Ejection of structural zinc leads to inhibition of γ-butyrobetaine hydroxylase

Anna M. Rydzika, Jürgen Brema, Weston B. Struwea, Grazyna T. Kochanb, Justin L. P. Beneschc, Christopher J. Schofielda

aDepartment of Chemistry, Chemistry Research Laboratory, University of Oxford, 12 Mansfield Road, Oxford OX1 3TA, United Kingdom
bStructural Genomics Consortium, University of Oxford, Old Road Campus Roosevelt Drive, Headington OX3 7DQ, United Kingdom

current address: Navarrabiomed-Fundacion Miguel Servet, C/Irunlarrea 3, Complejo Hospitalario de Navarra, 31008 Pamplona, Navarra, Spain.

Article history:
Received 25 July 2014
Revised 9 September 2014
Accepted 11 September 2014
Available online 19 September 2014

Keywords:
γ-Butyrobetaine hydroxylase
Carnitine
2-Oxoglutarate dependent oxygenase
Zinc ejection
Ebselen

Abstract

γ-Butyrobetaine hydroxylase (BBOX) is a 2-oxoglutarate and Fe(II) dependent oxygenase that catalyses an essential step during carnitine biosynthesis in animals. BBOX is inhibited by ejection of structural zinc by a set of selenium containing analogues. Previous structural analyses indicated that an undisrupted N-terminal zinc binding domain of BBOX is required for catalysis. Ebselen is a relatively potent BBOX inhibitor, an observation which may in part reflect its cardioprotective properties.

The 2-oxoglutarate (2OG) and ferrous iron dependent oxygenases are a large enzyme superfamily, which perform a wide range of oxidations, hydroxylations and demethylations. Structural studies have revealed 2OG oxygenases contain a conserved double stranded β-helix (DSBH) core which supports the residues responsible for Fe(II) and 2OG binding. However many 2OG oxygenases also contain other domains which also have the ability to bind metals, in particular zinc finger type domains. The functions of their zinc binding domains are likely diverse; in some cases they have been shown to be required for folding and catalysis, for example, in histone demethylase KDM4A the C-terminal zinc finger is required for activity and conformational stability and is likely involved in substrate binding. There are also examples of 2OG dependent oxygenases where zinc finger type domains are not required for catalysis; some of these are responsible for mediating protein–protein interactions, for example, the N-terminal zinc finger of prolyl hydroxylase protein domain 2 (PHD2) and the zinc binding domain(s) of the DNA hydroxylases (Tet enzymes).

Carnitine is indispensable for humans, and has roles including enabling the transport of long chain fatty acids through the mitochondrial membrane. Carnitine is associated with both health benefits and risks. Carnitine biosynthesis is conserved across animals and involves four enzyme catalysed steps. γ-Butyrobetaine hydroxylase (BBOX) is a 2OG and Fe(II) dependent oxygenase, which catalyses the final step in carnitine biosynthesis in animals, that is, hydroxylation of γ-butyrobetaine (GBB) to give L-carnitine (Fig. 1). Inhibition of BBOX is reported to aid recovery after cardiac dysfunction induced by ischemia/reperfusion. As observed both in solution and by crystallographic analyses, BBOX is a homodimer, with each monomer containing N- and C-terminal domains (Fig. 1). The C-terminal BBOX domain has a DSBH core fold and contains the active site, with the Fe(II), 2OG and GBB binding residues. The N-terminal domain contains the Zn(II) binding site, where the Zn(II) is chelated by a Cys3-His motif (Cys38, Cys40, Cys43 and His82), which is highly conserved in BBOX from different species (Fig. S1). The zinc binding domain of BBOX has been shown to be involved in dimerisation; however, the influence of the presence of Zn(II) on BBOX activity has not been previously investigated. Therefore, we were interested to test whether removal of structural zinc affects BBOX activity.

Selenium and sulfur based compounds are reported to be effective zinc ejectors, due to their demonstrated ability to interact with cysteine-residues involved in Zn(II) binding in many proteins. We therefore examined a set of potential zinc ejectors for their ability to inhibit BBOX (Table 1). BBOX activity was measured using an assay measuring release of fluoride from a fluorinated GBB analogue (Fig. S2). As a control an isoquinoline derivative...
BIQ (FG2216, IOX3) (8) was used, which has been previously reported to inhibit BBOX through chelating active site Fe(II) and interacting with specific active site residues.

Compounds (1)–(7) were found to be BBOX inhibitors; their IC50 values were found to be dependent on the time of preincubation prior to reaction (Table 1, Fig. S3), that is, consistent with a mechanism involving irreversible ejection of structural zinc. Interestingly, Ebselen (1) was the most potent inhibitor with an IC50 value of 0.5 μM. Ebselen is known to be a Zn(II) ejector, as shown on the example of metallothionein and human histone demethylase KDM4A. Ebselen is in late stage clinical development for stroke treatment.

Having shown that selenium and sulfur containing compounds are BBOX inhibitors, we examined Zn(II) release from BBOX induced by analogues (1)–(7) (Fig. 2). This was done employing a fluorescence based assay, wherein the amount of Zn(II) was quantified by measuring an increase of fluorescence upon binding of Zn(II) to a Zn(II) specific probe (Fig. S4). As expected compounds (1)–(7) caused Zn(II) release in a time and dose-dependent manner (Figs. 2, S5 and S6). PhSeCl (2), PhSeBr (3) and PhSeOOH (4) were the most effective zinc ejectors, consistent with their good inhibitory properties and decreases in their IC50 values with increasing preincubation times (Table 1). PhSeOH (5) and PhSeSePh (6) were apparently poor zinc ejectors, consistent with their relatively high IC50 values. A control demonstrated that no Zn(II) ejection was induced by BIQ (8), which binds to the active site iron of 2OG oxygenases including BBOX. Interestingly, thiram (7) displayed a sigmoidal dependence of Zn(II) release on time. Ebselen (1) displayed only moderate ability to eject zinc under the standard assay conditions. This observation suggests that the mode of BBOX inhibition by Ebselen (1) may be more complex than simple Zn(II) ejection.

Table 1  
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC50 BBOX [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 min</td>
</tr>
<tr>
<td>Ebselen (1)</td>
<td><img src="image" alt="Ebselen Structure" /></td>
<td>0.69 ± 0.11</td>
</tr>
<tr>
<td>PhSeCl (2)</td>
<td><img src="image" alt="PhSeCl Structure" /></td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>PhSeBr (3)</td>
<td><img src="image" alt="PhSeBr Structure" /></td>
<td>7.1 ± 1.7</td>
</tr>
<tr>
<td>PhSeOOH (4)</td>
<td><img src="image" alt="PhSeOOH Structure" /></td>
<td>35 ± 7</td>
</tr>
<tr>
<td>PhSeOH (5)</td>
<td><img src="image" alt="PhSeOH Structure" /></td>
<td>54 ± 11</td>
</tr>
<tr>
<td>PhSeSePh (6)</td>
<td><img src="image" alt="PhSeSePh Structure" /></td>
<td>55 ± 5</td>
</tr>
<tr>
<td>Thiram (7)</td>
<td><img src="image" alt="Thiram Structure" /></td>
<td>56 ± 15</td>
</tr>
<tr>
<td>BIQ (8)</td>
<td><img src="image" alt="BIQ Structure" /></td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

Note that the IC50 values depend on the preincubation time.
BBOX by BIQ, involving coordination of the active site Fe(II).

Binding of the sulfur/selenium containing compounds (1–7), including Ebselen (1) (Figs. 3 and S8), was not significantly changed in the presence of an Fe(II), suggesting that they do not bind at the BBOX active site.

We then carried out MS analyses to investigate potential covalent modifications of BBOX by the most potent inhibitors (1–4). We envisaged that BBOX-S-SeR bonds may survive the LC–MS conditions; indeed in the MS spectra of BBOX treated with 1 equiv of PhSeCl (2), PhSeBr (3) and PhSeOOH (4) a single covalent modification was observed, corresponding to a PhSe-group mass shift (+156) (Fig. 4), that is, reaction with a cysteinyl thiol. In the case of Ebselen (1), even after prolonged (24 h) incubation, only small amounts of covalent modification were detected (+274 peak, Fig. 4). Non-denaturing MS analyses (Fig. S10) suggested that Ebselen can bind more than once at low concentrations forming adducts stable under these conditions (80 V cone voltage). At high concentrations of Ebselen (10 equiv), BBOX underwent apparent multiple covalent modifications corresponding to 9 Ebselen molecules bound per BBOX molecule (Fig. S5). Thus, Ebselen may inhibit by both covalent and non-covalent mechanisms.

Overall, we have shown that selenium and sulfur containing compounds can inhibit BBOX catalysis. In most cases, the mechanism likely involves covalent modification of one or more cysteine residues causing subsequent release of structural Zn(II). The results imply that a functional zinc binding domain is required for BBOX catalysis, as in the case for some other 2OG dependent histone demethylases, for example, KDM4A. We have identified inhibitors of BBOX, with Ebselen—a clinical trial candidate for the treatment of acute stroke—being particularly potent. Because BBOX inhibition is reported to aid in recovery after cardiac dysfunction, the therapeutic effect of an Ebselen could thus, at least in part, be due to BBOX inhibition. Alternatively, BBOX inhibition may cause side effects in Ebselen treatment.

Figure 2. Ejection of zinc from BBOX by analogues (1)–(8). Time course of Zn(II) ejection from BBOX by inhibitor (1)–(8) at 0.4 mM (A). Dose–response curves for zinc ejection from BBOX by (1)–(8) as measured after 1 min (B) and 20 min (C) of incubation of BBOX.

Figure 3. Binding of compounds (1)–(8) to BBOX as assayed by intrinsic fluorescence quenching of BBOX (Fig. S8). (A) comparison of binding of analogues (1)–(8). (B) Binding of Ebselen (1) and BIQ (8) in the presence and absence of Fe(II); the results imply Ebselen does not bind to the active site Fe(II).

Figure 4. MS analyses for binding of inhibitors (1)–(4) to BBOX. MS under denaturing reveals analogues (2)–(4) likely covalently modify BBOX when used in a 1:1 enzyme: inhibitor ratio (mass difference +156). Note relatively little binding was observed for Ebselen (1) (mass difference +274).
addition of a mixture containing all components to a solution of BBOX and the inhibitor (preincubated for 0, 10 or 20 min prior to the start of the reaction). Reactions were quenched after 10 min by addition of the TBS-fluorescein probe in DMSO (40 μl) to a final concentration of 5 μM. The plate was then sealed and incubated for 60 min at room temperature, prior to addition of 10 μl of 50 mM Hepes buffer pH 7.0. The resultant signal was read up to 5 min after addition of Hepes buffer using an Evision Multilabel plate reader (Perkin Elmer) fitted with FITC FP 480/30 (480 nm, bandwidth 30 nm) and FITC FP 535/40 emission (535 nm, bandwidth 40 nm) filters. For each reaction, controls containing all reagents but without BBOX, or without inhibitor were recorded. The normalised fluorescence signal was defined as the observed fluorescence signal minus the control signal. Percentage activity was calculated as a ratio of normalised signal for reaction containing inhibitor to reaction containing no inhibitor. Each reaction was carried out in quadruplicate. Errors were calculated as standard deviations from four separate measurements. KIC data were fitted using XLfit software (IDBS Solutions) using 4-parameter logistic model (sigmoidal dose–response with variable slope).


36. Zinc(II) concentrations were determined by measuring the increase in fluorescence upon binding of Zn(II) to the Zn(II)-specific fluorophore FluoZin-3 probe and variable concentration of the tested compound in 50 mM Tris-HCl buffer pH 7.5 with a final volume of 50 μl in 96-well plates (Greiner, black, bottom: flat, clear). A calibration curve was obtained between 0 and 10 μM Zn(II) in the presence of 10 μM BBOX (the shape of the curve was shown to be affected by the presence of BBOX). Readings were performed in 60 cycles at 1% of the plate per cycle.

37. Fluorescence was measured using a Pharasar FS plate reader (BMG Labtech) using the following parameters: excitation 485 nm, emission 520 nm. Assays generally contained 10 μM BBOX, 10 μM FluoZin-3 probe and variable concentration of the tested compound in 50 mM Tris-HCl buffer pH 7.5 with a final volume of 50 μl in 96-well plates (Greiner, black, bottom: flat, clear). A calibration curve was obtained between 0 and 10 μM Zn(II) in the presence of 10 μM BBOX (the shape of the curve was shown to be affected by the presence of BBOX). Readings were performed in 60 cycles at 1% of the plate per cycle.

38. Fluorescence was measured using a Pharasar FS plate reader (BMG Labtech) using the following parameters: excitation 280 nm, emission 350 nm, 5 μM BBOX, 20 μM Fe(II) with variable concentration of tested compounds. Assays were performed in 50 mM Tris buffer pH 7.5 containing 200 mM NaCl in a total volume of 50 μl per well in 96-well plates (Greiner, black, bottom: flat, clear). Measurements were done in triplicate, error bars represent standard deviations.

The concentration of inhibitor was plotted against 1/IC50, where 1/IC50 is the observed decrease in fluorescence signal and IC50 is maximal measured fluorescence signal (initial fluorescence measurement for BBOX alone).

Samples for mass spectrometry were prepared by treatment of BBOX (10 μM) in 50 mM Tris-HCl buffer pH 7.5, containing 200 mM NaCl with inhibitor to final concentration of 10 μM (total vol. 50 μl). Samples were run on Waters LCT Premier XE Spectrometer equipped with electrospray interface coupled to an Agilent 1100 Series HPLC with a C18 autosampler inlet system. The column used was Merck Chromolith 2 C18-2 × 5 mm column (Merck). Samples were kept at -4 °C before injection and run with a gradient from 5% B to 100% B in 5 min (solvent A-water, 0.15% formic acid; B-acetonitrile).