Key Role of Conserved Histidines in Recombinant Mouse \(\beta\)-Carotene 15,15′-Monooxygenase-1 Activity

Received for publication, January 12, 2005, and in revised form, June 2, 2005
Published, JBC Papers in Press, June 10, 2005, DOI 10.1074/jbc.M500409200

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Alignment of sequences of vertebrate \(\beta\)-carotene 15,15′-monooxygenase-1 (BCMO1) and related oxygenases revealed four perfectly conserved histidines and five acidic residues (His172, His337, His368, His374, Asp376, Glu460, Glu514, Glu457, and Glu435 in mouse BCMO1). Because BCMO1 activity is iron-dependent, we propose that these residues participate in iron coordination and therefore are essential for catalytic activity. To test this hypothesis, we produced mutant forms of mouse BCMO1 by replacing the conserved histidines and acidic residues as well as four histidines and one glutamate non-conserved in the overall family with alanines by site-directed mutagenesis. Our in vitro and in vivo data showed that mutation of any of the four conserved histidines and Glu457 caused total loss of activity. However, mutations of non-conserved histidines or any of the other conserved acidic residues produced impaired activity. Therefore, the conserved histidines and Glu457 are absolutely required for the catalytic mechanism of BCMO1. Because the mutant proteins are impaired in iron binding, these residues are concluded to coordinate iron required for catalytic activity. These data are discussed in the context of the predicted structure for the related bacterial apocarotenoid oxygenase.

\(\beta\)-Carotene 15,15′-monooxygenase-1 (BCMO1) is the initial enzyme step in the biosynthesis of vitamin A in animals, symmetrically cleaving \(\beta\)-carotene to produce two molecules of all-trans-retinal (1). In the last several years, a number of BCMO1 orthologs have been cloned and biochemically characterized (2–7). BCMO1 belongs to a family of oxygenases of diverse activities, including lignonostilbene dioxygenase in bacteria (8–10); an epoxycarotenoid-cleaving enzyme required for abscisic acid biosynthesis in plants (11); and mammalian RPE65, a retinal pigment epithelial protein required for the production of the visual chromophore 11-cis-retinal in the visual cycle (12). The most recent addition to the characterized carotenoid oxygenases is cyanobacterial apocarotenoid 15,15′-oxygenase PCC 6803 (ACO) (13). Despite their unique functionalities and the obvious importance of these proteins, structural studies on this family have been hindered by technical difficulties, and only recently has the crystal structure of eubacterial ACO been published (14).

Originally, Drosophila BCMO1 was identified by homology to a previously characterized plant 9-cis-epoxycarotenoid dioxygenase (15) and by its ability to cleave \(\beta\)-carotene (4). Subsequently, mouse BCMO1 was identified by our laboratory based on its homology to mammalian RPE65 (3). Because the biochemical function and mechanism of BCMO1 have been studied in detail (3, 5, 7), it is functionally the best characterized mammalian member of the family and could serve as a model for studying putative vertebrate oxygenases of more elusive function such as RPE65 and BCMO2 (16).

Although there is a weak overall identity (<10%) among these proteins, there are four histidines and five acidic residues that are absolutely conserved and a well conserved acidic stretch (which we call the “signature” sequence) that is common to the superfamily (see Fig. 1). In the original characterization of its activity, iron was found to be an essential cofactor for intestinal BCMO1 (17, 18) as well as for recombinant proteins (4). Iron is also required for the activity of other members of the family such as lignonostilbene dioxygenase (8). Therefore, we propose that four conserved histidines and five acidic residues would be the best candidates as putative metal-binding residues in BCMO1.

In this study, we demonstrate the necessity of the conserved histidines and acidic residues in the catalytic mechanism of BCMO1, supporting our hypothesis that these residues are necessary for iron coordination in BCMO1. Our data agree well with the crystal structure prediction for an iron(II) center coordinated by four conserved histidines and fixed by three glutamates in the related ACO (14) and provide empirical evidence for the validity of this prediction.

Experimental Procedures

Site-directed Mutagenesis—A panel of mutant BCMO1 proteins was made using a previously described pBAD/BCMO1 construct (3) as a template. This construct contains the BCMO1 open reading frame with a C-terminal V5 epitope and polyhistidine tag. The 15 residues of mouse BCMO1 under study (His49, Asp52, His55, Glu140, His172, His174, His237, etc.) were replaced for alanine using the single-step mutagenesis method of Champe & Jones (19). The BCMO1 open reading frame was ligated into pBAD/TOPO, a TOPO cloning vector (Invitrogen), and the recombinant plasmid was transformed into competent E. coli DH5α. For each construct, a total of 30000–40000 transformants were screened for the presence of the plasmid via antibiotic selection using ampicillin (100 μg/ml). DNA was isolated from individual transformants using the QIAprep Spin Miniprep Kit (Qiagen) and the sequences of each construct were confirmed via DNA sequencing. The open reading frames of the resulting constructs were subcloned into the pET22b vector, a high-copy-number plasmid that permits expression in E. coli in the absence of isopropyl β-D-thiogalactopyranoside induction. The subcloning procedure was carried out by the single-step method described by colleagues in the department.

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This paper is available on line at http://www.jbc.org
were grown in LB broth supplemented with 100 µg/ml ampicillin. These cultures were used to inoculate 500-ml cultures. At mid-log phase, the culture was harvested by centrifugation at 20,000 rpm for 15 min at 4 °C. The pellet was resuspended in B-PER detergent (Pierce; one-twenty-fifth of the volume of initial culture) and placed on ice, soluble proteins were isolated by centrifugation at 20,000 × g for 15 min at 4 °C. Recombinant His-tagged protein was purified using Talon CellThru resin (Clontech). The B-PER extract was applied to the resin and incubated with gentle agitation for 1 h at 4 °C. The resin was extensively washed with 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl and 10 mM MgCl2 and then transferred to a column containing B-PER resin (Pierce). Solubilized protein was supplemented with EDTA-free Complete protease inhibitor. The purified enzyme was stored in 40% (v/v) glycerol at −20 °C until use. To use, protein was concentrated on Amicon Ultra-15 filters with a molecular weight cutoff of 30,000.

In Vivo Assay of the Enzymatic Activity of Mouse BCMO1 and Mutants—pBADM/BMO1, pBADM-Ser104, and pBADM-Ser104 lactate dehydrogenase (RTS) constructs were transformed into E. coli strain TOP10 as described previously (3), with small modifications. Fifty cultures were used to inoculate 500-ml cultures. At mid-log phase, the absorbance was measured; and cells were grown for another 3 h at 0° C; the absorbance was measured; and cells were grown in LB broth supplemented with 150 mM NaCl, 4% formaldehyde, 0.5% Tween, 1% pyrogallol, and 1.18 M NaCl, 4% formaldehyde was added to stop the reaction, and the incubation was continued for 10 min at 37 °C. Then, 500 µl of acetonitrile was added, the solution was vortexed and put on ice at 4 °C for 5 min. The upper acetonitrile phase was collected, and 100 µl was injected and analyzed by reverse phase HPLC.

HPLC Analysis of β-Carotene and All-trans-retinal—β-Carotene, all-trans-retinal, and a-tocopherol acetate (internal standard for in vivo assays) were separated on a 4.6 × 250-mm Supelco Supelcosil LC318 C18 5-µm column with a flow rate 1 ml/min and simultaneous UV detection at 451, 383, and 292 nm (Agilent 1100 HPLC series). The initial conditions consisted of acetonitrile and 0.015% ammonium acetate (60:40) and were held for 5 min (0 min for in vivo method), followed by a linear gradient to 50:50 acetonitrile/isopropanol alcohol over 10 min (5 min for the in vivo method), which was held for an additional 10 min. β-Carotene and all-trans-retinal were quantified from their peak area using standard curves obtained with 220 and 0.5–20 ng of material, respectively, in 60:40 acetonitrile/water buffer.

ELISA for Measurement of Immunoreactive BCMO1—Direct ELISA was used to determine BCMO1 concentrations in the partially purified protein preparations obtained from bacteria by one-step His tag affinity purification. Additional purification steps led to a total loss of enzymatic activity. As the standard, the BCMO1 construct was amplified using a His tag linear template generation set (Roche Applied Science). The resultant PCR product with regulatory elements and a C-terminal His tag was subcloned into the pCRTopo vector and used as a template for the in vitro transcription and translation system (Rapid Transcription/Translation System, Roche Applied Science). Recombinant β-carotene was recovered from inclusion bodies by solubilization in 6 M guanidine HCl, refolding by the rapid dilution method in 0.5 M arginine, and subsequent dialysis and concentration (22). Purified protein was stored in 40% (v/v) glycerol at −20 °C, and the same batch of purified protein was used as a standard for all ELISAs. 96-Well OptiPlate-96FHB (PerkinElmer Life Sciences) plates were coated with 2–100 ng of standard and 0.5–4 µg samples/well in 100 µl of imidazole-buffered saline (IBS) containing 1 mM tris(2-carboxyethyl)phosphine hydroxylchloride and one Complete protease inhibitor tablet/50 ml. Plates were incubated overnight at 4 °C, rinsed with IBS wash buffer (KPL, Inc., Gaithersburg, MD), and blocked with 300 µl of 10% Blok-Hen II (Aves Labs Inc., Tigard, OR), 10% fetal bovine serum, and 10% SuperBlock solution (Pierce) in IBS at room temperature. After a brief rinse with IBS, the plates were incubated in alkaline phosphatase-conjugated goat anti-chicken IgY (1:1200 in IBS with 20% glycerol, 1% BSA, and one complete protease inhibitor tablet/ml) at room temperature for 1 h. The plates were washed and incubated with the BCMO1 antibody (raised against the mouse BCMO1 peptide sequence NYIRKIDPQTLETLEK (Aves Labs, Inc.)) in IBS with 20% fetal bovine serum and SuperBlock for 1 h at room temperature. After washing with IBS, the plates were incubated for 1 h at room temperature in alkaline phosphatase-conjugated goat anti-chicken IgY (1:1200 in IBS with 20% fetal bovine serum and SuperBlock). The plate was then thoroughly washed with Bidistilled water followed by 200 µM Tris-HCl (pH 7.5). The reaction was stopped with 200 µM Tris-HCl (pH 12.0) and 100 µM FeSO4, and the resulting absorbance was measured at 405 nm and 650 nm. Fluorescence was recorded with 355/460-nm filters. All samples were run in duplicate.

Iron Determinations—Proteins were purified as described above for in vitro assays. Samples (0.5 ml) were diluted 16-fold with buffer containing 37 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 100 mM FeSO4 and then reconstituted to the original volume using Amicon Ultra filters with a molecular weight cutoff of 30,000. Analytical reagent blanks were prepared by running buffers through the whole procedure using the same filters, glassware, plasticware, and pipette tips used to process the protein samples. Each sample and the corresponding blank of the same weight (ranging from 0.5 to 1.3 g) were placed in acid-cleaned quartz tubes and dry-ashed (20). Samples were diluted to a final volume of 3 ml using 18 megohm-cm purity water and analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES). A Leeman PS3000 spectrometer was used (combination simultaneous and sequential high resolution echelle-based ICP-AES system). Detailed instrumental operating conditions as well as calibration information are summarized elsewhere (24). NIST-SRM 1640 Trace Elements in Natural Water was run as standard along with all samples to ensure analytical accuracy; iron concentrations determined were in good agreement with the certified value of 34.4 ± 1.6 ng/g.

Miscellaneous—Total protein concentration was measured routinely using the Advanced protein assay reagent (CytoSkeletal, Denver, CO). The NanoOrange protein quantification kit (Molecular Probes, Inc., Eugene, OR) was used for measurement of diluted protein solutions (<1 µg/ml).

RESULTS

Identification of Perfectly Conserved Amino Acid Residues—Alignment of all known family members from taxonomically diverse organisms revealed ~40 residues to be absolutely conserved.
conserved not only in BCMO1 and RPE65, but also in ACO and BCMO2 proteins (Fig. 1). Included among these are four histidines and five acidic residues (not including two in the signature sequence) that we propose to be putative iron-binding residues in all family members. To determine the role of these four histidines and five acidic residues in the catalytic mechanism of BCMO1, we constructed a set of alanine substitution mutants (Fig. 2). We also replaced each of four non-conserved histidines and one non-conserved acidic residue (Glu450) with alanines. These residues are present only in the mammalian members of the family (BCMO1, BCMO2, and RPE65). If our hypothesis is correct, these latter residues

![Image](alignment_of_carotenoid_oxidase_family_members.png)

**Fig. 1.** Alignment of carotenoid oxygenase family members. Histidine and acidic residues conserved throughout the superfamily are in boldface; the signature sequence is underlined. Asterisks, identity; double dots, strong similarity; single dots, weak similarity. The chloroplast recognition signal was removed from the amino acid sequence of VP14 dioxygenase, and the last 24 amino acids of mouse BCMO1 were removed. GenBank™/EBI accession numbers are as follows: mouse BCMO1, AF271298; mouse BCMO2, AJ290392; mouse RPE65, NM_029987; Zea mays VP14, ZMU95953; Synechocystis lignostilbene dioxygenase (ACO), D90914.

![Alignment of carotenoid oxygenase family members](alignment_of_carotenoid_oxidase_family_members.png)

**Role of Histidines in Mouse BCMO1**

![Alignment of carotenoid oxygenase family members](alignment_of_carotenoid_oxidase_family_members.png)
Production of Mutant Forms of BCMO1 and in Vivo Enzymatic Activity—We made use of β-carotene-accumulating pACBETA-transformed E. coli (20) as an in vivo system for measurement of enzymatic activity. In these experiments, pBAD mutant constructs were transformed into competent E. coli cells. Cultures were split at mid-log phase; one portion was induced, and the other was not induced. After several hours, induced cultures expressing the wild-type protein and some of the mutants were completely bleached, whereas cultures expressing other mutants did not show bleaching (data not shown). To quantify the amount of all-trans-retinal formed or β-carotene consumed by the enzyme, all-trans-retinal and β-carotene extracted from induced cells were analyzed by reverse phase HPLC. Cells were grown in the dark at 30 °C for 3 h after induction, which was the bleach end point for the wild-type protein as judged visually. However, the amount of all-trans-retinal extracted from cells did not linearly correlate with the amount of β-carotene cleaved or the time after induction. This was probably due to further metabolism of all-trans-retinal in the bacterial cells. Therefore, all-trans-retinal production could not be used as a measure of activity in this system. On the other hand, the loss of β-carotene could be used to determine activity by comparing the β-carotene produced by the uninduced portion of the same culture used as a control with that in the induced portion of the culture. The catalytic activity is expressed as the ratio (%) of β-carotene extracted from the induced culture to β-carotene extracted from the uninduced culture, normalized to cell density. It should be emphasized that the presence of all-trans-retinal in cell extracts is an important enzymatic characteristic of the functionally active expressed protein and is an important qualitative correlate of activity.

Results for all 15 mutant proteins expressed in the in vivo system are presented in Fig. 3. pBAD/BCM01 (wild-type) was used as the positive control and pBAD/LacZ as the negative control in our scale of enzymatic activity. We found that all four mutants with replaced conserved histidines (H172A, H237A, H308A, and H514A) neither lost β-carotene nor accumulated all-trans-retinal and thus failed to convert β-carotene to all-trans-retinal, as detected in cell extracts. Mutation of Glu469 to Ala also eliminated catalytic activity (~100%, no loss of β-carotene). Replacement of the conserved Glu457 led to a substantial reduction in activity (~80% of β-carotene remaining). On the other hand, mutation of any of the four non-conserved histidines (H49A, H58A, H174A, and H309A) as well as the conserved Glu314 and non-conserved Glu450 did not lead to a dramatic decrease in activity in comparison with wild-type BCMO1. The amount of β-carotene extracted from induced cells compared with uninduced cells was 17.6 ± 1.2% for wild-type BCMO1 and up to 42.6 ± 2.2% for the H309A mutant (Fig. 3). Although these mutations influenced the catalytic activity of the enzyme, they clearly did not abolish it. It is likely that these amino acids are not necessary for metal ion coordination or can be compensated for. The change in the signature sequence Glu469 to Ala led to a significant decrease in activity (52.3 ± 4.9%) in 3 h, and cells expressing the E469A construct were bleached if left growing overnight. In cells expressing either the D52A or E140A mutant, all-trans-retinal was still produced, but the amount of β-carotene degraded was <50%. To investigate further the importance of the last two acidic residues, we constructed two double mutants (D52A/E140A and E140A/H49A) and tested them for activity. The loss of β-carotene was insufficient to make a conclusive comparison with single mutants; however, we found that the E140A/H49A mutant was still able to produce all-trans-retinal, whereas the D52A/E140A mutant failed to do so (Fig. 4). Therefore, these two acidic residues in combination are required for catalytic activity.

To confirm that the different mutants produce comparable amounts of protein in vivo, immunoblots of extracts from 13 cell lines transformed with mutant BCMO1 constructs were analyzed. All cells except for those transformed with the empty pBAD/LacZ construct expressed comparable amounts of pro-
Role of Histidines in Mouse BCMO1

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The Role of Histidines in Mouse BCMO1 Expression and Activity

Introduction

Biological oxidation of retinal (all-trans-retinal or 11-cis-retinal) to all-trans-retinoic acid is catalyzed by the enzyme rat brain cytochrome P450 oxidoreductase (BCMO1) (2,3). The BCMO1 protein has been purified from mouse liver microsomes (4) and human liver cytosol (5). In recombinant mutant proteins purified by metal affinity chromatography, we could maintain activity for several hours of preparation. However, the enzyme was too unstable, and the activity was lost within 24 h. This study was therefore designed to compare the biochemical characteristics of wild-type BCMO1 and enzymatically active mutants revealed that the decrease in activity compared with the wild-type enzyme was primarily due to changes in $V_{\text{max}}$, the largest change being observed for the D52A and E140A mutants. The greatest increase in $K_m$ (4.7 times) was observed for the D52A mutant, although the significance of this finding is still to be shown. We were not able to obtain kinetic curves for the E457A and E469A mutants. As expected from the in vivo experiments, no activity was detected for mutants with replaced conserved histidines or for the E405A mutant. The H49A mutant was active but unstable upon purification, so we were unable to determine reliable biochemical parameters for this enzyme. The H174A and H309A mutants demonstrated low activity in the in vivo system, probably due to their close location to conserved histidines (Hi372 and His1083), and were not assayed in vitro. These changes most probably affected protein folding but not iron binding directly.

Metal Analyses of BCMO1 and Its Inactive Mutant Versions—To address directly whether enzymatically inactive mutants lose the ability to bind iron, preparations of these proteins were probed for the presence of iron by ICP-AES. Purified BCMO1 without added iron showed much less activity (25%) than when iron was added into the assay mixture. Therefore, purified proteins were recharged with 100 nM FeSO4 in 20 mM imidazole buffer and tested for activity prior to ICP-AES analysis.

Table I

| Enzyme       | $K_m$ (μM) | $V_{\text{max}}$ (pmol substrate/μg enzyme) |
|--------------|------------|------------------------------------------|
| WT BCMO1     | 1.2        | 104.8                                    |
| H49A         | Unstable*  | Unstable*                                |
| D52A         | 4.7        | 0.9                                      |
| E140A        | 2.7        | 8.3                                      |
| E314A        | 0.9        | 0.8                                      |
| E450A        | 2.3        | 83.0                                     |
| WT BCMO1 (Ref. 5) | 7        | 600.0                                    |
| WT BCMO1 (Ref. 7) | 1        | 0.4                                      |
| WT BCMO1 (Ref. 3) | 6        | 2.2                                      |

*Initial activity was comparable to that of wild-type BCMO1; however, the enzyme was too unstable, and the activity was lost within several hours of preparation.

Discussion

The BCMO1 protein by a specific ELISA method are the only practical ways to characterize this enzyme so far. Formation of product was linear up to 60 min (data not shown) and for 25–150 ng of enzyme preparation. Variation among triplicates within each experiment averaged 15%.

FIG. 6. Hanes plot for wild-type BCMO1 kinetics. The points on the plot represent the average of three independent substrate curves obtained with different enzyme preparations. Variation among triplicates within each experiment averaged 15%.

FIG. 5. Immunoblots of extracts from the cell lines transformed with mutant BCMO1 constructs. Mid-log phase cultures of E. coli cells were induced with 0.02% L-arabinose, grown for 3 h at 30 °C in the dark, and harvested. The cell cultures (3 ml, $A_{595} = 1.7$) were resuspended in 50 mM Tris-HCl (pH 8) and 300 mM NaCl with protease inhibitors (0.5 ml) and homogenized using a FastPrep instrument (Qiobiogene) according to the recommendations of the manufacturer.

10 mg of total lysate protein was loaded per lane. Lane 1, pBAD/wild-type BCMO1; lane 2, pBAD/LacZ; lane 3, pBAD/H48A; lane 4, pBAD/H55A; lane 5, pBAD/H174A; lane 6, pBAD/H309A; lane 7, pBAD/D52A; lane 8, pBAD/E140A; lanes 9 and 10, metal-affinity-purified recombinant BCMO1 (1.6 μg of protein/lane); lane 11, pBAD/E141A; lane 12, pBAD/E405A; lane 13, pBAD/E450A; lane 14, pBAD/H172A; lane 15, pBAD/H372A; lane 16, pBAD/H508A; lane 17, pBAD/H514A; lane 18, pBAD/H309A.

The concentration from the studies of Redmond et al. (3) and Paik et al. (7) were considerably lower compared with our data (Table I) and those of Lindqvist and Andersson (5). Because more extensive purification procedures lead to a total loss of activity, using insect cells to express protein (5) and quantifying the extensive purification procedures lead to a total loss of activity, using insect cells to express protein (5) and quantifying the
TABLE II

| Enzyme          | Iron after blank subtraction | Total protein | BCMO1 protein eq | Iron bound (% WT BCMO1) |
|-----------------|-----------------------------|---------------|------------------|-------------------------|
|                 | ppk                         | mg            | µg               | nmol iron / nmol BCMO1 eq |
| WT BCMO1        | 86.0 ± 5.5                  | 0.32          | 19.2             | 100.0 ± 7.1             |
| H172A           | 30.7 ± 7.8                  | 0.39          | 24.7             | 45.2 ± 11.9             |
| H237A           | 2.5 ± 4.6                   | 0.29          | 45.3             | 4.8 ± 7.2               |
| H308A           | 14.4 ± 3.8                  | 0.41          | 15.8             | 19.0 ± 4.8              |
| H514A           | ND                          | 0.7           | 16.7             | 0*                      |
| E405A           | 5.3 ± 4.9                   | 0.59          | 21.5             | 9.5 ± 9.5               |

*The amount of iron was not significantly different from that in corresponding blanks or water.

Discussion

Iron has long been known to be an essential cofactor in the enzymatic cleavage of β-carotene. Goodman and Huang (17) and Olson and Hayashi (18) demonstrated in 1966 that chelators of ferrous iron inhibit the β-carotene cleavage activity of the enzyme in intestinal extracts. The results presented herein identify the residues of BCMO1 responsible for coordination of the iron cofactor recognized by these workers and underscore the importance of iron in the catalytic mechanism of this enzyme. Given the absolute conservation of these residues among its paralogs, this characteristic of BCMO1 is likely to be paradigmatic for all members of this family.

Heme or iron-sulfur clusters were not found in BCMO1 (17, 18, 25), and thus, it was proposed that iron binds directly to functional groups in the enzyme. A variety of oxygen-activating non-heme iron-containing enzymes have been described recently (26). Two basic classes include mono- and dinuclear iron centers, and both heavily implicate histidines and acidic functionalities (27, 28). A common motif in oxygen-activating mononuclear iron centers consists of a two-histidine/one-carboxylate facial triad found in a variety of unrelated enzymes (29). A second class of enzymes employs binuclear non-heme iron clusters (26, 27). So far, most of the oxygen-activating non-heme diiron enzymes contain a pair of (D/E)XXH motifs in their amino sequences, but the integral membrane oxygenase alkane hydroxylase (AlkB), another diiron enzyme, lacks this motif and appears to use eight conserved histidines as ligands (30).

However, the recent solution of the ACO crystal structure, employing four histidines at the axis of a seven-bladed β-propeller chain fold, demonstrates yet another type of mononuclear iron center (14). Iron(II) is coordinated in a nearly perfect octahedral arrangement by four histidines. Such coordination is also known for 15-LOX (31) and certain non-heme centers of photosystem type II and photosynthetic bacterial reaction centers (32–34). The fifth position in the crystal structure is occupied by a water molecule, and it is proposed that the sixth position is assumed by dioxygen. The positions of three of the four histidines (His398, His384, and His440) are fixed by glutamates. Presumably, all members of this family, including BCMO1, share this iron center arrangement. In this study, we have presented the first direct empirical evidence supporting this crystal structure prediction.

We have demonstrated an important role for the conserved histidines (His172, His237, His308, and His144) and Glu465 in the catalytic mechanism of BCMO1. Replacement of any of these residues individually led to total loss of enzymatic activity, and as we expected, replacement of any of the four non-conserved histidines (H49A, H58A, H174A, and H309A) did not eliminate catalytic activity, although mutation of the non-conserved His174 and His309 led to a substantial decrease in activity in the in vivo assay. A reasonable explanation is that they are located adjacent to the conserved His172 and His308 and thus may indirectly influence the functioning of those residues. On the other hand, only one of the predicted well conserved acidic residues (Glu465) was crucial for catalytic activity, whereas replacement of Glu314 had no significant effect on activity, and the Glu465, Asp52, and Glu465 mutations had an intermediate effect in the in vivo assay. As we expected, replacement of the non-conserved Glu465 did not lead to any change in activity, whereas mutation of the signature sequence conserved glutamic acid (E469A) had also an
intermediate effect on catalytic activity.

The in vitro biochemical characterization of mutants was consistent with the in vivo results. Of those mutants with activity, the difference in catalytic activity compared with the wild-type enzyme was primarily due to a $V_{\text{max}}$ change, with the D52A and E140A mutants having the most substantial $V_{\text{max}}$ decrease (~100-fold). A significant increase in $K_m$ was seen only in D52A (~4.7-fold). The in vivo assay with the D52A/E140A double mutant showed that the combination of the two was absolutely required for activity. The changes in $K_m$ for D52A and $V_{\text{max}}$ for D52A and E140A may indicate a role in substrate binding and turnover. On the other hand, it may be that these residues participate in iron binding by being replaceable ligands and can be replaced with a reaction intermediate or another amino acid during catalysis in the wild-type protein and in single mutants (35).

To visualize the spatial positions of BCMO1 replacements, we plotted the BCMO1 paralogs of ACO residues on the ACO three-dimensional ribbon model (Fig. 7). Our data are in good agreement with predictions from the crystal structure. The four conserved histidines required for BCMO1 activity are in BCMO1 (paralog of BCMO1 Glu405) was found to be mutated in some cases of Leber's congenital amaurosis (36), a congenital disease of early-onset blindness. Based on the phenotype of the knockout mouse (12), these patients probably lack 11-

In summary, our mutagenesis and iron data together provide the first biochemical insight into the catalytic mechanism of BCMO1. It is likely that this role of the conserved histidines and glutamates in iron coordination is similar for the entire superfamily. Furthermore, our data provide clear experimental validation of the proposed structure of this family of enzymes.

**Acknowledgment**—We thank F. Ella Greene for analytical contributions related to the iron determinations.

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**Role of Histidines in Mouse BCMO1**

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