



Hypothesis

Is carotenoid ornamentation linked to the inner mitochondria membrane potential? A hypothesis for the maintenance of signal honesty

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ABSTRACT

Several mechanistic hypotheses have been proposed for how carotenoid pigmentation of integumentary structures can serve as an honest signal of individual quality. These hypotheses are founded on proposed links between carotenoids, immuno responsiveness, and oxidative stress, but an absence of biochemical information on the oxidative pathways of carotenoids has limited the sophistication of such hypotheses. Based on published evidence, we propose that the oxidation of carotenoids for the purpose of ornamentation in birds and reptiles is coupled to the inner mitochondria membrane. We predict that several carotenoid oxidation reactions yielding ornamental pigments occur on the inner mitochondrial membrane. Three of these reactions are proposed to occur within the ubiquinone biosynthesis cluster known as the *Coq* cluster consisting of approximately a dozen *Coq* members, tightly integrated and intimately associated with Complex I and III of the electron transport system. Ubiquinone and highly oxidized ornamental carotenoids share a stereochemically-conserved binding region suggesting that these two molecules may have shared similar pathways in the past. Carotenoids and ubiquinones may cooperate as redox participants in anti-radical reactions or independently in helping to maintain membrane or supra-complex stabilization during times of high-energy demand. Under this hypothesis, oxidation of carotenoids is coupled to the inner mitochondria membrane potential such that ornamental coloration reflects the efficiency of cellular respiration.

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1. Introduction

Among the best-studied ornamental traits in animals are bright yellow, orange, and red color displays produced by deposition of carotenoid pigments in integumentary structures such as eyes, bills, skin, or feathers [1,2]. Current theory proposes that bright carotenoid displays are honest signals of individual quality [3,4] and abundant empirical evidence in many bird species supports an association between the showiness of carotenoid coloration and individual performance [5]. The mechanisms that link carotenoid ornamentation to performance, however, remain poorly understood [6].

Carotenoids cannot be synthesized by vertebrates *de novo*, so they must be sequestered in the diet [7]. In many birds, carotenoids are metabolized before they are deposited [2]. The majority of these reactions are oxidations (carotenoids lose electrons) while some may involve reductive hydride (H^-) transfers (gains electrons). In

this paper we will consider only oxidation reactions. Because carotenoids readily receive and transfer electrons, carotenoids are believed to serve as antioxidants within animal systems [8,9], and empirical studies consistently find links between oxidative stress, immunocompetence, and carotenoid intake [10,11]. These observations have led to the development of the Resource Tradeoff Hypothesis for the maintenance of honest signaling via carotenoid pigmentation [12–14]. By this hypothesis, carotenoids are essential and limiting resources serving as immuno-stimulants, antioxidants, and integumentary colorants, and only individuals with large pools of carotenoid resources are able to both maintain oxidative homeostasis and produce brightly colored integuments [15]. Alternatively, the Shared Pathway Hypothesis proposes that production of ornamental traits is fundamentally linked to vital cellular processes needed for general organism functionality [16]. By this latter hypothesis, carotenoid ornamentation is a direct indicator of the physiological state of the individual and is not simply a function of resource availability [17–19].

A shortcoming of both the Resource Tradeoff Hypothesis and the Shared Pathway Hypothesis is lack of specific cellular mechanisms. The locations and circumstances under which carotenoids might serve as critical antioxidants remain to be resolved even for model

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species like the laboratory mouse (*Mus musculus*) and are completely unexplored in vertebrates with integumentary ornamentation. Such vagueness has made the Resource Tradeoff Hypothesis difficult to critically assess but easy to support with statistical associations. Similarly, the specific pathways that are shared between vital cellular processes and mechanisms of ornamental production have not been proposed for any color display.

In this paper we propose a biochemical model for the pathways that are shared in the production of cellular energy and the production of oxidized carotenoid pigments used in ornamentation. We propose that the oxidation of carotenoids occurs within the inner mitochondria membrane (IMM). We first highlight the similarities between carotenoids and ubiquinones in both structure and function. We next show that the pathways leading to UQ biosynthesis involve the same stereo-specific biochemical modifications required for carotenoid metabolism. We then explore the various core reactions of which ubiquinones are the principal redox participants. Finally, we introduce the IMM carotenoid oxidation hypothesis in detail providing supporting evidence from the literature. We conclude by considering the implications of linking ornamental coloration to cellular respiration for understanding both color signals and basic mitochondrial processes.

2. Structural and functional links between carotenoids and ubiquinone

Ubiquinone (UQ), also referred to as Coenzyme Q (CoQ_n), is best known for its role in aerobic respiration where it carries electrons and protons from the matrix side of the IMM to the inner mitochondria space (IMS). The movement of protons into the IMS provides the proton-motive force that drives ATP synthesis [20]. UQ has a multi-oxygenated aromatic planar ring and a ten-member (CoQ₁₀) isoprenoid or farnesyl tail (Fig. 1). The aromatic ring is redox active and the isoprenoid side chain is flexible enough to allow fluid movement within and across the bilayer leaflets and interaction with UQ binding sites. The redox-active aromatic end ring oscillates between the IMM surfaces accepting and transporting both protons and electrons [21,22]. Because UQ is especially

lipophilic, it does not engage in radical transfer reactions with water-soluble anti-oxidants such as vitamin C and glutathione (GSH). If it is found outside the normal electron transfer pathways, however, it can interact with other lipophilic radical partners such as vitamin E (α -tocopherol) and carotenoids [23]. The aromatic ring of UQ derives from L-Tyrosine and L-Phenylalanine, and the isoprenoid tail is synthesized via condensation of isoprene monomers originating from acetyl Co-A via the mevalonate pathway [24]. In carotenogenic bacteria, plants, and fungi, carotenoids are also assembled via the mevalonate pathway and so share a part of their biosynthesis with prenylated quinones [24–26]. Unlike carotenoids, UQ is very difficult to assimilate in the diet due to its higher lipophilicity [27].

Carotenoids are acquired by animals in their diets [1], and they fall into two major groups: xanthophylls and carotenes (Fig. 1). Two dietary carotenes (β , β -carotene, β , ϵ -carotene) as well as one dietary xanthophyll (β -cryptoxanthin) are pro-vitamin A carotenoids. Pro-vitamin A carotenoids are sequestered primarily for vitamin A homeostasis [28,29]. In all birds and some reptiles (turtles and most lizards), ingested carotenoids are subject to one or more rounds of oxidation leading to the production of some combination of canary xanthophylls, keto-carotenoids, and cotingins (involving the loss of 4, 8 or 12 electrons respectively across 2 end-rings) (Fig. 2). Several of these oxidation reactions of dietary carotenoids create the pigments that are used in oil droplets in the retina [30] and that are deposited to produce some of the most brilliant color displays in animals. Beyond precursor–product relationships, the biochemical natures of the oxidation reactions as well as the identities of the enzymes that catalyze these reactions remain completely unknown in animals. Like UQs, carotenoids actively interact with radicals undergoing both electron abstraction and electron transfer reactions, covering a wide range of anti- and pro-oxidant behavior [31].

2.1. Structural similarities of UQ and carotenoids

On first consideration, UQ and carotenoids appear to share few similarities in structure: UQ has an aromatic planar end-ring with a non-conjugated isoprenoid tail, and carotenoids have a weakly conjugated hexene end-ring with a conjugated isoprenoid backbone. UQ has an additional ketone at position C2 while carotenoids have a lipophilic dimethyl group in the same position. On closer inspection, however, the two ring systems share important similarities in molecular structure (Fig. 1). Using cotingin (a carotenoid pigment isolated from the feathers of the Pompadour cotinga, *Xipholena punicea* [32];) as a fully oxidized carotenoid to compare to UQ, we found that carbons C6–C5–C4–C3–C2 of the carotenoid ring system was stereochemically identical to the same sequence in UQ (Fig. 1). Astaxanthin (3,3'-dihydroxy-4,4'-diketo- β -carotene) mimics C6–C5–C4–C3 of UQ and retains the stereochemistry for binding (considering the four binding residues on UQ and electron transfer (ketone)). All three of these molecules are planar within the known binding sequence of UQ. This degree of similarity favors a potential for carotenoids and UQs to occupy similar binding sites. The single exception we found occurred at site Q₀ in Complex III (ubiquinol-cytochrome c oxidoreductase, E.C. 1.10.2.2) (discussed below). In general we found that binding sites for the UQ ring and tail system within the various ETC complexes were accommodating (with sufficient volume) and appeared electrostatically compatible (with no apparent charge incompatibilities). Ohshima et al. [33] found the quinone reduction site at Complex I was spacious enough to accommodate bulky exogenous UQ analogs. This latter site was comparable in many ways to sites we inspected visually from available ETC 3D Protein Data Bank files revealing quinone binding (3H1J, 2WQY, 2H88 et al.).

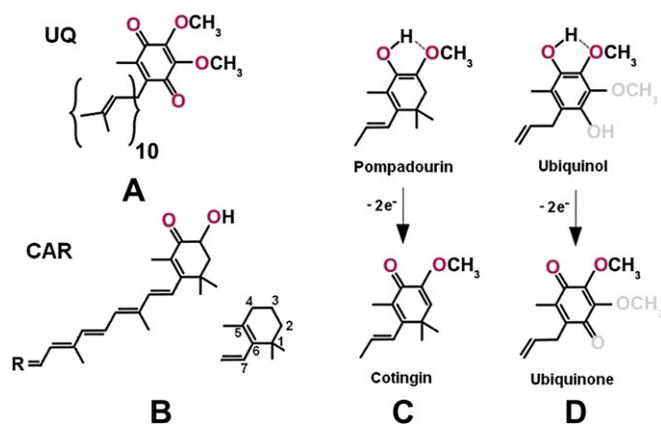


Fig. 1. Stereochemical representations of the end rings of ubiquinone (UQ), ubiquinol (UQH₂) and carotenoids (shown as one half a carotenoid in (B)). β -ionone ring positions are numbered. UQ (A) has mirror symmetry across the end ring with the exception of the methyl group, which determines the binding configuration of this pigment with UQ redox complexes. The stereochemistry of the methyl group is maintained in carotenoids (B). Carotenoids with oxygenated functional groups (B) are considered xanthophylls; the removal of these hydroxyl groups convert them to carotenes. Zeaxanthin can be converted to lutein (not shown) by relocation of the double bond within the β -ionone ring from position C5=C6 to C4=C5. Molecular mimicry can be seen in the oxidation of pompadourin to cotingin (C) when compared with the same oxidation of ubiquinol to ubiquinone (D).

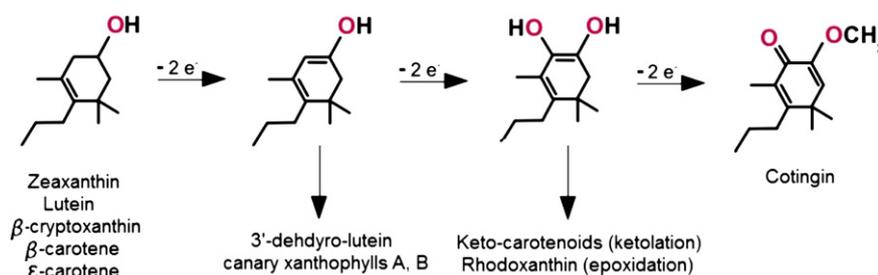


Fig. 2. The sequential oxidation of carotenoids from the least oxidized (dietary, far left) to the most highly oxidized (cotingin, far right). Evidence of the initial oxidation occurring within mitochondria under pro-oxidant conditions has been reported in mammals [50]. The initial oxidation products of dietary carotenoids are isoelectronic with either canary-xanthophyll A or B. The second round of oxidation leads to the production of more oxidized carotenoids such as rhodoxanthin and keto-carotenoids. Finally, in the neotropical Cotingas, a third round of oxidation, a reaction which take carotenoids to their peak oxidation state, leads to cotingin. All of these oxidations are potentially viable within the IMM and are discuss in the text in more detail.

2.2. Shared domains between UQ and carotenoids

Quinones and carotenoids have shared similar lipophilic domains for more than 3500 my. A large number of deeply rooted bacteria phyla are carotenogenic, and many of these have a ketolase to produce C4 keto-carotenoids [34]. We have discovered in our phylogenetic review of bacterial genomes from carotenogenic phyla that at least one carotenoid hydroxylase, known as crtD, has homology with Coq6, a hydroxylase found within the Coq biosynthesis cluster. We also observed that carotenoid enzymes often appeared to lie within or directly adjacent to operons occupied by primary oxidoreductases known to be active in electron transport. In *Arabidopsis*, electrons are transported directly from nascent carotenoids to the photosynthetic electron transport chain by plastoquinones [35]. There are numerous other examples of interactions of quinones and carotenoids across several carotenogenic bacteria phyla, including examples of electron transfer between the two. All of these observations support the ancient ties between UQ and carotenoids in fundamental redox biochemistry.

Prior to the rise of the cyanobacteria and the oxygenation of the biosphere, carotenoids evolved alongside low-potential quinones known as menaquinones (MK) that survive today in anaerobic bacteria [24]. Following the rise of oxygen of the biosphere approximately 1900 mya, later-evolving classes of proteobacteria (such as the α -proteobacteria which became mitochondria in eukaryotes) switched from anaerobic MKs to high-potential aerobic-UQs to adapt to increasing levels of molecular oxygen [36]. With the appearance of increased levels of molecular oxygen, quinones (and likely carotenoids as well) became more oxidized, thereby providing increasing protection against oxidation from rising levels of reactive oxygen. Simultaneously, the various redox centers within the ETC increased their midpoint oxidation potentials to accommodate the rise in carrier potential.

2.3. Shared function between UQ and carotenoids

UQ and carotenoids share a common basis for stabilizing radical intermediates allowing longer periods of time for redox recycling (repair) by locally cooperating anti-oxidants such as α -tocopherol (α TOH) [37]. A stable radical is less reactive and long-lived allowing sufficient time to be reduced by local anti-oxidants. Among the simplest radical reactions involving carotenoids is the transfer of an electron from a carotenoid to a nearby radical, thereby producing $\text{CAR}^{\bullet+}$, a positively charged radical with a half-life of up to 0.5 s [23,38–40]. Such radical stabilization by carotenoids is achieved across the conjugated polyene backbone of the carotenoid system [41]. UQ, on the other hand, is stabilized in radical form by its aromatic benzene ring nucleus, and presumably has extended

radical lifetimes in various compartments, most notably within circulating lipoproteins where they are quite active as anti-oxidants.

Both UQ and carotenoids are lipophilic redox-active pigments capable of both anti- and pro-oxidant behavior with a strong dependence on local redox participants [31,42–45]. Electrochemically, the midpoint potentials of UQ and carotenoids suggest that these molecules are redox coupled under physiologic conditions because both molecules reversibly interact with the $\text{O}_2/\text{O}_2^{\bullet-}$ couple and with each other [23,42,44].

Common redox reactions that are shared by UQ and carotenoids and likely to have a physiologic role *in vivo* are summarized in Table 1. The most notable anti-oxidant reaction shared by UQ and oxidized carotenoids such as astaxanthin is the direct quenching of superoxide ($\text{O}_2^{\bullet-}$). UQ and oxidized carotenoids both have enhanced oxidation potentials (high electron affinities) and are able to oxidize (remove an electron from) superoxide, thus quenching the radical directly. Interestingly, the potential to oxidize superoxide directly increases with redder (more oxidized) pigments suggesting a potential link of oxidative state to animal ornamentation [42,44].

Both UQ and carotenoids serve diverse functions. UQ is found in nearly all membranes outside of the mitochondria and serves several unique and independent functions in addition to providing anti-oxidant protection throughout the intracellular endomembrane system. UQ's primary function appears to be the shunting of electrons between NADH dehydrogenase to Complex III within the

Table 1

Radical reactions that are common between carotenoids and ubiquinones and likely to be physiologically important *in vivo*. The quenching superoxide through direct oxidation is a reaction uniquely shared between carotenoid and ubiquinones. Typical anti-oxidant behavior of carotenoid and ubiquinone involves giving up an electron to a radical (carbon centered) thereby reducing it and leaving a radical carotenoid and ubiquinone to be recovered by local anti-oxidants. Both carotenoids and ubiquinones have the capacity to reduce vitamin E (α -tocopherol) and therefore may interact with the well known ascorbate- α -TOC-Quinone relay of lipophilic radical reactions.

Name of reaction	Reaction equation ^a
Quenching of superoxide	$\text{ASTA} + \text{HO}_2^{\bullet} \Rightarrow \text{ASTA}^{\bullet-} + \text{O}_2 + \text{H}^+$ $\text{UQ} + \text{HO}_2^{\bullet} \Rightarrow \text{UQ}^{\bullet-} + \text{O}_2 + \text{H}^+$
Radical quenching (lipid)	$\beta\text{-CAR} + \text{R}^{\bullet} \Rightarrow \beta\text{-CAR}^{\bullet+} + \text{R}^-$ $\text{UQH}_2 + \text{R}^{\bullet} \Rightarrow \text{UQH}^{\bullet} + \text{RH}$
Regeneration of vitamin E (α -TOC)	$\text{CAR} + \alpha\text{TO}^{\bullet} \Rightarrow \text{CAR}^{\bullet+} + \alpha\text{TO}^-$ $\text{UQH}_2 + \alpha\text{TO}^{\bullet} \Rightarrow \text{UQH}^{\bullet} + \alpha\text{TO}^- + \text{H}^+$
Conjugation reactions (radical)	$\text{X}^{\bullet} + \text{GS}^{\bullet} \Rightarrow \text{GS-X}$
Conjugation reactions (Michael addition)	$\text{X} + \text{GSH} \Rightarrow \text{GS-X}$

^a ASTA = astaxanthin; UQ = ubiquinone; UQH₂ = ubiquinol; β -CAR = β -carotene; R = alkyl/peroxyl; X = adduct; α TO = α -tocopherol; GS = glutathione radical; GSH = glutathione.

electron transport chain. UQ has been found to serve as proton translocators in lysosomes [46] and as an electron accepting co-factor in numerous mitochondria enzymes including mitochondria glycerol-3-phosphate dehydrogenase (GPDH-M, E.C. 1.1.5.2), electron transfer flavoprotein dehydrogenase (ETFDH, E.C. 1.5.5.1), choline dehydrogenase (CHDH, E.C. 1.1.99.1) and dihydroorotate dehydrogenase (DHODH, E.C. 1.3.3.1). UQ also serves to link several ETC auxiliary redox proteins in the mitochondria where they shunt electrons to Complex III.

Specific roles for carotenoids in cellular processes of vertebrates are less clear, although there is mounting evidence that carotenoids do contribute to quenching radical reactions in the membranes in which they reside. There is also evidence that carotenoids can modulate and stimulate the cellular immune response [47,48]. The role of carotenoids in filtering light and quenching reactive singlet oxygen within the *macula lutea* has been well characterized in retinal biochemistry [49]. Physiologically, carotenoids may behave as either anti- or pro-oxidant depending on the redox partners with which they associate and the local physiologic conditions [31]. Unesterified carotenoids with C3 keto groups (canary xanthophylls) are likely candidates for Michael addition reactions with sulfhydryl groups such as those provided by glutathione and protein cysteines.

3. The IMM carotenoid oxidation hypothesis

We advance the hypothesis that in some reptiles and birds carotenoids and ubiquinones may share similar domains among potentially bi-functional enzymes including those found in the Coq biosynthesis cluster on the IMM [26]. Coq enzymes are proposed to oxidize and modify both UQ and carotenoids at specific stereochemical positions on the end rings. Substrate exposure and co-localization of both UQ and carotenoids provide the opportunity for such shared biosynthetic pathways, and such shared pathways would have significant implications for broadening our understanding of carotenoid metabolism in vertebrates. The three biosynthetic steps that we propose are shared between UQ and carotenoids are (1) ketolation (hydroxylation), (2) O-methylation, and (3) final oxidation to cotingin resulting in 2,3-desaturation (Fig. 2).

3.1. Production of canary xanthophylls

In vertebrates, the production of highly oxidized carotenoids begins with the conversion of dietary zeaxanthin and lutein to carotenoids that are isoelectronic with canary xanthophylls (Fig. 2). The first step in the pathway involves the initial loss of two electrons converting the β -ionone ring to an ϵ -ionone canary-xanthophyll C3 ketone. This initial reaction is responsible for the production of 3'-dehydro-lutein (3'-oxolutein), a carotenoid oxidation product found in the plasma of many vertebrates. Didehydro-lutein, a carotenoid isoelectronic with canary-xanthophyll B, and 3'-dehydro-lutein were found to be oxidized from dietary carotenoids within the mitochondria of mammals [50]. In other mammalian studies, the production of 3'-dehydro-lutein is proposed to be carried out by dehydrogenases based on finding equi-molar concentrations of (3R,6'S)- and (3R,6'R)-3'-dehydro-lutein [51].

Carotenoids isoelectronic with canary xanthophylls have been detected in numerous vertebrates tested for this metabolite [40,51–56]. Isoelectronic is used here to define a condition in which simple enzyme assistance (generally acid-base catalyzed) can overcome small activation energy barriers of substrate isomerization leading to more stable products. Because the reaction does not require the transfer of electrons, the isomerization is isoelectronic. Generally these isomerizations take the form of keto–enol

isomerizations, and account for many of the similar structures seen between carotenoids and UQ. Whether the pathway(s) to canary-xanthophylls is shared by the unknown ketolase in a second series of oxidations is uncertain because the enzymes involved are unknown. The pathway to canary-xanthophylls may be completely independent of the pathway to ketolation and may vary significantly across species.

3.2. Carotenoid C4 ketolation may be supported by a Coq hydroxylase

There are several potential biosynthetic routes to the production of oxidized ornamental carotenoids in birds. Evidence must be garnered from pigment profiles and a few data on circulating carotenoids [2]. To date not a single non-destructive carotenoid-modifying enzyme has been identified in birds [2,45]. Many sequential reactions happen rapidly, and intermediates may be strongly bound within an oligomeric arrangement of enzymes and hence be generally undetectable. The most popular route to avian keto-carotenoids (based on bacteria non-heme di-iron oxygenases) is via a double hydroxylation event at C4 on the dietary carotenoid β -ionone ring. In this catalytic scenario the carotenoid would be held within the catalytic site for two activated rounds of hydroxylation (presumably consuming two NAD(P)Hs and two O₂'s). Following the second hydroxylation and the formation of a gem-diol, the carotenoid would spontaneously dehydrate to a ketone at the C4 position. The latter reaction was first proposed by Hudon [57] within the catalytic site of an activated P450. One might expect this scheme to occasionally result in the deposition of singularly hydroxylated carotenoids in feathers and integument, but to date not a single C4 hydroxylated product has been proven to be a ketolated intermediate. Stradi et al. (1995) identified a C4 hydroxylated pigment known as isoastaxanthin (4,4'-dihydroxy- ϵ,ϵ -carotene-3'-dione) from goldfinches that appeared to be the product of a direct hydroxylation of C4 on the end ring of canary-xanthophyll B (note that enzyme assistance could convert isoastaxanthin to astaxanthin and vice versa because isoastaxanthin and astaxanthin are isoelectronic). There appears to be clear evidence for a C4 hydroxylation event, but whether the carotenoid stays for two rounds of hydroxylation remains unknown. Peroxide attack from a co-factor such as FAD or perhaps an unknown oxygenase could lead to a direct C4 ketolation, but because the ketolase remains unknown, any arguments claiming lack of detectable intermediates as proof of absence or of catalytic mechanisms to ketolation remains speculative [58].

A second pathway to ketolation involves the desaturation of the β -ionone ring at the C3–C4 position. Many hydroxylases share bi-functional behavior between hydroxylation and desaturation because the two reactions share similar mechanisms [59]. In the case of the proposed ketolase, a slight shift of the oxygen activated iron center (should the ketolase have an iron center) could alter hydroxylation and favor hydrogen abstraction leading to C3–C4 desaturation (as in fatty acid desaturations). The end product is a keto–enol isomer that can spontaneously transform to a ketone. If the C3–C4 desaturation occurs prior to the initial hydroxylation event, the product is capable of spontaneously relaxing into canary-xanthophyll (hydroxylation of this species would account for isoastaxanthin reported by Stradi et al. [60]). Although no intermediate products of C3–C4 desaturation have been found, desaturation of the β -ionone ring remains a viable alternative pathway considering the nature of hydroxylases and desaturases that commonly convert between these two reactions [61].

Finally, a hydroxylation event could be followed by a dehydrogenase reaction converting the C4 hydroxyl group to a ketone, a very common reaction in cells. This would require two enzymes in

close proximity (perhaps even fused) because there is little evidence in avian pigment profiles that singularly hydroxylated products are found. Here the absence of intermediates might carry more weight in arguing against this particular reaction sequence, but it would depend on the degree and nature of coupling between the hydroxylase and dehydrogenase.

Within the Coq biosynthesis cluster three UQ hydroxylases participate in the hydroxylation of the nascent UQ ring: Coq6, Coq? (unk), and Coq7 [24,26,62]. Coq7, a glutamate non-heme di-iron enzyme, is not a likely candidate for carotenoid hydroxylation because hydroxylation by this enzyme is directed at C5 on carotenoids and not C4 (relative to the isoprenoid polyene backbone and tail of carotenoids and UQ respectively). Both Coq6 and Coq? are, however, catalytically aligned with the C4 position of carotenoids. In both UQ and oxidized carotenoids an sp^2 hybridized C–H bond is oriented toward the axis of catalysis and the local topologies are identical (Fig. 1). Note that the sp^2 planar configuration at C4 would not apply to zeaxanthin. Both UQ and the proposed carotenoid are hydroxylated at the same equivalent position relative to the C6 β -ionone ring carbon. For carotenoids, the hydroxylation would be followed by keto–enol relaxation, perhaps enzyme assisted, to the known C4 ketolated product.

At this point in the synthesis the C4 ketolated product may escape the mitochondria, perhaps *en masse* across membranes in close contact with mitochondria (mitochondria associated membranes or MAMs) [24,26,62–65] or be shuttled by an unknown lipid transporter(s) [66]. Carotenoids leaving the mitochondria may be deposited within the secretory system where they are esterified and packaged into nascent VLDL particles. Alternatively they may be stored in nearby oil-droplets. For animals that use ketolated carotenoids as integumentary colorants or as retinal pigments, ketolation is the terminal step in the carotenoid oxidation process.

It is important to note that the UQ hydroxylase Coq? has not been identified. Evidence suggests that it is not likely a flavin-containing oxidoreductase, at least in the fungi *Saccharomyces cerevisiae* but this observation may or may not apply to vertebrates since *S. cerevisiae* is missing Complex I (NADH-coenzyme Q reductase, E.C. 1.6.5.3) [62,67]. Without knowledge of the identity of this enzyme, little can be deduced regarding mechanisms of hydroxylation. Phenyl-based aromatic hydroxylases originate from a number of enzyme families, including P450s as well as fatty acid hydroxylases/desaturases. Both types of enzymes hydroxylate aromatic rings via hydrogen atom extraction, a reaction compatible with carotenoid ketolation. Without further knowledge of the unknown Coq? hydroxylase, the compatibility or incompatibility of a single enzyme capable of hydroxylating both UQ and carotenoids remains speculative. It is interesting to note that ubiH, a required serine/threonine kinase for Coq? hydroxylase activity, was recently found linked to a core QTL map position on chromosome 5 (Tgu5) in a study on Zebra Finch (*Taeniopygia guttata*) beak color, which is pigmented with ketolated carotenoids [68].

3.3. C4 ketocarotenoids are further oxidized in some birds

In a few bird species belonging to the neotropical Cotingidae, carotenoid hydroxylation is followed by O-methylation of astaxanthin to pompadourin (for clarity we do not show the methylation reaction in Fig. 2). We propose that the methyl transferase Coq3, a member of the Coq UQ biosynthesis cluster, is the enzyme that O-methylates astaxanthin to produce pompadourin (Fig. 3). Given that this methylation reaction appears restricted to one family of neotropical birds, the substrate availability of the methylase following C4 ketolation may be a significant selective barrier to overcome. On the other hand, the channeling between the

methylase and the final site of oxidation to cotingin appears to be somewhat less restrained since O-methylation generally occurs concomitant to 2,3-desaturation. Coq3 has broad substrate specificity and utilizes S-Adenosyl methionine (SAM) as co-factor in the methyl transfer reaction [69]. It is likely that substrate channeling also plays a role in directing product out of the IMM. Substrate channeling of UQ between Complex I and III has been demonstrated in supra-complex assemblies of the ETC [22].

Once astaxanthin is O-methylated to pompadourin, it is either transported out of the IMM or channeled along the UQH₂ path to the Q_o binding site of Complex III (ubihydroquinone: cytochrome c oxidoreductase or cyt bc₁), one of only two principal sites in mitochondria for UQH₂ oxidation [22,23]. Complex III, which functions as a homodimer *in vivo*, consists of three core protein sub-units, an Fe–S Rieske protein, a cytochrome b complex (with both cytochrome b_L and b_H bound) and cytochrome c₁ which transfers electrons to cytochrome c for delivery to Complex IV. The proposed Q_o site is available on the IMS side of the IMM and sits close to the 2Fe2S Rieske cluster and the low potential heme cytochrome b_L.

The oxidation of UQH₂ at the Q_o site of Complex III is the rate-limiting step through this complex and appears to be dependent on the mid-point potential of a Rieske 2Fe2S center near Q_o [62]. Bound distally within the Q_o site of Complex III, UQH₂ yields two electrons in a bifurcated oxidation, delivering each electron to two independent acceptors. The first electron is transferred to a 2Fe2S Rieske iron sulfur center (ISC) and is the rate-limiting step in this oxidation [70]. The second electron, following relocation of the ubisemiquinone radical from the distal to the proximal site within Q_o, is transferred to a low potential cytochrome b heme (cyt b_L) [70,71]. The bifurcated nature of this two-electron oxidation is unique among all cytochrome-bc₁ family members. Thermodynamically it is more favorable for UQH₂ to send both electrons to the high potential 2Fe2S Rieske center (following 2Fe2S recycling) but conformational changes in the cytochrome b protein prevent the second electron from reaching the Rieske center and is transferred separately to the low potential cytochrome b_L, a component of the proton pumping Q cycle of Complex III (Fig. 3) [72–74].

Pompadourin is missing the C2 keto group that is found in UQ, suggesting it may not be compatible with competent cycling of bifurcated electron transfer within Complex III and therefore may not participate directly in the Q cycle of the complex. We suggest that the lack of a C2 keto group on the carotenoid may allow the carotenoid to sit longer in the distal Q_o site and deliver the second electron to the 2Fe2S Rieske center following cytochrome c₁ reduction [70,75,76]. Other possibilities for the second electron oxidation in carotenoids may involve homodimer inter-protein electron transfer [71,76,77] or other bypass reactions including oxidation by molecular oxygen forming superoxide [75,78].

3.4. Carotenoid metabolism in IMM

The above hypothesis—linking the biosynthesis of ubiquinone and the metabolism of carotenoid pigments—places controlled carotenoid oxidation in the IMM within liver hepatocytes. The liver is a likely location for carotenoid oxidation because it is active in lipogenesis within the secretory system. Liver cells are involved in the homeostasis of glycolysis, fatty acids, triglycerides, cholesterol and carotenoids and their transport into and out of circulation. Liver tissue has been proposed to be the tissue in which the ketolase is active during molt [79–81].

We advance the hypothesis that the hepatic IMM in birds provides a sufficient and sustainable oxidative environment with potentially compatible enzymes necessary for the sequential oxidation of dietary carotenoids to more highly oxidized forms. Along with these specialized and potentially bi-functional oxidative

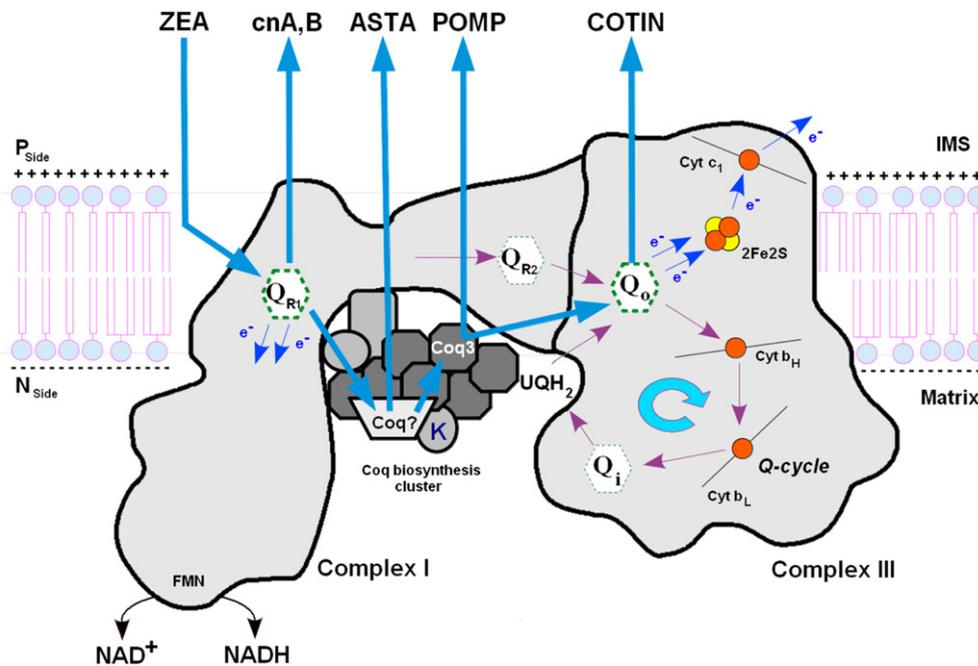


Fig. 3. Simplified diagram of Complex I and III depicting their assembly within a supercomplex (respirasome) showing the coenzyme Q (Coq) biosynthesis cluster associated with Complex I. Several of the Coq biosynthesis enzymes (Coq1–Coq10) are not labeled for clarity. Entering dietary carotenoids are proposed to be initially oxidized at site Q_{R1} (currently unknown except by experimental results – see text) in a reverse flow of 2 electrons to NAD^+ resulting in the conversion of zeaxanthin and lutein to oxidized carotenoids that are isoelectronic with canary xanthophylls (a second oxidation site for dietary carotenoids may occur at Q_{R2} but is not addressed here). Not shown is the proposed oxidative dehydration of lutein to 2,3-anhydrolutein catalyzed by the acidic P (positive) side of the IMM (requires an acidic environment for catalysis). Following initial oxidation at Q_{R1} carotenoids are proposed to follow substrate channels to the Coq hydroxylase Coq?, a hydroxylase that is currently unknown. Not shown is Coq6, the second candidate for carotenoid hydroxylation. Both Coq? and Coq6 are stereochemically competent to hydroxylate the equivalent position on carotenoids. K depicts the serine/threonine kinase (ubiH) required for hydroxylation (appears to be required for the phosphorylation of the C4 hydroxylase). Carotenoids such as β -carotene and β -cryptoxanthin (1/2 is carotene) may be desaturated at site Q_{R1} prior to hydroxylation or may be hydroxylated twice at Coq? forming a diol that spontaneously decomposes to a ketone (see text for details). At this point the ketolated product astaxanthin (ASTA) may be routed out of the IMM and back to the endoplasmic reticulum for export or storage in oil-droplets. The purple arrows depict the normal Q cycle path for electrons which are not expected to be supported with oxidized carotenoids (though this cannot be ruled out). In cotingas such as *Xiphophila punicea* (Pompador cotinga) ASTA presumably travels through substrate channels, likely gated, to Coq3, an O-methylase with broad substrate specificity. The resulting product is pompadorin (POMP), stereochemically equivalent to reduced UQH_2 (see Fig. 2). Following the methylation reaction POMP may be either routed out of the IMM to the ER or continue on to Q_0 , the most likely oxidation site for the conversion of POMP to cotingin (COTIN). As discussed in the text this latter reaction is likely to occur via a 2 electron transfer to the 2Fe2S Rieske iron sulfur center. The IMM is electropositive on the inner membrane space (IMS) and electronegative on the matrix side (N side). The Q cycle, responsible for cycling protons for the establishment of a proton-motive gradient is depicted within Complex III. Q_i is a binding site for UQ on the matrix side that participates in proton cycling. Not all redox centers are shown to improve clarity. Cardiolipin, discussed in the text, is depicted as phospholipids adjacent to the core complex proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enzymes this system would have in place an efficient import/export mechanism to transporting carotenoids into and out of the mitochondria rather than unidirectionally into the mitochondria for destruction by BCDO2. Carotenoids have been shown to preferentially concentrate in the outer and inner membranes of mitochondria in many cell types, but these results vary and are strongly dependent on species and tissue sampled. For example, in avian hepatocytes and lymphocytes, carotenoids are highly concentrated within their mitochondria following supplementation [82–84]. In bovine milk fat globules, carotenoids are highly concentrated in mitochondria and within the corpus luteum where they appear to be sequestered in luteal cells [85,86]. However, in mammalian mitochondria isolated from hepatocytes, carotenoids are generally absent in supplemented animals (see next section). After more than a century of carotenoid-based research, the physiologic roles supported by carotenoids and the intracellular locations in which they take place remain relatively undefined. The anti-oxidant and immuno-stimulating properties of carotenoids have been well documented, but the molecular mechanisms behind these events remain uncertain.

3.5. Carotenoids as pro-oxidants within mitochondria

In mammals [50] and fish [87], carotenoids have been found to interfere with electron transport and mitochondria functionality,

leading to the up-regulation of anti-oxidant enzymes and in many cases triggering apoptosis. Amengual et al. [50] demonstrated in a study using BCDO2 knockout mice that hepatic mitochondria became dysfunctional when mice were given a diet supplemented with zeaxanthin and lutein. Similar observations have also been made in studies of zebrafish, *Danio rerio* [87]. The impairment was proposed to be the result of interference with electron transport, presumably originating from radical carotenoid behavior within the IMM as evidenced by oxidized carotenoid metabolites. Carotenoids triggered significant mitochondrial anti-oxidant responses including the induction of manganese superoxide dismutase (Mn-SOD), phosphorylated MAPK and AKT, and other anti-oxidant systems as well as depolarizing the IMM potential. In wild-type mice having intact mitochondria BCDO2, Mn-SOD was induced 9-fold following carotenoid supplementation. Presumably carotenoids entered into oxidation reactions with unknown complexes resulting in increased superoxide leakage from the ETC followed by lipid peroxide and other ROS radical attacks. Similar oxidative stress effects have been characterized for numerous redox-sensitive drugs engaging in superoxide forming redox reactions with Complex I [88,89].

Interestingly [50] found that 3,3'-didehydrolutein formed from zeaxanthin and 2,3-anhydrolutein (end ring) formed from lutein were much more effective inducers of oxidative stress in BCDO2 knockout mice mitochondria (or intact HepG2 cells which do not

express BCDO2) than their parent dietary compounds. This latter result supports the argument that BCDO2 likely removes these oxidized pigments as waste before they can escape the mitochondria or interfere with the ETC on the IMM. 3,3'-didehydrolyutein, having two unsaturated α,β C3 ketone groups on each end-ring, may enter into a Michael addition reaction with nearby nucleophiles forming conjugates with glutathione (GSH), protein cysteinyl groups, or other available anion centers [88–92]. GSH is a critical anti-oxidant within the matrix of mitochondria and depletion of GSH levels would have immediate toxic consequences. These results support that argument that the first level of carotenoid oxidation in mammalian mitochondria is a conversion of zeaxanthin and lutein into oxidized products isoelectronic with both canary-xanthophyll A and B.

Despite these observations showing that carotenoid can be damaging in the mitochondria of mammals and fish, we advance the hypothesis that in birds there exists a redox window of tolerance within the IMM in which carotenoids may enter the mitochondria and support several biochemical processes. Birds have a much lower superoxide leakage rate from their ETC than to fish or mammals [93–95], which may allow carotenoids to enter the avian IMM under controlled redox conditions without the immediate danger of their transformation to pro-oxidant radical carotenoids or escaped reactive aldehydes. The high levels of carotenoids measured in the mitochondria of chickens [84] supports the idea that carotenoids can and do exist in quantities in the mitochondria.

4. General discussion

If our hypothesis linking carotenoids to the UQ pool is correct, then the oxidation of dietary carotenoids occurs within the IMM of hepatic cells. The transport of carotenoids into and out of the mitochondria has important implications for what is being signaled by carotenoid-based pigmentation in ornamented animals but also for a general role of carotenoids in the IMM serving as electron-scavengers or in yet uncharacterized roles. We propose that carotenoids entering the IMM are integrated locally within the UQ pool where they may serve as anti-radical anti-oxidants, superoxide quenchers (for highly oxidized carotenoids such as astaxanthin), or perhaps modulators of respirasome assembly. Antioxidant activity of carotenoids is likely to support the synergistic redox partners: α -tocopherol and ascorbate.

In our model, the initial oxidation of carotenoids to products isoelectronic with canary xanthophylls is carried out by unknown IMM protein(s). There is no way to determine at this time whether this first round of oxidation may be shared by the C4 ketolase or is independent of it. From this pool of initially oxidized carotenoids, C4 hydroxylation by either Coq6 or the unknown Coq? hydroxylase could lead to the formation of keto-carotenoids. Presumably redox gating along substrate channels will determine whether or not a carotenoid continues on to the next Coq enzyme for further oxidation. In cotingas, the neotropical group of birds that produce cotingins, the most oxidized carotenoids in vertebrates, C4 ketolase is followed by O-methylation. This latter reaction is proposed to be carried out by Coq3, a broad substrate-accepting O-methyl transferase that methylates an equivalent position on UQ. The O-methylated product, pompadourin, may either escape the IMM or continue along a pathway similar to newly synthesized UQH₂, finally being oxidized to cotingin at the proposed Q_o site of Complex III.

The availability of UQ is likely the condition most critical for maintaining a homeostatic flux of electrons through the ETC [22,96]. Moreover, the ratio of UQ to UQH₂ is the redox couple that is primarily responsible for maintaining the IMM potential ($\Delta\mu\text{H}^+$) [75,76]. The Coq biosynthesis cluster is required for Complex III

assembly in certain strains of *S. cerevisiae* [97] (Dr. P. Cobine *pers. comm.*). Given that for stability and function Complex I requires interactions with both the Coq biosynthesis cluster and Complex III, the interaction between Complex III and the Coq biosynthesis complex could couple efficient assembly of the supra-complexes to UQ as well as carotenoid access [22].

To maximize efficient ATP production, birds have evolved a spare capacity of ETC complexes compared to other tetrapods [93–95]. Excess ETC complexes are designed to meet instantaneous energy demands for activities such as powered flight. A consequence of such spare capacity is low radical superoxide leakage under metabolic resting conditions and low electron transport rates as seen in state 4 electron transport as described [93,94,98]. So long as electrons entering the ETC are equal to the electrons leaving, superoxide radical leakage from the electron transport chain is maintained at minimal levels. In the resting condition, the excess ETC Complexes of birds are more than adequate for maintaining superoxide leak-free electron flow through the ETC. Based on the physical arrangement of excess complex assemblies, it seems likely that carotenoids and ubiquinones have potential to interact in a cooperative manner. Opportunities for accessibility to ETC proteins may be more numerous in birds than in other vertebrates.

The implications of linking carotenoid metabolism to UQ and the function of the ETC in the IMM are substantial. For more than a decade, physiological ecologists have been reporting associations between circulating carotenoids, immune system parameters, oxidative state, various measures of individual performance, and ornamentation [11]. To date, the best explanation for these associations has been that carotenoids are crucial and limiting resources in their roles as antioxidants in cells [13]. Without knowledge of the pathways involved in carotenoid utilization, including carotenoid metabolism, researchers have treated the mechanisms of carotenoid utilization as a black box, tweaking conditions at the input phase and measuring output parameters [6]. By proposing specific mechanisms for the utilization and metabolism of ingested carotenoids, we have engaged in a first attempt to open the box and understand the mechanism of carotenoid utilization. If we are correct in our hypothesis, then the links between organismal functionality and carotenoid ornamentation arise not because of tradeoffs in the allocation of carotenoid pigments but because of links between carotenoid pathways and the systems for energy production that occur at the level of the IMM. The implications are that carotenoid pigmentation is potentially signaling the most fundamental aspect of the quality of the individual and that studies of carotenoid systems in ornamented birds might provide key new insights into fundamental processes in the IMM.

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