Loss-of-Function Mutation in Carotenoid 15,15′-Monooxygenase Identified in a Patient with Hypercarotenemia and Hypovitaminosis A1–3

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Abstract

The enzyme carotenoid 15,15′-monooxygenase (CMO1) catalyzes the first step in the conversion of dietary provitamin A carotenoids to vitamin A in the small intestine. Plant carotenoids are an important dietary source of vitamin A (retinol) and the sole source of vitamin A for vegetarians. Vitamin A is essential for normal embryonic development as well as normal physiological functions in children and adults. Here, we describe one heterozygous T170M missense mutation in the CMO1 gene in a subject with hypercarotenemia and mild hypovitaminosis A. The replacement of a highly conserved threonine with methionine results in a 90% reduction in enzyme activity when analyzed in vitro using purified recombinant enzymes. The Michaelis-Menten constant (Km) for the mutated enzyme is normal. Ample amounts of carotenoids are present in plasma of persons consuming a normal Western diet, suggesting that the enzyme is saturated with substrate under normal conditions. Therefore, we propose that haploinsufficiency of the CMO1 enzyme may cause symptoms of hypercarotenemia and hypovitaminosis A in individuals consuming a carotenoid-containing and vitamin A-deficient diet. J. Nutr. 137: 2346–2350, 2007.

Introduction

Plant carotenoids are an important dietary source of vitamin A (retinol and its esters) and the sole source of vitamin A for vegetarians. The first step in the conversion of dietary provitamin A carotenoids to vitamin A is the cleavage of the central carbon 15,15′-double bond in carotenoid substrates. This reaction is catalyzed by the cytoplasmic enzyme carotenoid 15,15′-monooxygenase (CMO1) previously termed β-carotene 15,15′-monooxygenase (1,2). The most common carotenoid substrate for CMO1 is β-carotene, which is cleaved by the enzyme to form 2 molecules of retinal (retinaldehyde). The retinal formed is further converted to retinol and subsequently to retinol esters in the epithelial cells of the intestinal mucosa and then transported in chylomicrons to the liver, the main organ for vitamin A storage (3). Importantly, numerous studies have shown that a substantial amount of the absorbed dietary carotenoids are not cleaved by the CMO1 enzyme in the human intestine, suggesting that the CMO1 enzyme is saturated during normal dietary conditions (4–8). Carotenoids that escape the CMO1 enzyme are also incorporated into chylomicrons together with other lipids and the majority of carotenoids circulating in the blood are associated with low and high density lipoprotein particles and hence are taken up in tissues via the LDL receptor (9–11). CMO1 has been shown to be highly expressed in the epithelial cells of a variety of extraintestinal tissues, which suggests that the enzyme may constitute a back-up pathway for vitamin A synthesis during times of insufficient dietary intake of vitamin A (12). An important feature of the enzyme is that only 1 unsubstituted β-ionone ring half-site is required for efficient 15,15′-double bond cleavage; hence, in addition to β-carotene, there are ~50 additional known provitamin A carotenoids found in nature that can each form 1 molecule of retinal (13).

Retinoids are vital for normal embryonic and fetal development as well as for normal physiological functions in children and adults. Vitamin A deficiency in humans leads to impaired dark adaptation, xerosis of the conjunctiva and cornea, keratomalacia, and impaired function of the immune system. In some developing countries, dietary or primary deficiency of vitamin A remains the major cause of blindness in young children and carries with it a high mortality rate (3,14).

Whether vitamin A deficiency in humans can be caused by primary failure of enzymatic cleavage of carotenoids to retinal is unknown. So far, only a few cases have been reported in which individuals were thought to be vitamin A deficient due to an impairment in the conversion of carotenoids to retinal in the intestine (5,15–20). These subjects had orange-yellowish skin and variable degrees of mild vitamin A deficiency and in all cases, the biochemical hallmark was high levels of circulating...
carotenoids. The fact that the patients had slightly lowered serum vitamin A levels and reportedly consumed diets normal to low-normal in carotene intake excludes dietary carotenemia. Importantly, all patients responded well to treatment with vitamin A for the mild deficiency. The accumulation of carotenoids in the skin of the patients was slowly alleviated by low-carotenoid diets. These reports all relate to nonvegetarian individuals and the effects of reduced vitamin A production due to a dysfunctional CMO1 enzyme would become more apparent in people who adhere to a strict vegetarian diet lacking preformed vitamin A, albeit rich in provitamin A carotenoids. The incidence of individuals with impaired conversion of carotenoids to vitamin A is unknown, because in most cases, Western diets contain sufficient amounts of preformed vitamin A and hence compensate for the reduced production of retinoids from carotenoids.

In 1970, one of the present authors (D.E.S.) (17) published a case in which the subject had increasing yellowness of the skin but otherwise appeared healthy. The patient had very high serum β-carotene concentrations; the mean of 10 tests was 14.8 μmol/L, whereas published normal values range from 0.9–3.7 μmol/L (17). The patient’s serum vitamin A level was low to low normal. Excessive intake of β-carotene–rich food was excluded as the cause of the hypercarotenemia and it was suggested that the symptoms were caused by an abnormality of the conversion of β-carotene into vitamin A. In this study, we identified a novel T170M mutation in the BCMO1 gene in this patient. The replacement of the highly conserved threonine with methionine results in a ~90% reduction in activity when analyzed in vitro using purified recombinant enzymes. We also describe 1 novel single nucleotide polymorphism (SNP) in apparently normal individuals.

Materials and Methods

**Mutation detection.** Genomic DNA was extracted from white blood cells 5 d after blood was collected from the patient using the Puregene DNA Isolation kit (Gentra Systems) and subsequently analyzed by PCR amplification and DNA sequencing. Exon-specific primers were designed to anneal to sequences in the flanking introns; hence, this method of screening allows us to identify mutations that affect classical intron-exon splicing junctions as well as those within exons. The description of the primers used for PCR amplification of BCMO1 exons is presented in Supplemental Table 1. For exons 5, 7, 8, 9, and 10 of the BCMO1 gene, the initial denaturation cycle lasted 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C, and then a final extension for 5 min at 72°C. The conditions were the same for exons 2, 3, 4, 6, and 11 except the annealing temperature was 60°C. For exon 1, the annealing temperature was 57°C and the extension time 60 s. The primers used to amplify part of the BCMO1 promoter were P218: 5′-AAAGAGCC-GTGCCGCAAATACAGTGCAATTCGG-3′; 5′-GGGATGGTACATGTTGACATTTCCAGCAGGGG-3′; and P5pr: 5′-CTTCTCGA-GTTTGGGATGCTCGTACGTTCC-3′, and anti-sense 5′-TGGTGAATCATAGGCTTCTCCGTCCAGGACATCGC-TTCTGCTCGGTACAGGGACGTCGCTGAG-3′. The -c-myc tagged 3′ part was then introduced into the respective pCMV-hCMO1 His-containing vectors using AccI/SalI restriction enzyme sites, resulting in an exchange of the His tag for a myc tag. The pFastBac1-hCMO1 T170 c-myc, pFastBac1-hCMO1 M170-His, and pFastBac1-hCMO1 M170 c-myc donor plasmids, for subsequent production of recombinant baculovirus, were constructed by transfer of the pCMV vector inserts into pFastBac1 (Life Technologies) using EcoRI and SalI.

**Insect cell culture, expression, and purification of recombinant human CMO1 protein.** These techniques were performed as previously described (21), except that 16,000 g supernatant was prepared by adding 1 L of each culture to 2 volumes of homogenization buffer (50 mmol/L NaCl and 5 mmol/L NaPO₄, pH 7.0) to the insect cell pellet and the cell membranes were disrupted by 20 strokes using a Kontes SZ 23 tissue grind pestle (Kontes) followed by centrifugation at 16,000 × g, 30 min at 4°C.

**Gel filtration chromatography.** The purified enzymes (5 μg) were applied on a Sephacryl S-300 (Pharmacia) column (7 × 280 mm) equilibrated with 50 mmol/L Tricine-KOH buffer (pH 8.0), containing 100 mmol/L NaCl, 1 mmol/L dithiothreitol, and in some experiments, 1% (w/v) octylthioglycoside (OTG) (Pierce) was included in the buffer. The column was run with a flow rate of 21 mL/h and 0.25-mL fractions were collected. Then 7.5 μL of each fraction was loaded on 10% SDS-PAGE and analyzed by immunoblotting for presence of CMO1 protein (21). The column was calibrated with proteins of known molecular weight, bovine serum albumin (67 kDa), catalase (232 kDa), and Blue Dextran (~2 × 10⁶ Da).

**CMO1 enzyme assay and HPLC analysis of reaction products.** The reactions were performed as described (21), except that in the in vitro kinetic analyses, the β-carotene concentration in the reactions varied between 1 and 32 μmol/L and the purified CMO1 enzyme amount varied between 0.25 and 1.6 μg per reaction. Equal amounts of wild-type and M170 CMO1 enzymes were used in the in vitro wild type/ M170 mixing experiments. In experiments with 16,000 g supernatants expressing CMO1 enzymes, 16 μL supernatant (460 μg total protein) and 8 μmol/L β-carotene were used in a 60-min reaction. The reactions were terminated and analyzed by HPLC as described (21). We calculated kinetic constants and measured protein concentrations as described (21). Protein levels of CMO1 protein in 16,000 g supernatant fractions were quantified after immunoblotting and exposure to HyBlot CL autoradiography film (Denville Scientific). The films were scanned using a Hewlett Packard ScanJet 5100c and blot images were then quantified using Multi Gauge Version 3.0 software (Fujifilm).

**Protein electrophoresis and immunoblotting blotting.** Protein electrophoresis and immunoblotting with undiluted hybridoma culture medium containing mouse anti-CMO1 antibody MAH 1–11 or mouse anti-c-myc antibody raised against a EQKLISEEDL peptide (gift from Dr. Mike Roth, UT Southwestern Medical Center at Dallas), or purified polyclonal rabbit anti-6xHis antibody (Research Diagnostics) diluted 1:10,000 in Tris buffered saline containing 0.2% Tween 20 was performed as previously described (21).

**Amino acid sequence analysis.** Amino acid sequences for CMO1 from 8 different species plus the amino acid sequence for apocarotenoid 15,15’-oxygenase (ACO) from cyanobacterium were included in a CLUSTALW multiple alignment (22). GenBank accession numbers for CMO1 sequences used: human, AF294900_1; chicken, CAB90825.1; mouse, AF271298_1; rat, BAB60807.1; dog, XP_546815.1; cow, AAY25023.1; pufferfish, CAF95764.1; chimpanzee, XP_523435.2; and cyanobacterium Synechocystis sp. 6803 ACO, P74334.

**Results**

**Identification of a heterozygous mutation in the gene encoding CMO1 in a patient with hypercarotenemia and hypovitaminosis A.** DNA sequence analysis of amplified exons encoding CMO1 in a patient with hypercarotenemia 2347

Mutation in human BCMO1 causes hypercarotenemia
showed the individual to be heterozygous for an ACG → ATG allele in exon 5 of the gene, resulting in a T170M substitution in CMO1 (Supplemental Fig. 1), thus replacing a highly conserved threonine with a methionine (Fig. 1). To investigate whether the T170M substitution represents a SNP, we analyzed exon 5 in the BCMO1 gene in 30 unrelated, apparently normal individuals. The results showed that all 30 subjects were homozygous for the C allele that encodes the threonine residue.

**Biochemical analysis of recombinant enzymes.** Enzyme assays revealed that the T170M mutation severely compromised the CMO1 enzyme activity, because the specific activity of M170 assays revealed that the T170M mutation severely compromised the CMO1 enzyme activity, because the specific activity of M170 was 0.75 nmol retinal-mg protein$^{-1}$ min$^{-1}$ compared with 7.02 nmol retinal-mg protein$^{-1}$ min$^{-1}$ for the wild-type enzyme, corresponding to a turnover number ($K_{\text{cat}}$) of 0.05 min$^{-1}$ and 0.45 min$^{-1}$, respectively (Table 1; Fig. 2). The Michaelis-Menten constant ($K_m$) was 6 μmol/L for both enzymes. When M170 and the wild-type enzymes were mixed in equal amounts, the $K_{\text{cat}}$ was 0.22 min$^{-1}$, i.e. ∼50% of wild-type activity; hence, the M170 enzyme did not exert a dominant negative effect on the wild-type CMO1.

In a previous publication, we determined that the purified wild-type enzyme migrated on a Sephadex S-300 gel filtration column as a ∼230-kDa oligomer using buffer conditions identical to those used for enzyme assays, i.e. including the detergent OTG (21). To determine whether the impaired enzyme activity of the M170 enzyme was due to a change in the protein’s oligomeric state, we subjected the purified wild-type and M170 enzymes to gel filtration chromatography in the enzyme assay buffer containing the detergent OTG. Both wild-type CMO1 (Fig. 3A) and CMO1-M170 (Fig. 3B) migrated as ∼230-kDa oligomers. To ascertain whether the CMO1 proteins are monomers or oligomers in a detergent-free solution, gel filtration chromatography experiments were also performed in enzyme assay buffer without OTG. Both wild type (Fig. 3C) and M170 (Fig. 3D) migrate as ∼65-kDa monomeric proteins under detergent-free conditions, suggesting that the OTG detergent creates detergent-protein micelles containing oligomeric CMO1 complexes. Furthermore, when assayed for enzyme activity, the wild-type and M170 enzyme fractions that were eluted from the column exhibited similar activities regardless of whether the enzymes had been chromatographed in the presence or absence of OTG detergent (data not shown).

The potential effect of the M170 enzyme on the wild-type enzyme was further examined by coexpressing c-myc-tagged wild-type CMO1 and His-tagged M170 enzymes, as well as expressing the 2 variants individually. The relative amounts of the wild-type and the mutant enzyme in the 16,000-g supernatants were determined by immunoblotting, utilizing antibodies against the CMO1 enzyme, c-myc, and 6xHis, respectively (Fig. 4B). The activity of the wild-type CMO1 16,000-g supernatant was 2.6 pmol retinal formed-mg protein$^{-1}$ min$^{-1}$, which represents 100% activity (Fig. 4A, lane 1). The results demonstrate that the activity of CMO1 M170 was ∼8% of wild-type CMO1 (Fig. 4A, lane 3), whereas the fraction that contained 85% of M170 and 15% of wild-type CMO1 enzyme had an activity corresponding to 19% of the wild-type protein (Fig. 4A, lane 2). Furthermore, when His-tagged wild-type CMO1 was coexpressed in Sf-9 cells with c-myc-tagged CMO1 M170 and subsequently Co$^{2+}$-column affinity purified, CMO1 M170 was not copurified with the wild-type enzyme (data not shown).

**Promoter sequence analysis.** Because the subject was found to be heterozygous for the T170M mutation, we wanted to explore the possibility of mutations in the BCMO1 promoter by sequence analysis of a 218-bp region (−91 to +27; +1 being the transcriptional start site) of the most proximal part of the promoter, which includes a potential TATA-box (23) as well as a potential response element for the nuclear PPAR. These sites in the proximal mouse BCMO1 promoter have been shown to be sufficient to drive both basal and specific promoter activity (24). The analysis revealed that both alleles in the patient were identical with the corresponding BCMO1 promoter region in the 2 human genomic sequences (NCBI accession nos. NT010498 and NW926528), which include the BCMO1 gene on chromosome 16q21–23 (data not shown).

**Novel BCMO1 SNP.** When we analyzed the BCMO1 gene in our patient DNA and the sample collection from 30 normal individuals, we discovered a novel R228C SNP in exon 6 (Fig. 5). The polymorphic T allele at position 228 (CGC → TGC) encodes

### TABLE 1 Kinetic constants of CMO1 variants

<table>
<thead>
<tr>
<th>CMO1 variant</th>
<th>$K_m$ [μmol/L]</th>
<th>$k_{\text{cat}}$ [min$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6.0 ± 1.9</td>
<td>0.45 ± 0.05</td>
</tr>
<tr>
<td>Wild type/M170</td>
<td>5.0 ± 1.5</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>M170</td>
<td>6.0 ± 1.4</td>
<td>0.05 ± 0.003</td>
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</tbody>
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1. Each $K_m$ and $k_{\text{cat}}$ value in the table represents the mean ± SD of 2–6 independent experiments performed in duplicate.

**FIGURE 1** Partial amino acid sequence alignment of 8 different CMO1 isoforms and 1 carotenoid cleaving enzyme from a cyanobacterium. The highly conserved threonine residue is indicated with an arrowhead and the His involved in binding of the catalytic iron in the active site is highlighted with black. The amino acid residues are numbered to the right of the sequences.

**FIGURE 2** In vitro kinetic analysis of wild-type and M170 CMO1 enzymes.
a protein with cysteine rather than arginine and was only found in heterozygous form. The frequency of the C allele (Arg) was 0.98 and that of the T allele (Cys) was 0.02. We also found the 2 previously characterized SNP, R267S, and A379V (NCBI’s dbSNP; refSNP ID rs12934922 and rs7501331, respectively). The individual with hypercarotenemia described in this article was homozygous for R228, S267, and A379.

Sequence analysis of genes involved in carotenoid metabolism and vitamin A biosynthesis. Because the subject was heterozygous for the T170M version, we analyzed other genes known to be involved in carotenoid metabolism and vitamin A synthesis. By using our standard protocol for primer design and PCR amplification, we analyzed exons and flanking regions in the genes encoding carotenoid 9\#10\#-monooxygenase, cellular retinol binding protein I and II, and retinol dehydrogenase types 11, 12, and 14. All these genes were found to be normal in the subject (data not shown).

Discussion

In this study, we reported the identification of a mutation in the BCMO1 gene in a subject with hypercarotenemia and mild hypovitaminosis A. The T170M mutation results in a ~90% loss of function in the CMO1 enzyme and is the first case to our knowledge of a loss-of-function mutation in the human BCMO1 gene.

Threonine at position 170 is one of the more conserved residues among the CMO1 isozymes of different species. Although the function of this residue is not clear, substitution of this small hydrophilic threonine with a bulky hydrophobic methionine results in a severe attenuation of enzymatic activity. T170 is positioned in the proximity of H172, which is most likely 1 of the 4 His that coordinate the iron atom in the catalytic center of the enzyme (25). It should be mentioned that the structure of ACO from the cyanobacterium Synechocystis sp. 6803 was recently characterized (26). ACO belongs to the same protein family as CMO1 and several other iron-containing carotenoid cleaving enzymes and the catalytic iron (II) ion that defines the active center in ACO is buried in a tunnel-like cavity that holds the substrate. One of the iron-coordinating His residues in ACO corresponds to H172 in CMO1 and this His residue forms a stabilizing hydrogen bond with the serine residue in ACO that corresponds to T170 in human CMO1 (Fig. 1). Hence, the methionine at position 170 of the enzyme may destabilize binding or change the position of the iron (II), thereby compromising the enzyme’s catalytic capacity. The observation that the M170 enzyme’s affinity for β-carotene is identical to the wild-type T170 enzyme suggests that this residue is not crucial for substrate binding.

The T170M mutation was only found in a single allele of the subject and no other mutations were identified. With our method of screening, we would have found any mutations that affected the exons or the classical exon-intron splice junctions. However, it is conceivable that the other allele contains a mutation that creates a splice donor or acceptor site distant from the exon-intron junctions. This would, in turn, cause the formation of an aberrantly spliced CMO1 messenger RNA, a hypothesis that could have been tested by RT-PCR, but CMO1 messenger RNA-containing tissue from the patient was not available. Another explanation would be that the wild-type allele could contain a mutation in the promoter region of the CMO1 gene, a hypothesis we tested by sequencing 218 bp of its proximal 5’-flanking region; however, this region was wild type in both alleles. This observation does not, however, exclude the possibility of the existence of a more distant promoter mutation.

In a previous study, we described that the purified recombinant CMO1 enzyme exists as a 230-kDa tetramer in solution as revealed by gel filtration chromatography (21). Because our aim then was to ensure that the protein fractions detected by immunoblotting were in a catalytically active form, we performed the
gel filtration experiments under buffer conditions identical to those used in the enzyme assays with 1% OTG detergent in the elution buffer; hence, experimentally created detergent-protein micelles could be the cause of the migration as a tetrameric complex. In an effort to revisit this question and to explain the observation that a heterozygous mutation causes a disease phenotype, we tested whether the M170 enzyme could have a dominant negative effect on the wild-type T170 subunit as has been demonstrated with several tetrameric enzymes, e.g. mitochondrial acetaldehyde dehydrogenase (27,28). If it is assumed that both T170 and M170 are present in equal numbers and that the 4-subunit assembly is random, the mix of different combinations would result in a total enzyme activity much less than 50% of wild-type activity if the M170 subunit has a dominant negative effect on the complex. On the other hand, if there is no interaction between the wild-type and mutant enzymes, the heterozygote will have a total activity of ~50% of the wild-type activity. Reconstitution experiments with purified T170 and M170 enzymes and coexpression of the 2 enzymes in cultured cells revealed an additive, not dominant negative, effect of the M170 enzyme on the wild-type T170 enzyme, suggesting that the polypeptide chains catalyze chemistry independent of each other. Furthermore, gel filtration chromatogaphy demonstrated that CMO1 is a monomeric enzyme in the absence of detergent. Taken together, these experiments strongly suggest that the CMO1 molecules operate as monomers independently of each other, which leads us to the question whether CMO1 deficiency is another example where a heterozygous loss-of-function allele causes a disease phenotype, i.e. haploinsufficiency.

Haploinsufficiency can be exemplified by the various forms of porphyria, which are inherited and acquired disorders in which the activities of the enzymes of the heme biosynthetic pathway are partially deficient (29). There are 8 enzymes involved in the synthesis of heme and an enzymatic defect in any of the 8 enzymes in the pathway, with the exception of the first enzyme, results in a 50% reduction in activity and causes a porphyria phenotype. Because ample amounts of β-carotene and other carotenoids are present in plasma of numerous species, including humans, our observations supports the notion that the CMO1 enzyme is normally saturated with substrate. Thus, even though we have found a heterozygous mutation in only 1 subject, our data suggest that CMO1 deficiency manifests itself in an autosomal dominant fashion.

In conclusion, haploinsufficiency of the CMO1 enzyme may cause symptoms of hypercarotenemia and hypovitaminosis A in individuals consuming a carotenoid-containing and retinol-inadequate diet. Especially susceptible would be individuals on a pure vegetarian diet lacking preformed vitamin A, a situation common in developing countries where the major source of vitamin A is dietary provitamin A carotenoids. Further studies of individuals with hypercarotenemia and hypovitaminosis A will be necessary to assess the correlation of mutations in CMO1 with these phenotypes.

**Literature Cited**