The human enzyme that converts dietary provitamin A carotenoids to vitamin A is a dioxygenase *

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*Running title: Reaction mechanism of human BCO1

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Background: The human enzyme β-carotene 15-15'-oxygenase (BCO1) has been thought to be a monooxygenase.

Results: Incubation of BCO1 and β-carotene in H218O-16O2 or H216O-18O2 medium yields 2 retinals both of which contain oxygen atoms originating solely from O2 gas.

Conclusion: BCO1 is a dioxygenase.

Significance: It is important to clearly establish an enzyme’s reaction mechanism especially when the name reflects the mechanism.

ABSTRACT

β-Carotene 15-15'-oxygenase (BCO1) catalyzes the oxidative cleavage of dietary provitamin A carotenoids to retinal (vitamin A aldehyde). Aldehydes readily exchange their carbonyl oxygen with water, making oxygen labeling experiments challenging. BCO1 has been thought to be a monooxygenase, incorporating oxygen from O2 and H2O into its cleavage products. This was based on a study that used conditions that favored oxygen exchange with water. We incubated purified recombinant human BCO1 and β-carotene in either 16O2-H218O or 18O2-H216O medium for 15 minutes at 37°C and the relative amounts of 18O-retinal and 16O-retinal were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). At least 79% of the retinal produced by the reaction has the same oxygen isotope as the O2 gas used. Together with the data from 18O-retinal-H216O and 16O-retinal-H218O incubations to account for non-enzymatic oxygen exchange, our results show that BCO1 incorporates only oxygen from O2 into retinal. Thus, BCO1 is a dioxygenase.

Vitamin A deficiency is the most common vitamin deficiency in the world and affects an estimated 190 million preschool-age children and 19.1 million pregnant women worldwide (1). In areas of endemic vitamin A deficiency, people obtain vitamin A almost exclusively as provitamin A carotenoids found in foods of plant origin (2). Provitamin A carotenoids are enzymatically converted to retinal (vitamin A aldehyde) (Figure 1A) by the enzyme β-carotene 15-15'-oxygenase (BCO1) (3). Hence, understanding the mechanism and regulation of this enzyme is important.

The reaction mechanism, and consequently, the nomenclature of BCO1 and other carotenoid cleavage oxygenases (CCO’s) have been controversial (4-6). The first report of a CCO was made in 1965 by Goodman and Huang (7), who showed that β-carotene was converted to retinal by cell-free rat intestinal homogenates in the presence of O2. The following year, the same group then showed using 3H labels that the hydrogens of the 15-15' double bond of β-carotene (the site of oxidative cleavage) are retained during the enzymatic oxidation reaction, and proposed that the reaction most likely has a dioxygenase mechanism (8). However, the label “dioxygenase” should only be used when oxygen labeling experiments have clearly established that only oxygen from O2 is incorporated by the enzyme into its oxidative cleavage products. BCO1 was
given the Enzyme Commission (EC) number 1.13.11.21 in 1972, designating a dioxygenase (9), 29 years before the first report of an oxygen labeling experiment. A monooxygenase mechanism was proposed for BCO1 in 2001 (10). In that study, α-carotene, purified chicken BCO1 and horse liver alcohol dehydrogenase (HLADH) were incubated in an 85% 17O2-95% H218O environment. HLADH was used to form retinols from the aldehydes, which readily exchange their carbonyl oxygen with water (11). The resulting products (retinol and α-retinol) were purified by high-performance liquid chromatography and silylated. Using gas chromatography-mass spectrometry (GC-MS), the authors found virtually equal enrichment of 17O and 18O in both silylated retinols, suggesting a monooxygenase mechanism (Figure 1 B). However, it is possible that the long reaction time (7.5 hours) and extensive processing favored oxygen exchange between the initial aldehyde products and the aqueous medium. Also, the HLADH reaction is reversible, and the enzyme displays dismutase activity (interconverting the aldehyde into alcohol and carboxylic acid) (6,12). This means that the aldehydes were never completely eliminated during the 7.5 hour incubation, and a significant amount of oxygen exchange with water may have occurred. Despite the inconclusiveness of this study, the enzyme’s EC number was changed to 1.14.99.36, classifying it as a monooxygenase (13), and subsequent literature has referred to the animal orthologs of the enzyme as β-carotene 15-15′-monooxygenase (BCMO1). Indeed, the National Center for Biotechnology Information named the gene **BCMO1** (14).

To elucidate the reaction mechanism of human BCO1, we conducted multiple oxygen labeling experiments with minimal reaction and processing times to minimize oxygen exchange between retinal and water. Our results demonstrate that BCO1 is not a monooxygenase, but a dioxygenase.

**EXPERIMENTAL PROCEDURES**

*Chemicals-* β-Carotene (≥97%), all-trans-retinal (≥98%), H218O (97% atom) and 18O2 gas (99% atom) and Dowex 50WX4 were purchased from Sigma-Aldrich.

**Synthesis of 18O-retinal**- All-trans-retinal (20 nmol), H218O (200 equivalents), 2 mL acetonitrile and 60 mg of Dowex 50WX4 (hydrogen form) were stirred at room temperature in a closed vial protected from light for 1.5 hours. This is based on the method of Kawanishi et al. (15). The solids were removed by decantation, and the retinoids were then extracted with 3x2 mL hexanes. The hexane extracts were combined and stored at -80°C. The final product is 91% 18O-retinal as measured by LC-MS/MS (below).

**Freeze-drying of purified recombinant human BCO1**- Purified recombinant human BCO1 was prepared according to our previously published method (16). The purified enzyme preparation catalyzed the oxidative cleavage of β-carotene with a Vmax = 197.2 nmol retinal/mg BCO1 × h, Km = 17.2 μm and catalytic efficiency kcat/Km = 6098 M−1 min−1. Ten μg of purified recombinant human BCO1 and 40 μL of 5x reaction buffer (500 mM Tricine-KOH, pH 8.0 at 37°C, 2.5 mM dithiothreitol, 20 mM sodium cholate, 75 mM nicotinamide) (16) were combined in a 10-mL amber headspace vial, and the vial was capped and flash-frozen in liquid nitrogen. The headspace vials were stored in dry ice for 30 minutes during transport to the freeze-dryer. The caps of the headspace vials were then fitted with individual syringe needles for venting, and the vials were placed in the jar of the manifold freeze dryer (Labconco). Freeze-drying was done for 16 hours at 0.14 mBar. The syringe needles were removed, and the headspace vials were stored at -80°C until use. Each vial of freeze-dried enzyme produces about 60 pmol of retinal from 4 nmol of β-carotene with the in vitro BCO1 activity assay system described in the following section.

**In vitro BCO1 activity assay in 16O2-H218O**- The in vitro enzyme assay using purified recombinant human BCO1 was based on our previously published method (16). The freeze dried enzyme-reaction buffer mixture in the headspace vial (described in previous section) was dissolved in H218O to a final volume of 160 μL and placed in a 37°C shaking water bath. The reaction was initiated by adding 40 μL of β-carotene substrate solution (containing 4 nmol β-carotene, 0.3 μL Tween-40 and 20 nmol α-tocopherol) prepared in H218O. The reaction was allowed to proceed in the
water bath with gentle shaking and the vial exposed to air (which contains oxygen as 99.8% \textsuperscript{16}O\textsubscript{2} (17)) for 15 minutes. The quenching with 37% formaldehyde in the original method had to be omitted since the latter contains \textsuperscript{16}O\textsubscript{2}. Instead, the reactions were quenched with 300 μL of acetonitrile, and the lipophilic compounds were extracted with 3x1 mL of hexanes under red lights. The combined hexane extracts were dried under N\textsubscript{2}, re-dissolved in 100 μL 3:1 (v/v) acetonitrile-H\textsubscript{2}\textsuperscript{18}O, filtered through a 0.22 μm syringe-driven filter, and injected into the HPLC. The whole process from the start of the reaction to the elution of the retinal peak in the HPLC takes about 50-60 minutes.

In vitro \textit{BCO1} activity assay in \textsuperscript{18}O\textsubscript{2}-H\textsubscript{2}\textsuperscript{16}O- The enzyme–reaction buffer solution (10 μg of purified recombinant human \textit{BCO1}, 40 μL of 5x reaction buffer and water to a total volume of 160 μL) was placed in a headspace vial and degassed by exposure to water aspirator vacuum for 2 minutes. The headspace vial was purged with nitrogen gas, then connected to the \textsuperscript{18}O\textsubscript{2} gas cylinder and placed in a 37°C water bath. Forty μL of \textit{β}-carotene substrate solution, prepared in degassed water (H\textsubscript{2}\textsuperscript{16}O), was then injected into the vial using a syringe. The reaction was allowed to proceed in the water bath with gentle shaking for 15 minutes. The reaction was quenched by injecting 300 μL of acetonitrile into the vial before the \textsuperscript{18}O\textsubscript{2} gas flow was turned off. The reaction mixture was then extracted and processed as in the previous section, except that the extract residue was re-dissolved in acetonitrile-H\textsubscript{2}\textsuperscript{16}O.

Control experiments- To account for the oxygen exchange between water and retinal, we incubated 60 pmol all-\textit{trans}-retinal (\textsuperscript{18}O-retinal) in the reaction mixture prepared in H\textsubscript{2}\textsuperscript{18}O containing active \textit{BCO1}, as described above for “In vitro \textit{BCO1} activity assay in \textsuperscript{16}O\textsubscript{2}-H\textsubscript{2}\textsuperscript{18}O.” We also performed an analogous incubation using our synthesized \textsuperscript{18}O-retinal in H\textsubscript{2}\textsuperscript{16}O.

\textit{UHPLC-MS/MS method-} The reaction mixture was separated by an Agilent 1290 UHPLC system (Agilent Technologies) using a Zorbax Extend 2.1 x 50 mm, 1.8 μm C18 column (Agilent Technologies). The flow rate was 0.8 mL/min, and the column temperature was 40 °C. The composition of solvents was as follows: A = 0.1% formic acid in water; B = 0.1% formic acid in acetonitrile. A linear eluting gradient was applied as follows: isocratic 60% B for 0.5 min, gradient from 60-78%B over 3 minutes, gradient from 78-100%B over 1.5 min, isocratic 100% B for 2 min and re-equilibration to 60%B over 2 minutes.

The HPLC was interfaced with an Agilent 6550 Q-TOF mass spectrometer (Agilent Technologies) using an electrospray ionization (ESI) source operated in positive ion mode. The MS instrumental parameters included: sheath gas temperature, 400°C; flow rate, 12 L/min; drying gas temperature, 150°C; flow rate, 15 L/min; 3 Hz MS/MS acquisition; 10 Hz MS reference scans; 30 psig nebulizer; Vcap, 2000 V; nozzle voltage, 2000 V; fragmentor, 350 V; ion funnel settings for small molecules. MS/MS transitions were acquired by collision-induced dissociation (CID) of all-\textit{trans}-retinal standard (m/z=285.218) and \textsuperscript{18}O-retinal (m/z=287.226) and found to optimize at a collision energy of 7.5 eV. Source and CID gas was high purity (>98%) nitrogen. Calibration was performed using ESI-L tuning mix (Agilent Technologies G1969-85000) and within-run reference compound was hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine, m/z 922.010 (Agilent Technologies HP-0921).

Quantification of retinal oxygen isotopologues- The fragmentation patterns of \textsuperscript{18}O-retinal and \textsuperscript{16}O-retinal are virtually the same (Figure 3). The parent retinals were not used for quantification to minimize errors arising from other naturally occurring isobaric species, which constitute about 2.2% based on the natural abundance of \textsuperscript{13}C (17). MS/MS was used to discriminate between the retinal analytes from these isobaric species, which will give different fragmentation patterns.

For quantification of the retinal oxygen isotopologues, the MS/MS fragments m/z= 119.086, 175.150, 105.070, 133.101, 163.101, 195.163, 231.162, for \textsuperscript{18}O-retinal and m/z= 119.086, 175.143, 105.070, 133.101, 161.092, 193.159, 229.158 for \textsuperscript{16}O-retinal were summed to generate one extracted ion chromatogram for each parent retinal species. These daughter ions were selected because they were the dominant fragment ions. Also, the last three daughter ions listed for \textsuperscript{18}O-retinal and \textsuperscript{16}O-retinal differ by 2 amu,
indicating that these fragments bear the oxygen atom.

RESULTS

For the BCO1-β-carotene reaction in $^{16}$O$_2$-H$_2^{18}$O medium, the retinal product obtained (about 60 pmol) after a 15-minute reaction was only about 3-10% $^{18}$O-retinal (Figure 1C). This range reflects what we obtained from three experiments done on different days. A sample LC-MS chromatogram is shown in Figure 2A. The small relative amount of $^{18}$O-retinal we observed in $^{16}$O$_2$-H$_2^{18}$O medium suggests that BCO1 is a dioxygenase (Figure 1B). If the enzyme is a dioxygenase, then theoretically, it should produce only $^{16}$O-retinal, and the $^{18}$O-retinal we observed was due to oxygen exchange with water. To verify this, we incubated 60 pmol of $^{18}$O-retinal with BCO1 in H$_2^{18}$O under the same conditions. The % $^{18}$O-retinal formed was similar (5-13%) to that produced in the reaction of BCO1 and β-carotene (Figure 1C). This confirms that the $^{18}$O-retinal we were detecting was coming from the oxygen exchange of retinal with water, and not from the enzyme incorporating oxygen from water during the oxidative cleavage reaction.

We then conducted the BCO1-β-carotene reaction in $^{18}$O$_2$-H$_2^{16}$O medium. Consistent with our previous experiments, the majority of the retinal product obtained contains the same oxygen isotope as that of O$_2$ (79-85% $^{18}$O-retinal). As in the previous section, this range reflects what we obtained from experiments done on different days. A sample LC-MS chromatogram is shown in Figure 2B, and the MS/MS traces for m/z = 285.218 and 287.226 (corresponding to $^{16}$O-retinal and $^{18}$O-retinal, respectively) are shown in Figure 3. To verify that the $^{16}$O-retinal (15-21%) we observed was due to oxygen exchange with water, we also incubated $^{18}$O-retinal (91% $^{18}$O-retinal) with BCO1 in H$_2^{16}$O under the same conditions. We observed 67-84% $^{18}$O-retinal, corresponding to a 7-24% net exchange (Figure 1C). Consistent with the previous section, these values strongly suggest that BCO1 reacts with β-carotene in an $^{18}$O$_2$-H$_2^{16}$O to form only $^{18}$O-retinal, and the small relative amount of $^{16}$O-retinal is due to oxygen exchange with water.

We also performed the BCO1-β-carotene incubation in $^{16}$O$_2$-H$_2^{18}$O for 7.5 hours, and the retinal product obtained was 50% $^{18}$O-retinal, which verifies that such a long incubation time will indeed lead to a false identification of the enzyme as a monooxygenase.

The $^{16}$O$_2$-H$_2^{18}$O and $^{18}$O$_2$-H$_2^{16}$O experiments strongly suggest that BCO1 incorporates only oxygens from O$_2$ into retinal formed from the oxidative cleavage of β-carotene, and the minor amount of retinal with the same oxygen isotope as water is formed by non-enzymatic oxygen exchange. Thus, BCO1 is a dioxygenase and not a monooxygenase as had been previously thought.

If the parent retinals are used for quantification, the values differ by only 0-6% from the MS/MS calculation (Table 1), and the data still lead to the same conclusion that BCO1 incorporates only oxygen from O$_2$ into retinal formed from oxidative cleavage of β-carotene.

DISCUSSION

At this point, there is a very limited amount of literature on other CCO’s with which to compare our results. Most of the functionally characterized CCO’s are from plants, and these enzymes have been called “dioxygenases” despite the lack of conclusive oxygen labeling experiments (6,18). This error can be traced back to the lignostilbene “dioxygenases.” As of 1997, these enzymes were called as such even though no oxygen labeling experiments were carried out (6,19-23). At best, these studies showed that these enzymes require O$_2$. This error in naming was propagated into the CCO’s in 1997, when the first CCO to be cloned and characterized, maize Viviparous 14, was called a dioxygenase based on its sequence similarity to lignostilbene “dioxygenase” and not on oxygen labeling experiments (24-25). Even if the lignostilbene oxygenases were truly established to be dioxygenases back then, a sequence similarity is not necessarily a substitute for oxygen labeling experiments. Interestingly, the first report of an oxygen labeling experiment for a stilbene oxygenase (which was also identified because of sequence similarity to the plant CCO’s) showed a monooxygenase reaction mechanism (26).

Of the more than 200 putative CCO’s to be found in sequence databases (5), there are only four other oxygen labeling experiments done apart from the aforementioned 2001 BCO1 study. The oxygen labeling experiments on water-stressed
leaves of Xanthium strumarium in 1984 (27) looked at only one cleavage product, and the Arabidopsis thaliana study in 2006 (24) was deemed inconclusive because of the failure to show a consistent labeling pattern for the two cleavage products (Kloer and Schulz give a detailed critique of these two studies (5)). An oxygen labeling experiment was done with Microcystis PCC 7806 cells, which generate β-cyclocitral and crocetindial from oxidative cleavage of β-carotene (28). However, the results between the $^{16}\text{O}_2$-$\text{H}_2^{18}\text{O}$ and $^{18}\text{O}_2$-$\text{H}_2^{16}\text{O}$ incubations were contradictory, and the authors acknowledge that the longer processing time for crocetindial may have favored oxygen exchange. Another oxygen labeling study done with a purified recombinant marine bacterial CCO that also cleaves β-carotene to retinal also shows a dioxygenase mechanism (29), consistent with our results.

Unlike other enzyme names such as “isomerase” or “lyase”, the names “dioxygenase” and “monooxygenase” both indicate a specific reaction mechanism. Thus, the mechanism should be elucidated first before the name of an oxygenase is assigned. For oxygenases that yield aldehydes, oxygen exchange with water should be minimized and accounted for. BCO1 was called a dioxygenase in 1972 without an oxygen labeling experiment, and a monooxygenase in 2001 despite an inconclusive study. Our results demonstrate that BCO1 is a dioxygenase.
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FOOTNOTES
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The abbreviations used are: BCO1-β-carotene 15-15′-oxygenase, CCO-carotenoid cleavage oxygenase

FIGURE LEGENDS

FIGURE 1. Human BCO1 is a dioxygenase. A). The putative reaction mechanisms of BCO1. A monooxygenase incorporates an oxygen atom from O2 in one retinal molecule, and an oxygen atom from water into the other (10). A dioxygenase incorporates only atoms from O2 into the cleavage products (8). B) Theoretical percentages of 18O-retinal that will be obtained for oxygen labeling experiments with BCO1 as a monooxygenase and as a dioxygenase. C) Summary of results of oxygen labeling experiments with purified recombinant BCO1. The numbers separated by commas are the % 18O enrichment of the retinal product from individual experiments done on different days. Due to limited supply of 18O2, only two BCO1-β-carotene reactions were done in 18O2-H216O. Retinal obtained from the BCO1-β-carotene reaction contains predominantly the same oxygen isotope as O2. Control incubation of active BCO1 with 18O-retinal in H216O and 16O-retinal in H218O account for the oxygen exchange that occurred in the corresponding BCO1-β-carotene reactions. Thus, BCO1 incorporates solely oxygen from O2 during the oxidative cleavage of β-carotene, and is therefore a dioxygenase. The isotopic purity of the 16O-retinal standard is based on natural abundance of 16O (17) and verified by LC-MS/MS. Isotopic purity of synthesized 18O-retinal measured by LC-MS/MS.

FIGURE 2. LC-MS chromatograms for the reaction mixture of BCO1 and β-carotene in 16O2-H218O and 18O2-H216O. Purified recombinant human BCO1 (10 µg/200 µL) was incubated with β-carotene (20 µM) for 15 minutes at 37°C, and the reaction mixture was analyzed by LC-MS/MS. The LC-MS chromatograms from the reaction mixture in A) 16O2-H218O and B) 18O2-H216O are shown. The traces shown are the sum of the fragments from the MS/MS fragmentation of 18O-retinal (m/z=287.226) (blue trace), and 16O-retinal (m/z=285.218) (orange trace). MS/MS fragments used for quantification are listed in Materials and Methods.

FIGURE 3. MS/MS traces for the fragmentation of 16O-retinal and 18O-retinal obtained from the reaction of BCO1 and β-carotene in 18O2-H216O. The MS/MS trace for 16O-retinal (m/z=285.218) is shown in the upper panel, and that for 18O-retinal (m/z=287.226) in the lower panel.
Table 1. Comparison of retinal quantification by MS and MS/MS. The values using the parent ions obtained from LC-MS are in regular text, and those obtained by using the summation of daughter ions obtained from LC-MS/MS are in italics. Quantification by either method supports our conclusion that the retinal generated by BCO1 from β-carotene contains the same oxygen isotope as that of O₂.

|                 | \(^{16}\text{O}_2\text{-H}_2^{18}\text{O}\) | \(^{18}\text{O}_2\text{-H}_2^{16}\text{O}\) |
|-----------------|-----------------------------------------------|-----------------------------------------------|
| BCO1 + β-carotene | 3, 6, 10, 2, 6, 12                           | BCO1 + β-carotene 79, 85, 80                   |
| BCO1 + \(^{16}\text{O}\)-retinal | 5, 7, 13, 11, 6, 15                           | BCO1 + \(^{18}\text{O}\)-retinal (91%, 89%) 67, 84 |

\(\%^{18}\text{O}\)-retinal
Reaction mechanism of human BCO1

Figure 1
A.

Monooxygenase

\[ \begin{align*}
\beta\text{-carotene} & \xrightarrow{O_2} \text{H}_2O \\
\text{retinal} & \xrightarrow{O_2} \text{H}_2O
\end{align*} \]

Dioxygenase

\[ \begin{align*}
\beta\text{-carotene} & \xrightarrow{O_2} \text{H}_2O \\
\text{retinal} & \xrightarrow{O_2} \text{H}_2O
\end{align*} \]

B. Theoretical results

|   | \(^{16}\text{O}_2\text{-H}_2^{18}\text{O}\) | \(^{18}\text{O}_2\text{-H}_2^{16}\text{O}\) |
|---|---------------------------------|---------------------------------|
| Monooxygenase | 50 | 50 |
| Dioxygenase | 0 | 100 |

C. Experimental results

| \(^{16}\text{O}_2\text{-H}_2^{18}\text{O}\) | \(^{18}\text{O}_2\text{-H}_2^{16}\text{O}\) |
|---|---------------------------------|
| BCO1 + \(\beta\text{-carotene}\) | BCO1 + \(\beta\text{-carotene}\) |
| 3, 6, 10 | 79, 85 |
| BCO1 + \(^{16}\text{O}\)-retinal (\(\geq 99\%\) atom) | BCO1 + \(^{18}\text{O}\)-retinal (91% atom)* |
| 5, 7, 13 | 67, 84 |

* BCO1 + \(^{18}\text{O}\)-retinal incubated in \(^{16}\text{O}_2\text{-H}_2^{16}\text{O}\).
Figure 2
A

B
Figure 3
The Human Enzyme that Converts Dietary Provitamin A Carotenoids to Vitamin A is a Dioxygenase
Carlo dela Sena, Kenneth M. Riedl, Sureshbabu Narayanasamy, Robert W. Curley, Jr., Steven J. Schwartz and Earl H. Harrison

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