzymes involved in flavonoid biosynthesis [anthocyanidin synthase (ANS) [24] and flavonol synthase (FLS) [13]] and human enzymes involved in oxygen sensing [prolyl hydroxylase domain 2 (PHD2) and factor inhibiting hypoxia-inducible factor (FIH)]. The results demonstrate that in all cases the predominant product is that resulting from greater than 90% incorporation of a single oxygen atom from dioxygen into succinate.

2. Materials and methods

Chemicals were purchased from Sigma Chemical Company, Poole, UK, except for Tris base (ICN Biomedicals), di-ammonium iron(II) sulphate (BDH). Argon was from BOC and ¹⁸O₂ was from CK Gases. Synthetic peptides were from Peptide Protein Research Ltd, Fareham, UK.

Enzymes were purified according to reported procedures: AlkB [25], FLS [13], ANS [24], FIH [15], N-terminally truncated PHD2 [26].

Standard assay conditions for each enzyme were used, the method used for excluding ¹⁶O₂ is described below [13,15,24–26]. AlkB assays were carried out using 1-methyladenosine as a substrate. FLS assays were carried out using (±)-*trans*-dihydroquercetin (DHQ) substrate. ANS assays were carried out with both (±)-naringenin and (±)-*trans*-DHQ as substrates, whereas FIH and PHD2 assays were carried out using synthetic peptides corresponding to the target regions of their protein substrate, hypoxia-inducible factor (DESGLPQLTSYDC-EVNAPI for FIH and DLDLEMLAPYIPMDDDFQL for PHD2).

Reactions carried out under ¹⁸O₂ (g) were set up in a Belle Technology glove box under an atmosphere of argon (0.4–0.8 ppm O₂). Buffer, water, solid 2OG, L-ascorbate and (NH₄)₂SO₄ · FeSO₄ · 6H₂O and all required plastics were ported into the box at least 16 h before use. Enzyme was thawed from –80 °C and ported into the box just prior to setting up incubations. Assays were set up in 5 mL polycarbonate tubes in two drops, one containing enzyme-Fe(II), the other containing L-ascorbate, 2OG and substrate. Tubes were then sealed with a rubber vaccine closure, allowing removal and evacuation of these tubes, followed by refilling with ¹⁸O₂ gas by injection of a syringe of gas through the vaccine closure. ¹⁸O₂ gas was collected in a glass cylinder, sealed at one end with a vaccine closure, over a reservoir of water degassed with He(g) (100 mL/min for 30 min). The cylinder was filled twice with ¹⁸O₂ gas, with only the second being used for the enzyme incubation.

Incubations in H₂¹⁸O were achieved by the use of concentrated solutions of reagents and buffers. The two-drop approach (enzyme/iron and substrate/cofactor) described above was employed but the total volume of the two was less than 5 μL. The reaction was started by addition of 95 μL H₂¹⁸O.

After 30 min incubation with shaking at 37 °C (or 30 °C for ANS and FLS) the samples were quenched with an equal volume of methanol. These mild quenching conditions were employed in order to minimise exchange of the labelled succinate product with solvent. Samples were injected immediately on to a Waters 2790 HPLC fitted with a Machery-Nagel, nucleosil 100-C-18 Nautilus column (125 × 2 mm) running isocratically in a 0.05% (v/v) formic acid aqueous solution, eluting via a flow splitter into a Micromass LCT mass spectrometer in negative ion mode (capillary voltage 2100 V, cone voltage 24 V).

3. Results

An LC/MS technique was developed to study the level of incorporation of oxygen into the succinate product of the enzymes. This enabled the retention of succinate on the C-18 column (Fig. 2A), thus separating it from other small molecules in the reaction such as buffer salts, which passed over the column in the void volume, and also from unreacted 2OG. 2OG was not observed by LC/MS, perhaps due to complexation with a metal ion resulting in an unretained salt, invisible to negative ion ESI-MS.

Analysis of the succinate product of all the enzymes used yielded similar results from incubation under $^{18}\text{O}_2$, namely that

the predominant species (90% or greater) in the negative ion mass spectrum of succinate was that containing a single ^{18}O atom ($[\text{M} - \text{H}]^- = 119$) (Fig. 2C). The very low levels of unlabelled succinate ($[\text{M} - \text{H}]^- = 117$) observed could result from incomplete removal of $^{16}\text{O}_2$ from the enzyme samples, but the possibility of low levels of exchange (<5%) cannot be ruled out. Addition of unlabelled succinate post-quenching to an incubation of AlkB with 1-methyladenosine resulted in an increase in intensity of the 117 Da peak in the mass spectrum, indicating that these species were in fact due to succinate. Table 1 gives the levels of ^{18}O incorporation into succinate in the different experiments. In the cases of ANS (with (±)-naringenin as substrate) and those of PHD2 and FIH, the level of incorporation of ^{18}O into the enzymatic products was also analysed. In each case it was found to be >99% in accord with literature results [13–15]. The corresponding analysis was not carried out with AlkB as the oxygenated product, formaldehyde, undergoes facile exchange [22,23]. Similarly, when ANS and FLS used (±)-DHQ as a substrate, the product was not analysed as the enzyme reaction includes a dehydration process in which the hydroxyl group derived from dioxygen is lost [13].

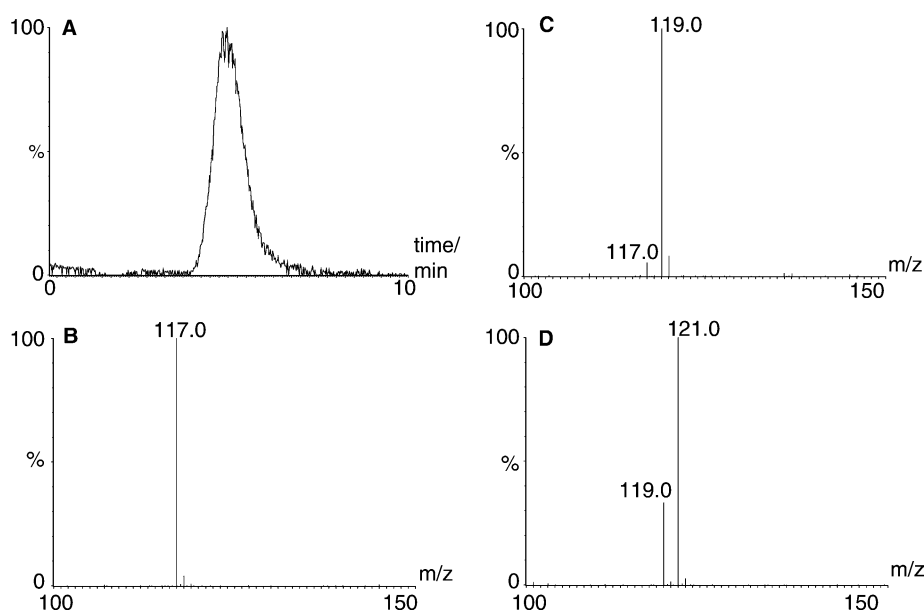


Fig. 2. TIC chromatogram and mass spectra of the succinate products of the PHD2 reaction, which are typical of others. (A) Elution of succinate from the C18 column; (B) mass spectrum of succinate from a reaction under $^{16}\text{O}_2$; (C) mass spectrum of succinate from a reaction under $^{18}\text{O}_2$; (D) mass spectrum of succinate from a reaction under $^{18}\text{O}_2$ and H_2^{18}O .

Table 1
Percentage incorporation of ^{18}O into succinate product from reaction under a $^{18}\text{O}_2$ atmosphere

Enzyme	Substrate	Incorporation of [^{18}O] into succinate (%)	Incorporation of [^{18}O] into product
AlkB	1-methyladenosine	>95	–
ANS	(±)- <i>trans</i> -DHQ	>90	–
ANS	(±)-naringenin	>95	>90 [13]
FLS	(±)- <i>trans</i> -DHQ	>95	–
FIH	DESGLPQLTSYDCEVN*API	>95	>90 [15]
PHD2	DLLEMLAP*YIPMDDDFQL	>95	>98 [14]

Values were calculated by comparison of the integration of the peaks at 117 Da for succinate and 119 Da for [^{18}O]-succinate. Where appropriate, values of incorporation into alcohol products are given. * indicates residue that is hydroxylated in peptides: at the pro-S β-position of asparagine for FIH [30] and at the *trans*-4 position of proline for PHD2 [14].

When incubations were carried out in the presence of H_2^{18}O , ($\text{H}_2^{18}\text{O}:\text{H}_2^{16}\text{O} = \text{ca. } 19:1$ in the final incubation mixture), incorporation of ^{18}O into succinate was observed, but no incorporation of label into the peptide products. In the case of PHD2, a high level of incorporation (approximately 80%) of ^{18}O from H_2^{18}O was seen into succinate, but for FIH, the level was no higher than 50%. This incorporation was thought to result from exchange of oxygen from bulk solvent with that of the ketone carbonyl of 2OG. It is possible that this exchange occurs while the 2OG is bound to the iron(II) in the active site, and that the different levels of exchange reflect differences in catalytic mechanism. However since the acid catalysed exchange of the carbonyl oxygens of aldehydes and ketones in solution is well-precedented [21], it seems probable that the differences in exchange levels in the presence of labelled water reflect, at least in part, the differences in incubation conditions. Incubations of PHD2 and FIH under H_2^{18}O but in the absence of prime peptide substrate (i.e., uncoupled turnover conditions) showed that succinate containing one ^{18}O atom was produced by PHD2. Under these conditions FIH did not catalyse the production of succinate. The lack of uncoupled turnover displayed by FIH was in accord with literature reports [15,27].

In order to rule out the possibility that the oxygens of succinate exchanged with the bulk solvent, incubations containing succinate but no 2OG were carried out under H_2^{18}O conditions and showed only the presence of a peak at 117 Da. Experiments with both FIH and PHD2 were carried out under conditions where both $^{18}\text{O}_2$ and H_2^{18}O were present and showed peaks for labelled succinate at 119 Da (incorporation of one atom of ^{18}O) and also at 121 Da (incorporation of two atoms of ^{18}O), supporting the hypothesis that bulk solvent exchanges only with the ketone carbonyl of 2OG in solution (Fig. 2D).

4. Discussion

These results utilising enzymes from both prokaryotic (AlkB) and eukaryotic (FIH, PHD2, ANS and FLS) sources suggest that complete incorporation of ^{18}O from $^{18}\text{O}_2$ into one carboxylate of succinate is a typical feature of catalysis by 2OG oxygenases, as is the case for the carboxylate of the homogenisate product of the mechanistically related enzyme 4HPPD [21]. For some hydroxylations, e.g., those catalysed by PHD2 and FIH, high levels of incorporation into the alcohol product are also observed in the same assays. In the case of some prokaryotic enzymes however, e.g., deacetoxy/deacetyl cephalosporin C synthase and clavaminic acid synthase (at least with some substrates) less than stoichiometric incorporation of oxygen into the alcohol product occurs [9,10]. However, with deacetoxy/deacetyl cephalosporin C synthase, prior work has established that a single oxygen from dioxygen is incorporated into succinate during catalysis [9], as was the case for the more typical reaction catalysed by thymine-7-hydroxylase [11] catalysis. When using (\pm)-DHQ as a substrate, ANS catalysis also leads to sub-stoichiometric incorporation of oxygen from dioxygen into its flavonoid product, but this reaction is a special case in which the nascent hydroxyl is lost by dehydration [13]. When ANS catalyses a typical hydroxylation, e.g., the hydroxylation of (\pm)-naringenin, high levels of incorporation occurred [13]. The deacetoxycephalo-

sporin C synthase (DAOCS, a bacterial enzyme that only catalyses the deacetoxycephalosporin C synthase component of the two reactions of its fungal homologue deacetoxy/deacetylcephalosporin C synthase) reaction might also be regarded as being atypical; there is crystallographic evidence that the oxidative rearrangement catalysed by DAOCS proceeds via a divergence from the more general reaction scheme in which a penicillin displaces succinate from a ferryl intermediate [28]. Clavaminic acid synthase is also unusual in that it catalyses three reactions and has an HXE rather than and HXD Fe(II) binding motif [29]. Thus, given the previous reports and the results presented here it seems likely that incorporation of one atom of molecular oxygen into the succinate product is a general feature of catalysis by 2OG oxygenases. It is also clear that there is mechanistic variation after formation of the reactive oxidising intermediates, but even in cases where this enables exchange of oxygen during hydroxylation, there is still (almost) complete incorporation of dioxygen derived oxygen into succinate.

Acknowledgements: We thank the BBSRC, the Wellcome Trust, and the EPSRC. M.P. was the recipient of a Commonwealth Fellowship from the Association of Commonwealth Universities.

References

- [1] Schofield, C.J. and Zhang, Z.H. (1999) Structural and mechanistic studies on 2-oxoglutarate-dependent oxygenases and related enzymes. *Curr. Opin. Struct. Biol.* 9, 722–731.
- [2] Hausinger, R.P. (2004) $\text{Fe}^{\text{II}}/\alpha$ -ketoglutarate-dependent hydroxylases and related enzymes. *Crit. Rev. Biochem. Mol. Biol.* 39, 21–68.
- [3] Costas, M., Mehn, M.P., Jensen, M.P. and Que Jr., L. (2004) Dioxygen activation at mononuclear non-heme iron active sites: enzymes, models, and intermediates. *Chem. Rev.* 104, 939–986.
- [4] Prescott, A.G. and Lloyd, M.D. (2000) The iron(II) and 2-oxoacid-dependent dioxygenases and their role in metabolism. *Nat. Prod. Rep.* 17, 367–383.
- [5] Kivirikko, K.I., Myllyla, R. and Pihlajaniemi, T. (1989) Protein hydroxylation: prolyl 4-hydroxylase, an enzyme with four cosubstrates and a multifunctional subunit. *FASEB J.* 3, 1609–1617.
- [6] Price, J.C., Barr, E.W., Tirupati, B., Bollinger Jr., J.M. and Krebs, C. (2003) The first direct characterization of a high-valent iron intermediate in the reaction of an α -ketoglutarate-dependent dioxygenase: a high-spin Fe^{IV} complex in taurine/ α -ketoglutarate dioxygenase (TauD) from *Escherichia coli*. *Biochemistry* 42, 7497–7508.
- [7] Price, J.C., Barr, E.W., Glass, T.E., Krebs, C. and Bollinger Jr., J.M. (2003) Evidence for hydrogen abstraction from C1 of taurine by the high-spin Fe^{IV} intermediate detected during oxygen activation by taurine: α -ketoglutarate dioxygenase (TauD). *J. Am. Chem. Soc.* 125, 13008–13009.
- [8] Mehn, M.P., Fujisawa, K., Hegg, E.L. and Que Jr., L. (2003) Oxygen activation by nonheme iron(II) complexes: α -keto carboxylate versus carboxylate. *J. Am. Chem. Soc.* 125, 7828–7842.
- [9] Baldwin, J.E., Adlington, R.M., Crouch, N.P. and Pereira, I.A.C. (1993) Incorporation of ^{18}O -labelled water into oxygenated products produced by the enzyme deacetoxy/deacetylcephalosporin C synthase. *Tetrahedron* 49, 7499–7518.
- [10] Lloyd, M.D. et al. (1999) Product-substrate engineering by bacteria: studies on clavaminic synthase, a trifunctional dioxygenase. *Tetrahedron* 55, 10201–10220.
- [11] Holme, E., Lindstedt, G., Lindstedt, S. and Toft, M. (1971) ^{18}O Studies of the 2-ketoglutarate-dependent sequential oxygenation of thymine to 5-carboxyluracil. *J. Biol. Chem.* 246, 3314–3319.
- [12] Wu, M., Begley, T.P., Myllyharju, J. and Kivirikko, K.I. (2000) Mechanistic studies on prolyl-4-hydroxylase: demonstration that

- the ferryl intermediate does not exchange with water. *Bioorg. Chem.* 28, 261–265.
- [13] Turnbull, J.J., Nakajima, J., Welford, R.W.D., Yamazaki, M., Saito, K. and Schofield, C.J. (2004) Mechanistic studies on three 2-oxoglutarate-dependent oxygenases of flavonoid biosynthesis – anthocyanidin synthase, flavonol synthase, and flavanone 3 β -hydroxylase. *J. Biol. Chem.* 279, 1206–1216.
- [14] McNeill, L.A. et al. (2002) The use of dioxygen by HIF prolyl hydroxylase (PHD1). *Bioorg. Med. Chem. Lett.* 12, 1547–1550.
- [15] Hewitson, K.S. et al. (2002) Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J. Biol. Chem.* 277, 26351–26355.
- [16] Kikuchi, Y., Suzuki, Y. and Tamiya, N. (1983) The source of oxygen in the reaction catalyzed by collagen lysyl hydroxylase. *Biochem. J.* 213, 507–512.
- [17] Liu, A., Ho, R.Y., Que Jr., L., Ryle, M.J., Phinney, B.S. and Hausinger, R.P. (2001) Alternative reactivity of an α -ketoglutarate-dependent iron(II) oxygenase: enzyme self-hydroxylation. *J. Am. Chem. Soc.* 123, 5126–5127.
- [18] Ryle, M.J., Liu, A., Muthukumar, R.B., Ho, R.Y., Koehntop, K.D., McCracken, J., Que Jr., L. and Hausinger, R.P. (2003) O₂- and α -ketoglutarate-dependent tyrosyl radical formation in TauD, an α -keto acid-dependent non-heme iron dioxygenase. *Biochemistry* 42, 1854–1862.
- [19] Lindblad, B., Lindstedt, G., Tofft, M. and Lindstedt, S. (1969) The mechanism of α -ketoglutarate oxidation in coupled enzymatic oxygenations. *J. Am. Chem. Soc.* 91, 4604–4606.
- [20] Baldwin, J.E., Adlington, R.M., Schofield, C.J., Sobey, W.J. and Wood, M.E. (1989) The role of α -ketoglutarate in cephalosporin biosynthesis. *J. Chem. Soc., Chem. Commun.*, 1012–1015.
- [21] Lindblad, B., Lindstedt, G. and Lindstedt, S. (1970) The mechanism of enzymic formation of homogentisate from *p*-hydroxyphenylpyruvate. *J. Am. Chem. Soc.* 92, 7446–7449.
- [22] Falnes, P.Ø., Johansen, R.F. and Seeberg, E. (2002) AlkB-mediated oxidative demethylation reverses DNA damage in *Escherichia coli*. *Nature* 419, 178–182.
- [23] Trewick, S.C., Henshaw, T.F., Hausinger, R.P., Lindahl, T. and Sedgwick, B. (2002) Oxidative demethylation by *Escherichia coli* AlkB directly reverts DNA base damage. *Nature* 419, 174–178.
- [24] Turnbull, J.J., Prescott, A.G., Schofield, C.J. and Wilmouth, R.C. (2001) Purification, crystallization and preliminary X-ray diffraction of anthocyanidin synthase from *Arabidopsis thaliana*. *Acta Cryst. D* 57, 425–427.
- [25] Welford, R.W., Schlemminger, I., McNeill, L.A., Hewitson, K.S. and Schofield, C.J. (2003) The selectivity and inhibition of AlkB. *J. Biol. Chem.* 278, 10157–10161.
- [26] McNeill, L.A., Bethge, L., Hewitson, K.S. and Schofield, C.J. (2005) A fluorescence-based assay for 2-oxoglutarate-dependent oxygenases. *Anal. Biochem.* 336, 125–131.
- [27] Koivunen, P., Hirsilä, M., Günzler, V., Kivirikko, K.I. and Myllyharju, J. (2004) Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. *J. Biol. Chem.* 279, 9899–9904.
- [28] Valegard, K., Terwisscha van Scheltinga, A.C., Dubus, A., Ranghino, G., Oster, L.M., Hajdu, J. and Andersson, I. (2004) The structural basis of cephalosporin formation in a mononuclear ferrous enzyme. *Nat. Struct. Mol. Biol.* 11, 95–101.
- [29] Zhang, Z.H., Ren, J.S., Stammers, D.K., Baldwin, J.E., Harlos, K. and Schofield, C.J. (2000) Structural origins of the selectivity of the trifunctional oxygenase clavaminic acid synthase. *Nat. Struct. Biol.* 7, 127–133.
- [30] McNeill, L.A., Hewitson, K.S., Claridge, T.D., Seibel, J.F., Horsfall, L.E. and Schofield, C.J. (2002) Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the β -carbon of asparagine-803. *Biochem. J.* 367, 571–575.