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Comparison of the substrate selectivity and biochemical properties of human and bacterial γ -butyrobetaine hydroxylase†

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2-Oxoglutarate and iron dependent oxygenases have potential for the stereoselective hydroxylation of amino acids and related compounds. The biochemical and kinetic properties of recombinant γ -butyrobetaine hydroxylase from human and *Pseudomonas* sp. AK1 were compared. The results reveal differences between the two BBOXs, including in their stimulation by ascorbate. Despite their closely related sequences, the two enzymes also display different substrate selectivities, including for the production of (di)hydroxylated betaines, implying use of engineered BBOXs for biocatalytic purposes may be productive.

Ferrous ion and 2-oxoglutarate (2OG) oxygenases are a ubiquitous enzyme family that play biologically important roles including in the regulation of protein biosynthesis, nucleic acid repair and fatty acid metabolism in many aerobic organisms.^{1–3} They are also involved in the biosynthesis of many secondary metabolites, where they often catalyse modifications of amino acids and peptides. γ -Butyrobetaine hydroxylase (BBOX) catalyses the final step in the biosynthesis of carnitine,^{4,5} *i.e.* the stereoselective hydroxylation⁶ of γ -butyrobetaine (GBB) (Fig. 1A), in many eukaryotes and some bacteria.^{7–9} In animals, carnitine mediates fatty acid transport into mitochondria and has other roles including maintaining the acyl-CoA/CoA homeostasis.^{10,11} The role of carnitine in bacteria such as *Pseudomonas* sp. AK1 is not established; it appears that at least some bacteria lack the ‘complete’ carnitine biosynthesis pathway, which in animals originates from *N*-trimethyllysine, possessing only the final BBOX catalysed

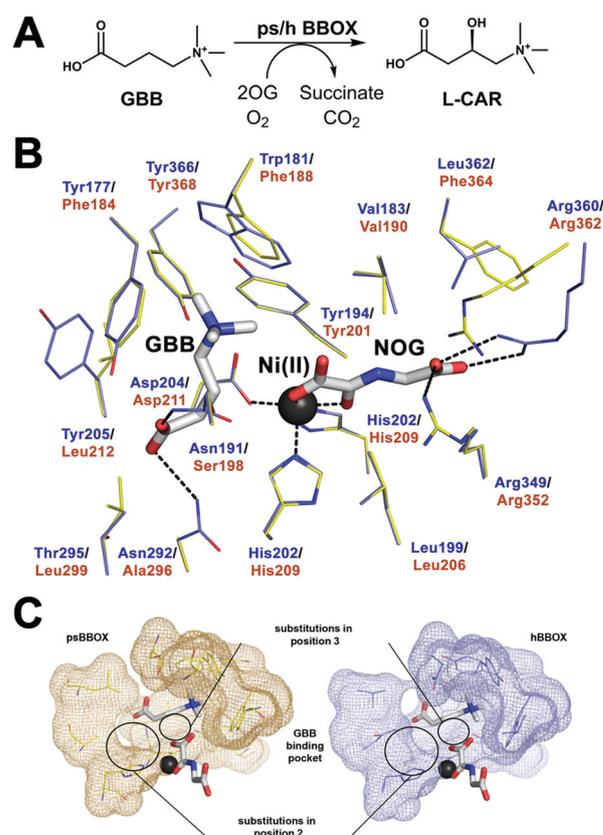


Fig. 1 (A) Reaction catalysed by BBOX. (B) Overlay of the GBB and 2OG binding pockets of hBBOX (blue/blue labels, PDB id: 3O2G) with a model of psBBOX (yellow/red labels). (C) Comparison of the surface of the GBB binding pockets as observed in the psBBOX model (left, with GBB, NOG and metal modeled based on hBBOX structure) and a hBBOX structure (right, PDB id: 3O2G) imply different spatial constraints for GBB analogue binding in the two enzymes), revealing more space for 2-substitutions in the case of psBBOX.

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step.⁴ BBOX is a therapeutic target in humans; the BBOX inhibitor Mildronate is used to decrease fatty acid oxidation in patients after myocardial infarction.^{12,13} The mechanism of

BBOX inhibition by mildronate has been studied, and shown to involve its oxidation to give several products, including ones arising from a Stevens type rearrangement.^{14,15}

2OG oxygenases show promise for use as biocatalysts, as shown by work on proline hydroxylases.^{16–19} In some cases they have been shown to have relatively lax substrate and product selectivities,¹⁷ suggesting they may be suitable for engineering to enable production of scaffolds of choice. Pioneering work on BBOX employed native enzyme isolated from both animal and *Pseudomonas* sp. AK1 sources.^{20,21} Crystallographic analyses of human BBOX reveal it has a core double-stranded β -helix fold, with surrounding elements that support the active site Fe(II), 2OG and substrate binding residues,¹⁴ as characteristic for the 2OG oxygenase superfamily.² BBOX also contains an N-terminal zinc binding domain, which has not been observed in other 2OG oxygenases. Human and psBBOX have ~30% sequence identity, and alignment of the psBBOX and hBBOX sequences reveals that most, but not all, crucial residues involved in substrate and metal binding are conserved (Fig. S4†). Comparison of the human BBOX structure with a model of psBBOX, implies that the overall folds and Fe(II) and 2OG binding residues are conserved, but that there are differences in the GBB binding residues. Specifically, Asn-191 and Asn-292, which are involved in the binding of the GBB carboxylate in hBBOX, are apparently replaced by Ser-191 and Ala-296 in psBBOX. Notable differences between hBBOX and psBBOX also occur within the ‘aromatic cage’, which binds the GBB trimethylammonium cation in hBBOX. In particular, the aromatic cage residues Tyr-177 and Trp-181 of hBBOX appear to be substituted by Phe-188 and Phe-184 in psBBOX (Fig. 1B). These differences suggested that the biocatalytic properties of hBBOX and psBBOX may be different. Here we describe comparative studies on the substrate specificity and kinetics of recombinant *Pseudomonas* sp. AK1 and human BBOXs.

Human BBOX (hBBOX) was prepared as reported.¹⁴ We then developed an efficient procedure for preparation of

recombinant BBOX from *Pseudomonas* sp. AK1 (psBBOX); recombinant psBBOX with an N-terminal hexa-His tag was purified to >90% purity in three chromatographic steps (by SDS-PAGE, Fig. S1–S3†).

Initially the cofactor requirements for the two BBOXs were investigated using an NMR based assay.¹⁴ The kinetics of 2OG oxidation was measured by analysis of succinate formation, and those for GBB hydroxylation by quantifying carnitine formation. For both BBOXs GBB hydroxylation was shown to be tightly coupled to that of 2OG oxidation to succinate (Fig. S5†). Only a low level (<10% of that in the presence of GBB) of 2OG conversion to succinate was observed in the absence of GBB (Fig. S6†). The initial kinetic characterisations, however, revealed differences between two BBOXs. The 2OG K_M was lower for hBBOX than psBBOX (153 μM vs. 532 μM , Table 1, Fig. S7†). This is notable because the activity of hBBOX in cells is proposed to be regulated by 2OG availability²² – it has an unusually high K_M for 2OG. The two BBOXs also differ in their dependencies on GBB (Table 1, Fig. S8†). For psBBOX the GBB K_M value of 2.5 mM (accounting for substrate inhibition at high concentration of GBB) or 163 μM (when only the Michaelis–Menten range was fitted) were much higher than for hBBOX, where a GBB K_M of 4 μM was measured. Further, psBBOX displayed only moderate substrate inhibition at high concentrations of GBB (>0.6 mM). In contrast hBBOX is inhibited by GBB at relatively low GBB concentrations (>20 μM) (Fig. S8†). Ascorbate (at 0.5 mM) was found to stimulate hBBOX (as for some, but not all, other 2OG oxygenases²³), but not psBBOX activity, consistent with the likelihood that ascorbate is not present in *Pseudomonas* cells (though it is possible that psBBOX will be stimulated by a prokaryotic ascorbate equivalent) (Fig. S9†).

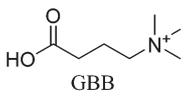
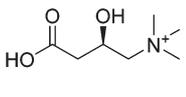
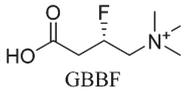
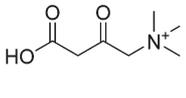
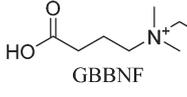
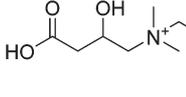
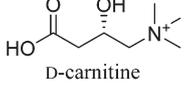
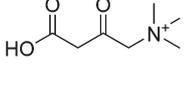
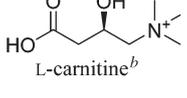
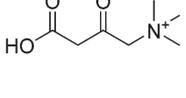
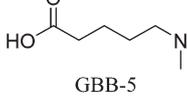
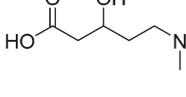
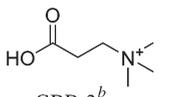
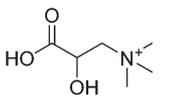
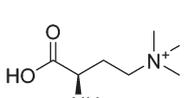
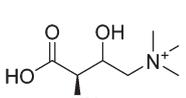
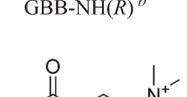
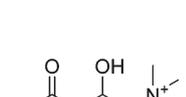
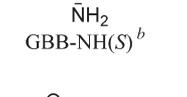
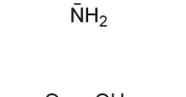
Having compared cofactor requirements of human and psBBOX, we proceeded to compare their substrate selectivities. Initially, we tested the reactivity of BBOXs with respect to oxidation of L-carnitine (L-CAR) and D-carnitine (D-CAR) (Table 2).

Table 1 Comparison of kinetic properties of psBBOX and hBBOX

Substrate	Structure	Enzyme	K_M [μM]	V_{max} [$\mu\text{M s}^{-1}$]	k_{cat} [s^{-1}]	K_I [μM]
2OG		psBBOX	532 ± 135	0.35	3.5 ± 0.3	—
		hBBOX	153 ± 44	0.080	1.6 ± 0.1	—
GBB		psBBOX	2474 ± 1466 (163 ^b)	1.10 ± 0.6	21.3	125 ± 74
		hBBOX	4.2 ± 3.9	0.04 ± 0.02	0.83	24 ± 14
GBBF		psBBOX	623 ± 102	0.47 ± 0.05	0.59	—
		hBBOX ^a	20 ± 9	0.11 ± 0.02	0.14	136 ± 44
GBBNF		psBBOX	412 ± 48	1.00 ± 0.07	5.0	—
		hBBOX ^a	20 ± 9	0.12 ± 0.04	0.30	226 ± 137

^a Data for hBBOX are from ref. 25. ^b Value measured from data within the Michaelis–Menten range. Assays were performed in the following conditions: 0.5 mM ascorbate, 0.2 mM KCl, 0.05 mM Fe(II), 1 mM 2OG (for GBB, GBBF and GBBNF K_M measurements), 0.1 mM GBB (for 2OG K_M measurements), 0.05–0.8 μM hBBOX/psBBOX (as described in ESI), 10% D₂O in 50 mM Tris-*d*₁₁ pH 7.5.

Table 2 Initial rates of BBOX catalysed hydroxylations of GBB analogues

Substrate	Product	Enzyme	Initial rate of hydroxylation [$\mu\text{M s}^{-1}$]	Initial rate of succinate formation [$\mu\text{M s}^{-1}$]	Ratio of succinate formation to hydroxylation
 GBB		psBBOX	0.29	0.32	1.1
		hBBOX	0.13	0.16	1.2
 GBBF		psBBOX	0.028	0.059	2.1
		hBBOX ^a	0.027	0.063	2.3
 GBBNF		psBBOX	0.21	0.24	1.1
		hBBOX ^a	0.083	0.097	1.2
 D-carnitine		psBBOX	0.018	0.054	3.0
		hBBOX	0.055	0.127	2.3
 L-carnitine ^b		hBBOX	0.0048	0.0760	15.8
 GBB-5		psBBOX	0.004	0.035	8.8
		hBBOX	0.016	0.029	1.8
 GBB-3 ^b		hBBOX	0.075	0.087	1.2
 GBB-NH(R) ^b		psBBOX	0.006	0.032	5.3
 GBB-NH(S) ^b		psBBOX	0.006	0.018	3.0
 GBB-OH ^b		psBBOX	0.048	0.069	1.4

^a Data for hBBOX are from ref. 25. ^b GBB-NH(R), GBB-NH(S) and GBB-OH were observed to be substrates only for psBBOX, whereas GBB-3 and L-carnitine were observed to be substrates only for hBBOX. Assays were performed employing 0.1 mM GBB analogue, 0.5 mM 2OG, 0.5 mM ascorbate, 0.2 M KCl, 0.05 mM Fe(II), 0.4 μM hBBOX/psBBOX, 10% D₂O in 50 mM Tris-*d*₁₁ pH 7.5.

hBBOX hydroxylates D-CAR to give 3-keto-GBB (which can readily undergo decarboxylation). D-CAR was found to be much poorer substrate for psBBOX than hBBOX (Fig. S10[†]), but clear evidence for formation of 3-keto GBB was observed by NMR. D-CAR was found to stimulate 2OG turnover beyond the level of D-CAR oxidation with both BBOXs (Fig. S10[†]). L-CAR was found

to be a poor hBBOX substrate, also being oxidised to an 3-keto GBB, however it was not a psBBOX substrate within our detection limit (Fig. S11[†]).

We then compared the activities of the BBOXs to catalyse oxidation of fluorinated GBB analogues, (3*S*)-fluoro GBB (GBBF)²⁴ and fluoromethyl GBB (GBBNF),²⁵ which are hBBOX

substrates.^{24,25} Both GBBF and GBBNF were found to be psBBOX substrates.²⁵ For both BBOXs, GBBNF was a better substrate than GBBF, consistent with crystallographic analyses which revealed little room for C-3 substitutions (Fig. 1C). The coupling ratio between 2OG and GBB analogue oxidation was good for GBBNF, but uncoupling was observed when GBBF was a substrate (Fig. S12–14†). With both BBOXs GBBNF displayed lower K_M values than GBBF (Table 1, Fig. S15 and 16†). psBBOX was not inhibited by GBBF nor GBBNF, at least up to 0.5 mM concentration of substrate. hBBOX was inhibited by low concentrations of GBBF, but much less inhibition was observed in case of GBBNF (Fig. S15 and 16†). Because GBB substrate inhibition was not observed at low concentrations with psBBOX, the lack of substrate inhibition of psBBOX by GBBF or GBBNF is unsurprising.

The observation that GBBNF is a better substrate for psBBOX than hBBOX, prompted us to examine substrate analogues with different chain lengths. Structural analogues of GBB are reported to be substrates for hBBOX.¹⁴ 3-Trimethylaminopropionate (GBB-3) and 5-trimethylaminovalerate (GBB-5) were found to be hydroxylated by hBBOX at C-2 and C-3, respectively. Similar experiments with psBBOX revealed that only GBB-5 was a substrate (within limits of detection), being hydroxylated at C-3, as for hBBOX (assignments were made by ¹H and ¹H–¹³C HSQC NMR spectra, Fig. S17†). In the case of hBBOX, GBB-3 was a reasonably good and GBB-5 a fair substrate, with GBB-3 being hydroxylated at 58% and GBB-5 at 12% of the initial GBB hydroxylation rate. GBB-5 was found to be a poor psBBOX substrate (1–2% of initial hydroxylation rate compared to GBB) (Table 2). GBB-3 was not hydroxylated even at a high concentration of psBBOX (13 μM). Both GBB-5 and GBB-3 stimulated uncoupled 2OG turnover by psBBOX (Fig. S18 and S19†), showing that GBB-3 and GBB-5 bind to the active site, however, predominantly in an unproductive manner. Neither hBBOX nor psBBOX oxidised 6-trimethylamino-hexanoate (GBB-6) or 2-trimethylaminoacetate (GBB-2).

BBOX is closely related to trimethyllysine hydroxylase (TMLH), which is the 2OG oxygenase that catalyses the first step of carnitine biosynthesis in animals.⁴ It was therefore of interest to investigate if the BBOXs can catalyse amino acid hydroxylation. Neither BBOX was able to catalyse hydroxylation of trimethyllysine. However, psBBOX but not hBBOX, was found to catalyse hydroxylation of both (*R*)- and (*S*)-analogues of 2-amino GBB (GBB-NH(*R*) and GBB-NH(*S*), respectively) to give products arising from C-3 hydroxylation as assigned by NMR (Fig. S20,† Table 2). The lack of activity with hBBOX with GBB-NH(*R*) and GBB-NH(*S*) is in agreement with the structural studies indicating that the hBBOX GBB binding site is too small to accommodate C-2 substituted GBB analogues. We then tested (2*S*)-hydroxy GBB (GBB-OH) as a substrate for BBOXs. GBB-OH was found to be a substrate for psBBOX only, giving the 2,3-dihydroxy GBB (Fig. S22†). GBB-OH was a better substrate for psBBOX than the 2-amino derivatives, *i.e.* GBB-NH(*R*) and GBB-NH(*S*) (the initial hydroxylation rate was 8 times higher than for amino derivatives, Table 2, Fig. S23†). Interestingly, the scaffold of 2,3-dihydroxy GBB is the same as

the natural product Anthopleurine²⁶ ((2*R*,3*S*)-dihydroxy- γ -butyrobetaine, Fig. S24†),^{27,28} which is produced as an alarm hormone in sea anemone *Anthopleura elegantissima*. This result raises the question of whether a BBOX related enzyme could be responsible for hydroxylation step in the biosynthesis of anthopleurine in sea anemone.

Finally, we tested a set of trimethylammonium containing compounds, some of which were close BBOX substrate analogues, *e.g.* acetylcholine (Fig. S25†). However, none of these were found to be hydroxylated by either hBBOX or psBBOX.

Conclusions

In conclusion, the results clearly demonstrate that while hBBOX and psBBOX share key properties, there are also clear differences between them, including their differential dependencies on ascorbate. As predicted based on the hBBOX crystal structure, there are differences in their substrate analogue selectivities, with psBBOX being more tolerant of modifications with increased steric demand in the aromatic cage responsible for the trimethylammonium binding. The substrate analogue results imply that engineering of the BBOX activity, might be productive, including with respect to the production of vicinal diols and amino-alcohols, such as present in some natural products, such as Anthopleurine.

Acknowledgements

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