The Regulation of Coenzyme Q Biosynthesis in Eukaryotic Cells: All That Yeast Can Tell Us

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Abstract

Coenzyme Q (CoQ) is a mitochondrial lipid, which functions mainly as an electron carrier from complex I or II to complex III at the mitochondrial inner membrane, and also as antioxidant in cell membranes. CoQ is needed as electron acceptor in β -oxidation of fatty acids and pyridine nucleotide biosynthesis, and it is responsible for opening the mitochondrial permeability transition pore. The yeast model has been very useful to analyze the synthesis of CoQ, and therefore, most of the knowledge about its regulation was obtained from the *Saccharomyces cerevisiae* model. CoQ biosynthesis is regulated to support 2 processes: the bioenergetic metabolism and the antioxidant defense. Alterations of the carbon source in yeast, or in nutrient availability in yeasts or mammalian cells, upregulate genes encoding proteins involved in CoQ synthesis. Oxidative stress, generated by chemical or physical agents or by serum deprivation, modifies specifically the expression of some COQ genes by means of stress transcription factors such as Msn2/4p, Yap1p or

Hsf1p. In general, the induction of COQ gene expression produced by metabolic changes or stress is modulated downstream by other regulatory mechanisms such as the protein import to mitochondria, the assembly of a multi-enzymatic complex composed by Coq proteins and also the existence of a phosphorylation cycle that regulates the last steps of CoQ biosynthesis. The CoQ biosynthetic complex assembly starts with the production of a nucleating lipid such as HHB by the action of the Coq2 protein. Then, the Coq4 protein recognizes the precursor HHB acting as the nucleus of the complex. The activity of Coq8p, probably as kinase, allows the formation of an initial pre-complex containing all Coq proteins with the exception of Coq7p. This pre-complex leads to the synthesis of 5-demethoxy-Q₆ (DMQ₆), the Coq7p substrate. When de novo CoQ biosynthesis is required, Coq7p becomes dephosphorylated by the action of Ptc7p increasing the synthesis rate of CoQ₆. This critical model is needed for a better understanding of CoQ biosynthesis. Taking into account that patients with CoQ₁₀ deficiency maintain to some extent the machinery to synthesize CoQ, new promising strategies for the treatment of CoQ₁₀ deficiency will require a better understanding of the regulation of CoQ biosynthesis in the future.

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When coenzyme Q (CoQ) was discovered in 1957 by F.L. Crane [Crane et al., 1957] as a novel component of the respiratory electron chain system, the future and crucial role of CoQ was not completely revealed. However, CoQ discovery was not an incidental event; it was part of a general study to analyze mitochondrial components [Crane, 2007]. Obviously, the importance of CoQ as a mitochondrial electron carrier was reinforced when CoQ was chosen as the lipid electron carrier by the electro-osmotic theory [Mitchell, 1961]. After that, the important function carried out by CoQ in mitochondria to provide energy to the cell has been demonstrated. This function obscures that CoQ is involved in other cell functions that are needed for an adequate metabolism as they are not visible under normal levels of CoQ. However, it is likely that patients affected by CoQ deficiency exhibit adequate energy generation, which implies that other CoQ-dependent functions are responsible for their characteristic phenotype non-related with bioenergetics. In this sense, CoQ is required as electron acceptor in some reactions of β -oxidation [Frerman, 1988] and nucleotide synthesis [Jones, 1980; Nagy et al., 1992], it regulates the opening of the mitochondrial potential transition pore [Fontaine et al., 1998; Fontaine and Bernardi, 1999; Walter et al., 2000], and it is also required by the uncoupler protein UCP1 to transfer protons at the matrix [Echtay et al., 2000]. Out of the mitochondria, CoQ has been described as a direct lipophilic antioxidant [Do et al., 1996; Bentinger et al., 2007] that can also help to recycle other lipid or water soluble antioxidants such as vitamin E [Beyer, 1994; Kagan et al., 1998] or ascorbic acid [Santos-Ocaña et al., 1995, 1998; Gómez-Díaz et al., 1997a, b; Arroyo et al., 2004].

CoQ₁₀ deficiency diseases are a subgroup of rare disorders included in the family of mitochondrial diseases [Quinzii et al., 2007a]. CoQ₁₀ deficiencies can be classified in 5 groups according to the specific affection of organs and systems [Quinzii et al., 2008], all related to a decrease of ATP availability and a mitochondrial dysfunction. In the last years, patients with mutations in genes directly associated with the CoQ₁₀ synthesis have been studied [Lopez et al., 2006; López-Martín et al., 2007; Mollet et al., 2008; Lagier-Tourenne and Tazir, 2008; Duncan et al., 2009; Heeringa et al., 2011; Salviati et al., 2012] which allowed a classification into primary (*PDSS1*, *PDSS2*, *COQ2*, *COQ4*, *COQ6*, *COQ9*, and *ADCK3*) or secondary CoQ₁₀ deficiency (when other genes non-related to CoQ₁₀ are affected) [DiMauro, 2006].

In general, patients with CoQ₁₀ deficiency show a wide range of CoQ₁₀ content [Quinzii et al., 2007a, b] that correlates with the severity of the phenotype. In patients with

a high deficiency, there is a general problem of energy availability that is usually incompatible with birth or leads to death at young age. Moderate levels of CoQ₁₀ allow development up to birth, and some patients reach juvenile or adult ages with several types of clinical symptoms, some of them produced by an energy shortage, but others are related with defects in additional functions of CoQ₁₀ such as the synthesis of pyridine-nucleotides [López-Martín et al., 2007]. Low CoQ₁₀ levels are also detected in patients with mitochondrial diseases non-related to genes involved in CoQ₁₀ biosynthesis (secondary deficiency), in patients with neurodegenerative diseases [Shults et al., 2002; Battino et al., 2003; Mancuso et al., 2006; Stack et al., 2008] and also in aged people [Turunen et al., 2004].

Oral supplementation is so far the best approach to increase CoQ₁₀ levels in patients [Ogasahara et al., 1989; Rotig et al., 2000; Salviati et al., 2005]. However, several studies have reported that the improvement obtained by oral supplementation does not generally apply to all cases and depends on the symptoms detected in patients and the genetic origin of the deficiency [Musumeci et al., 2001; Lamperti et al., 2003; Aure et al., 2004; Quinzii et al., 2005; Artuch et al., 2006; Mollet et al., 2008; Lagier-Tourenne and Tazir, 2008]. The treatment of patients has been afforded by the use of CoQ₁₀ analogs such as MitoQ [Tauskela, 2007] or idebenone [Meier and Buyse, 2009]. The main effect of those molecules is to improve the antioxidant protection [Becker et al., 2010; Smith and Murphy, 2010], but only a low effect has been obtained at the respiratory chain [Plecita-Hlavata et al., 2009].

A complementary alternative may be to increase the endogenous CoQ₁₀ synthesis in all tissues. Some approaches based on the use of peroxisome activators through PPAR- α such as di(2-ethylhexyl) phthalate (DEHP) [Bentinger et al., 2003] or polyisoprenoid epoxides [Bentinger et al., 2008a, b] have been described. However, both approaches have not been tested in humans and do not affect the CoQ₁₀ biosynthetic pathway specifically. In all cases, it is tempting to speculate that induction of the endogenous CoQ biosynthesis could be the best solution since the CoQ₁₀ biosynthetic machinery is present to some extent in patients independently of the severity of the deficiency. Finding targets to increase the CoQ₁₀ biosynthesis will be our goal in the next years, but it requires a better understanding of the regulation of CoQ₁₀ biosynthesis. This is the focus of this review: to analyze the state of the art of regulation of CoQ biosynthesis. However, although human CoQ₁₀ deficiency has been an emerging topic in the last decade, most of the information has been obtained from the yeast model *Sac-*

Fig. 1. Biosynthetic pathway of CoQ in yeast. The pathway of CoQ₆ biosynthesis starting from the first molecule with the quinone structure (polar ring and isoprene chain). This molecule is produced by the action of Coq1p and Coq2p that are not included in the scheme. Coq6p is a monooxygenase that catalyzes the DHHB synthesis and probably the synthesis of 2-methoxy-6-hexaprenyl-1,4-benzoquinone; Coq3p is an O-methyltransferase that cata-

lyzes 2 O-methylations, Coq5p catalyzes a C-methylation and Coq7p catalyzes the hydroxylation of DMQ₆. For the decarboxylation of 4-methoxy-3-hydroxy hexaprenyl benzoate, the responsible enzyme has not been found. Other proteins such as Coq4p and Coq8p are required to synthesize CoQ₆ but do not show catalytic activity.

S. cerevisiae. This review focuses on this organism, but known details of CoQ₁₀ biosynthesis in mammals or human are also included. For a better understanding of the CoQ biosynthetic pathway, a general scheme for yeast is depicted in figure 1.

CoQ and the Bioenergetic Metabolism

Taking into account the main function of CoQ as a component of the respiratory chain, it is reasonable to hypothesize a connection between the regulation of CoQ biosynthesis and the respiratory function. Obviously, this points to a big difference between human and yeast models; yeasts are facultative aerobic fermenters, they can

usually grow as fermenters in glucose even in presence of oxygen, but can also grow as respiratory organism in the absence of fermentable carbon sources [Gancedo, 1998]. This allows studying the transition from fermentation to respiration as a model of CoQ₆ biosynthesis activation since this molecule is needed preferentially during respiration. Results from Sippel et al. [1983] demonstrated this relationship; higher levels of glucose in the culture media clearly decrease the amount of CoQ₆ in yeasts. This amount was inversely correlated with the amount of a quinone precursor, 3,4-dihydroxy-5-hexaprenyl benzoate (DHHB), that was previously described as a precursor of CoQ₆ biosynthesis in eukaryotic cells [Goewert et al., 1981]. The repression produced by an excess of glucose is a typical feature of some yeasts such as *S. cerevisiae* which

is mediated via the repression of Snf1p kinase [Gancedo, 1998], a protein complex that belongs to the family of AMPK kinases [Santangelo, 2006]. Snf1p is kept inactive in the presence of glucose, but at levels under 0.5%, the repression is lost, and Snf1p induces the expression of downstream genes that are required for the use of other carbon sources such as ethanol, lactate or glycerol. At this point, it is very interesting to analyze the effect of cAMP [Sippel et al., 1983], since high levels of CoQ₆ are correlated with high levels of cAMP. The addition of cAMP in the presence of 10% glucose shows CoQ₆ levels similar to those obtained with non-repressing conditions. That supports the idea that phosphorylation may be a regulatory element to consider in CoQ₆ biosynthesis. It is possible to argue that PKA activation may be a general regulatory element for mitochondrial metabolism, but the specific accumulation of DHHB before the first methylation reaction of the pathway points to a specific mechanism involving the phosphorylation of Coq proteins (see fig. 1).

The decrease of glucose concentration in yeast growing in YPD medium and the raising of ethanol modify considerably the metabolic state of yeast. When glucose is exhausted, ethanol starts to be consumed, but it requires a modification of metabolism that produces a shift named post-diauxic-shift or PDS [Pedruzzi et al., 2000]. This growth stage initiates a respiratory metabolism that needs a higher amount of CoQ₆. It has been reported that CoQ₆ and DMQ₆ (5-demethoxy-coenzyme Q₆) levels are affected in yeast cultured in YPD: initially, DMQ₆ is accumulated until PDS initiation, and then DMQ₆ decreases and CoQ₆ becomes the predominant quinone in the yeast [Padilla et al., 2009]. These facts support the idea of a DMQ₆ to CoQ₆ conversion after PDS [Padilla et al., 2009]. In the same study, this change of quinone concentration is correlated with the expression of several COQ genes such as COQ3, COQ4, COQ5, COQ7, and COQ8. A similar result has been found in high-throughput mRNA expression studies [DeRisi et al., 1997] for all COQ genes.

DMQ₆ is not the only accumulated intermediate in yeast during the growth in YPD. 4-Hydroxy-3-hexaprenyl benzoate (HHB) is also accumulated at a high proportion (~80%) against total quinones during the exponential phase of growth [Poon et al., 1995] but is a minor component at stationary phase. This fact agrees with the idea that CoQ₆ biosynthesis shows at least 2 different intermediates, the first at the initial stages of the specific quinone ring formation (HHB) and also a second molecule (DMQ₆) close to the last stage of synthesis. HHB may be produced during the initial exponential phase because

there is high availability of energy produced by fermentation, and DMQ₆ is produced at the initial PDS growth to accumulate an intermediate that is ready to be converted in CoQ₆ when it is required. In this sense, DMQ₆ is accumulated in wild-type yeast and does show neither mitochondrial electron transport nor antioxidant protection properties [Padilla et al., 2004].

The simplest method to detect respiratory-deficient yeasts is the growth in glycerol as carbon source. This is useful to study CoQ₆ biosynthesis because the change of glucose to glycerol-based media induces the expression of COQ5 [Hagerman et al., 2002], although in the particular case of COQ5, the growth in oleic acid as carbon source produces a higher induction than glycerol. Oleic acid degradation in yeast requires its oxidation in peroxisomes, while CoQ₆, unlike in other organisms, is not required as an electron acceptor [Hiltunen et al., 2003; Antonenkov et al., 2010]. Therefore, the higher requirements of CoQ₆ under oleic acid growth must be related to the mitochondrial electron transport and also to the protection of peroxisome membranes against the oxidative stress generated in this cellular compartment. However, in the previously cited study [Hagerman et al., 2002], the determination of CoQ₆ under glycerol growth was not performed. Recently, it has been reported that the growth in glycerol increases the accumulation of CoQ₆ in wild-type yeast [Padilla et al., 2009] with the simultaneous reduction of DMQ₆. In the same study, the glycerol growth induces the expression of several COQ genes such as COQ5, COQ7 and COQ8, the relative mRNA level being 3 times higher than that obtained from glucose growth. The COQ4 gene is another gene that is upregulated by growth in YPG, similar to some other mitochondrial proteins such as subunits of the complex IV [Belogradov et al., 2001]. In summary, some COQ genes are upregulated when yeast is grown under respiratory conditions. However, little is known about the transcription factors involved in this regulation. There is one study that specifically analyzes transcription factors involved in the synthesis of CoQ₆ [Hagerman and Willis, 2002], but unfortunately, this study only focuses on the COQ5 gene. This gene is regulated by Hap2p, a subunit of the HAP complex (heme activated-glucose repressed CCAAT-binding complex) that is a global regulator of respiratory gene expression (fig. 2). Also, COQ5 expression is down-regulated by Mig1p, a transcription factor that collaborates in glucose repression. Mig1p is phosphorylated by Snf1p (which is active with low glucose and dephosphorylated by Glc7p). Other studies devoted to a general transcriptional analysis have shown that COQ2 is upregulated

Table 1. COQ gene regulation by transcription factors involved in the bioenergetic metabolism

Transcription factor	Documented regulation	Potential regulation
Hap1p	<i>COQ2</i> [Harbison et al., 2004]	<i>COQ2</i>
Hap2p	<i>COQ5</i> [Hagerman and Willis, 2002]	<i>COQ2, COQ6, COQ7</i>
Hap3p		<i>COQ2, COQ6, PTC7</i>
Hap4p		<i>COQ2, COQ6, PTC7</i>
Hap5p		<i>COQ2, COQ6, PTC7</i>
Mig1p	<i>COQ5</i> [Hagerman and Willis, 2002]	
Gcn4p	<i>COQ5, COQ8</i> [Moxley et al., 2009], <i>COQ7</i> [Staschke et al., 2010]	<i>COQ8, COQ1, COQ3, COQ4, COQ9, PTC7</i>
Adr1p		<i>COQ2, COQ7, COQ9</i>

by Hap1p, a transcription factor that produces a response based on the levels of heme and oxygen [Harbison et al., 2004; Hickman and Winston, 2007]. Using the platform YEASTRACT [Monteiro et al., 2008; Abdulrehman et al., 2011], it is possible to find potential promoter sequences recognized by transcription factors involved in the mitochondrial respiratory metabolism (table 1) such as Hap3p, Hap4p, Hap5p, and Adr1p. Curiously, the amino acid starvation through the transcription factor Gcn4p induces the expression of most COQ genes (documented or potential regulation). Glutamate is considered as the node of metabolism required for the biosynthesis of 16 amino acids. Since the source of glutamate is α -ketoglutarate, a Krebs cycle intermediate, the activation of mitochondria and then the respiratory metabolism is a required factor to increase the amino acid biosynthesis. A general view of this regulatory process is shown in figure 2.

Little is known about the effect of the bioenergetic metabolism in mammalian or human cells in CoQ biosynthesis. The most relevant data comes from nutritional interventions such as caloric restriction (CR). CR in yeast induces a life span extension by the activation of the respiratory metabolism [Lin et al., 2002; Ocampo et al., 2012]. A similar effect was found in HeLa cells cultured in serum from rats subjected to CR conditions: at the same time, CR promotes mitochondrial biogenesis and improves the bioenergetic efficiency [Lopez-Lluch et al., 2006]. An increased mitochondrial biogenesis was produced in muscle from human individuals subjected to CR conditions [Civitarese et al., 2007], although in both cases CoQ₁₀ level was not measured. However, CoQ determination in 2 CR-treated murine models, mice [Lass et al., 1999] and rats [Kamzalov and Sohal, 2004], demonstrates that CoQ level decreases along the life, but it is

Fig. 2. Summary of the transcriptional regulation of CoQ₆ biosynthesis depending on carbon sources and the bioenergetic metabolism. CoQ₆ biosynthesis is modulated by several transcription factors related to the response to different fermentable and non-fermentable carbon sources, and also by amino acid deprivation. Green factors indicate activation and red factors correspond to repression. Black numbers indicate documented expression and grey numbers correspond to a putative expression of the respective COQ genes and PTC7.

higher compared to control in aged CR animals. In mammalian cells, the activation of the mitochondrial metabolism by CR and probably by other interventions increases the amount of CoQ.

CoQ Biosynthesis and Oxidative Stress

The antioxidant protection is a second function of CoQ [Frei et al., 1990; Bentinger et al., 2007]. In yeast, the better characterized antioxidant role of CoQ₆ was the protection of phospholipid peroxidation detected in COQ3 null mutants (*coq3*) growing in the presence of linolenic acid [Do et al., 1996]. That effect applies to all CoQ₆-deficient strains [Schultz and Clarke, 1999]. The incubation of wild-type yeast with linolenic acid upregulates several genes such as COQ3 and COQ7 [Padilla et al., 2009] and also drives the DMQ₆ conversion to CoQ₆. It is very interesting that this conversion requires the hydroxylase activity of Coq7p and the O-methyltransferase activity of Coq3p. However, the translational or post-translational nature of this regulation was not determined. Other oxidant compounds such as hydrogen peroxide did not affect COQ gene expression [Padilla et al., 2009]. Using the platform YEASTRACT [Monteiro et al., 2008; Abdulrehman et al., 2011], it is possible to find potential pro-

Table 2. COQ gene regulation by transcription factors involved in oxidative stress response

Transcription factor	Documented regulation	Potential regulation
Msn2/4p	COQ1 [Lai et al., 2005] COQ6 [Berry and Gasch, 2008]	COQ1, COQ2, COQ4, COQ6, COQ7, COQ9
Yap1p	COQ1 [Thorsen et al., 2007]	COQ3, COQ4, COQ5, COQ6, COQ7, COQ8
Hsf1p	COQ4, COQ6 [Eastmond and Nelson, 2006]	

motor sequences recognized by transcription factors involved in the induction of antioxidant defenses such as Msn2p, Msn4p, Yap1p, and Hsf1p (table 2). Msn2/4p are activated under stress conditions, Yap1p is specific to the antioxidant response to H₂O₂ and Cd²⁺ and Hsf1p produces a response against heat shock. All COQ genes are upregulated (documented or potential regulation) by a general situation of stress, but only COQ1 shows a specific response to H₂O₂ and only COQ4 and COQ6 for hyperthermia.

In this case, in mammalian cells, it is possible to find 2 examples of the oxidative stress role to promote CoQ biosynthesis upregulation. Serum deprivation is a method widely used to produce a moderate oxidative stress treatment that increases mitochondrial reactive oxygen species (ROS) production and Ca²⁺ release [Kuznetsov et al., 2008]. This method is comparable to others that induce cell death based on oxidative stress treatments [Kuznetsov et al., 2011], apoptosis being the most important consequence of serum deprivation. Previously, several studies have demonstrated that exogenous CoQ₁₀ shows a protective role against oxidative stress. This is the case of CEM-C7H2 cells that undergo apoptosis after serum deprivation conditions but are protected in presence of exogenous CoQ₁₀ [Fernández-Ayala et al., 2000]. This protection is produced by the inhibition of ceramide release and caspase-3 activation through the inhibition of plasma membrane-bound neutral sphingomyelinase [Navas et al., 2002]. The mild oxidative stress produced by serum deprivation not only induces apoptosis but also increases CoQ₁₀ levels in the plasma membrane [Barroso et al., 1997]. It suggests a regulatory function of serum deprivation probably through oxidative stress signals. However, in those studies only plasma membrane CoQ₁₀ levels were measured but not the total amount or the synthesis rate. It is possible that another mechanism such as CoQ₁₀ transport from mitochondria or other endomem-

branes [Fernandez-Ayala et al., 2005; Padilla-López et al., 2009] was the origin of CoQ₁₀ accumulation instead of an increased synthesis. More convincing results regarding the molecular mechanism of oxidative stress to induce CoQ biosynthesis were obtained from the effect of the chemotherapeutic drug camptothecin (CPT). This drug is a topoisomerase inhibitor that induces DNA damage and oxidative stress [Gorman et al., 1997] and therefore apoptosis. Initially, the oxidative stress generated is unlikely to be associated with DNA damage, but the effect of CPT is inversely related with reduced glutathione content [Troyano et al., 2001]. CPT treatment in several cancer cell lines increases the amount of total CoQ₁₀ [Brea-Calvo et al., 2006], this increase being dose-dependent and inhibited by the presence of antioxidants. The CPT mechanism to increase CoQ₁₀ biosynthesis requires the activation of the COQ7 gene by the transcription factor NF-κB [Brea-Calvo et al., 2009]. COQ7 in yeast is an important component of the CoQ biosynthetic complex catalyzing a reaction of hydroxylation and also shows a crucial function on the regulation of the CoQ biosynthetic complex activity in yeast.

Evidence of a CoQ Biosynthetic Complex in Yeast

Biosynthesis of CoQ requires the participation of at least 9 genes in yeast directly involved in the synthesis of the quinone ring. A crucial question in the field has been to identify whether Coq proteins adopt a linear pathway as in *E. coli* [Gibson and Young, 1978] or require the formation of a protein complex. Several lines of evidence strongly support the second option, the existence of a biosynthetic complex of CoQ₆.

Nature of Precursors in Null and Point Mutants

A common property for null COQ mutants is the accumulation of HHB (fig. 1) from COQ3 to COQ9 genes even for regulatory proteins such as Coq4p [Poon et al., 1997; Gin et al., 2003; Johnson et al., 2005]. However, some point mutants in COQ genes accumulate the expected intermediate or diagnostic precursor. In the COQ7 gene, several point mutants such as *qm30* or *e2519* [Padilla et al., 2004] or E194K [Tran et al., 2006] accumulate DMQ₆ instead of HHB. The DMQ₆ synthesis requires the participation of several Coq proteins, and it is located in the final steps of the biosynthetic pathway (fig. 1), supporting the idea of the existence of a complex to produce CoQ₆. Several point mutants in the COQ series such as COQ4, COQ5, COQ8 [Baba et al., 2004; Marbois et al.,

2009; Xie et al., 2011] are able to partially restore the steady state of other Coq proteins. In the case of *COQ4* or *COQ8*, the lack of diagnostic precursors could be expected given that these proteins play a regulatory role. More interesting is the case of Coq5p, a C-methyltransferase. This effect suggests that most Coq proteins show a dual role, catalytic and structural, namely some mutations can remove the enzymatic function, but the presence of protein in mitochondria supports the complex assembly. Another important fact is analyzing the effect produced by null mutations on the presence of other Coq proteins in yeast mitochondria.

Steady-State Levels of Coq Proteins in Mitochondria

The steady-state levels of Coq proteins constitute an interesting approach to analyze the complex existence. If a possible component of a multi-enzymatic complex is absent in mitochondria by a null mutation in another gene, it constitutes an indicator of the complex existence. The first study performed to analyze the steady-state levels of Coq proteins was focused on the protein Coq3p [Hsu et al., 2000], basically thanks to the existence of specific antibodies and the possibility to measure the O-methyltransferase activity. It was the first of numerous analyses conducted in the laboratory of Dr. C.F. Clarke [Baba et al., 2004; Gin and Clarke, 2005; Hsieh et al., 2007; Marbois et al., 2009; Xie et al., 2011, 2012]. With these data, it was possible to establish 3 types of Coq proteins: (a) proteins always present in mitochondria independent of the null mutant analyzed, such as Coq1p, Coq2p, Coq5p, and Coq8p, (b) proteins that show a lower expression, such as Coq3p and Coq4p, and (c) proteins that completely disappear, such as Coq6p, Coq7p and Coq9p. With the premise that if a subunit of a complex cannot be assembled, it will be removed and degraded from mitochondria [Arlt et al., 1998; Rugarli and Langer, 2012], the proteins unaffected in null mutants (Coq1p, Coq2p, Coq5p and Coq8p) must be independent of the complex formation. In the case of Coq1p and Coq2p, it is possible that their contribution to the complex formation is the synthesis of a quinone-like precursor that is required to start the complex assembly (fig. 3A, B). More difficult to explain is the function of Coq5p and Coq8p. These proteins are likely required to initiate the complex and can be located at the mitochondria even if the complex is not produced.

Components of the CoQ₆ Biosynthesis Complex

The presence of a Coq protein in mitochondria itself does not demonstrate the participation in a biosynthetic

complex and does not allow to deduce the complex composition. It was possible to obtain information using new approaches such as detection of mitochondrial complexes by size exclusion chromatography (SEC) and BN-PAGE. Initially, a 700-kDa complex containing Coq3p, Coq4p, Coq6p, and Coq9p but not Coq1p or Coq5p was detected using SEC [Marbois et al., 2005; Hsieh et al., 2007]. The detection of O-methyltransferase activity in the same fractions demonstrates the presence of Coq3p and also validates the method. BN-PAGE analysis confirmed the presence of Coq3p, Coq4p, Coq5p, and Coq9p in the 700-kDa complex [Tauche et al., 2008; Marbois et al., 2009]. A second complex was detected by SEC at a high molecular weight (1,300 kDa) containing Coq3p, Coq4p and Coq7p [Tran et al., 2006; Marbois et al., 2009].

In summary, the CoQ₆ complex assembly is produced in 2 steps; the initial one is the formation of a pre-complex of 700 kDa containing most of the Coq proteins that are affected in null mutants, with the exception of Coq7p (fig. 3C). The pre-complex accumulates the precursor of Coq7p, DMQ₆ (fig. 3D). The second step is the full complex assembly after the addition of Coq7p (fig. 3E, F) and produces CoQ₆. Coq8p remains as an intriguing component that must play an extremely important role in complex formation although there is no evidence of its presence in both complexes. Coq8p overexpression studies point out the crucial function of this protein in CoQ₆ biosynthesis.

Overexpression Studies

Suppression studies by gene overexpression are very useful tools to analyze the structure or composition of multi-enzymatic complexes. In the specific case of CoQ₆, this analysis was performed with *COQ8* overexpression in several null mutant strains. The first null mutant analyzed was the *COQ7* null mutant (*coq7*) that was subjected to *COQ8* and *COQ4* gene overexpression because at this time both genes did not show any catalytic activity [Padilla et al., 2009]. However, only *COQ8* overexpression allowed the accumulation of DMQ₆ in *coq7* mutants. This was the starting point of a set of experiments clarifying the structure of the CoQ₆ biosynthetic complex. It was used to demonstrate the role of Coq6p as C5-hydroxylase. The addition of vanillic (4-hydroxy-3-methoxy benzoate) acid together with *COQ8* overexpression restored the CoQ₆ synthesis in a *COQ6* null mutant strain (*coq6*) [Ozeir et al., 2011]; vanillic acid bypassed the lack of Coq6p, but *COQ8* overexpression made possible the complex stabilization required to modify vanillic acid to CoQ₆. Without the addition of exogenous molecules, it

Fig. 3. Model of the biosynthetic complex for CoQ₆. The model is a summary of already published data and non-well-defined ideas shown in the text. **A** The precursor. The first quinone-like molecule is the 4-hydroxy-3-hexaprenyl benzoate (HHB) that is produced by the action of Coq1p and Coq2p in mitochondria. Both proteins are required to synthesize CoQ₆ but are not present in the complex. HHB is accumulated at the exponential phase of growth during fermentation. **B** The nucleation. Coq4p recognizes HHB and starts the nucleation process. The action of Coq4p must be triggered by the mere accumulation of Coq4p produced when cells reach the PDS. However, previous activation by a kinase cannot be excluded. **C** Formation of the 700-kDa pre-complex. During the PDS,

the activity of Coq4p bound to HHB starts the recruitment of Coq proteins to produce the 700-kDa complex or pre-complex. **D** Pre-complex activity. Because the pre-complex does not contain Coq7p, the activity of the pre-complex facilitates the accumulation of DMQ₆ during PDS. **E** The role of Ptc7p on the final assembly. Coq7p has been phosphorylated previously and cannot be a component of the pre-complex due to steric hindrance or charge repulsion with some components of the pre-complex. The phosphatase Ptc7p dephosphorylates Coq7p, erasing the repulsion from the pre-complex. **F** The full complex assembly. Coq7p bound to the pre-complex catalyzes the penultimate step of CoQ₆ biosynthesis after the formation of the full 1,300-kDa complex.

was possible to analyze the accumulation of diagnostic intermediates and the steady-state levels of Coq proteins after *COQ8* overexpression [Xie et al., 2012]. That method validates the DMQ₆ accumulation in *coq7* mutants produced by complex stabilization, and it also demonstrates the C-methyltransferase activity of Coq5p. The same approach applied to *coq3* and *coq4* mutants (*COQ8* overexpression) seems to be ineffective because it only produces the accumulation of HHB. For *coq3* mutants, it yields the expected results, but for *coq4* mutants, it supports the idea of an initial function of Coq4p in the assembly of the complex.

These lines of evidence demonstrate that CoQ₆ biosynthesis in yeast is the result of the assembly of a multi-enzymatic complex composed of some Coq catalytic proteins and also other Coq regulatory proteins. This complex seems to be nucleated around the first quinone molecule (HHB) and Coq4p (fig. 3A, B). Most of the Coq

proteins are bound to the initial HHB-Coq4p to produce the 700-kDa pre-complex (fig. 3C) that accumulates DMQ₆. Coq7p is bound to the pre-complex when high levels of CoQ₆ are required. The remaining question to solve is the nature of the mechanism which makes possible the full complex containing Coq7p (fig. 3F) that finally produces CoQ₆.

Regulation of CoQ Biosynthesis by Phosphorylation

The protein encoded by *COQ7* seems to be a regulatory element in CoQ₆ biosynthesis. Coq7p catalyzes the hydroxylation of DMQ₆ (fig. 1). DMQ₆ is accumulated in wild-type strains [Sippel et al., 1983; Padilla et al., 2004, 2009] and in some point mutants of *COQ7* growing in glucose. The model of CoQ₆ biosynthesis indicated above shows that Coq7p does not seem to be required for the

700-kDa pre-complex, but it requires the participation of Coq7p to produce CoQ₆ in the 1,300-kDa complex (fig. 3F). Coq7p has been described as a protein that can be phosphorylated by Coq8p [Tauche et al., 2008; Xie et al., 2011], but recently Coq7p phosphorylation has been associated with the metabolic state of yeast [Martin-Montalvo et al., 2011]. The introduction of 3 phosphosite modifications in Coq7p detected by a NetPhos analysis [Ingrell et al., 2007] leads to non-phosphorylatable proteins (Ser20, Ser28, Thr32 to Ala20, Ala28, Ala32). The expression of the triple modified version of *COQ7* in a *coq7* null mutant dramatically increases CoQ₆ up to 250%. When CoQ₆ is needed, the dephosphorylation of Coq7p must be a requisite to facilitate the interaction of Coq7p with the pre-complex rendering the full 1,300-kDa complex (fig. 3F).

In *S. cerevisiae*, 7 of the 33 phosphatases [Sakumoto et al., 1999] encoded in the yeast genome are mitochondrial phosphatases [Claros and Vincens, 1996], but in most cases the function is known with the exception of Ptc7p, which belongs to the PPM family [Martin-Montalvo et al., 2013]. Ptc7p dephosphorylates Coq7p in vivo and in vitro [Martin-Montalvo et al., 2013], and it is expressed in non-fermentable carbon sources and in oxidative stress conditions. Coq7p phosphorylated in vitro by PKA is dephosphorylated by Ptc7p, corroborating that the lack of *PTC7* leads to the accumulation of phosphorylated Coq7p in vivo. A direct relationship between Coq7p and Ptc7p was demonstrated when the non-phosphorylatable version of Coq7p (Coq7p-AAA) was expressed in a *PTC7* null mutant strain (*ptc7*) [Martin-Montalvo et al., 2013]. This overexpression produces a high amount of CoQ₆ (250% compared to control) since phosphatase is not needed when Coq7p is constitutively dephosphorylated. This supports completely the function of Ptc7p as Coq7p-phosphatase and points out the Ptc7p functions as a regulator of CoQ₆ biosynthesis in particular and as regulator of mitochondrial metabolism in general.

Discussion and Future Perspectives

The biosynthesis of CoQ is regulated in order to match mainly 2 cellular processes, the bioenergetic metabolism and the antioxidant defense. At the transcriptional level, higher CoQ requirements caused by the carbon source in yeast or the availability of nutrients in yeasts and mammalian cells modify dramatically the expression of genes involved in CoQ synthesis. In a similar way, oxidative stress generated by different agents such as chemical re-

agents or serum deprivation induces the specific expression of genes involved in CoQ synthesis. Clearly, the induction of *COQ* gene expression increases the amount of CoQ, but this expression is modulated by new regulatory steps associated with the protein import to mitochondria and mainly with post-translational modifications such as the CoQ biosynthetic complex assembly and a phosphorylation cycle. Complex assembly is a process involving 3 steps: nucleation, pre-complex assembly and full complex assembly. The phosphorylation of Coq7p and the consequent dephosphorylation by the phosphatase Ptc7p is the mechanism explaining the last step. This model for regulation of CoQ₆ biosynthesis introduces a target to improve the CoQ biosynthesis, the triangle composed by the subject of phosphorylation, Coq7p, the phosphatase, Ptc7p, and the kinase, probably Coq8p.

However, CoQ biosynthesis is a topic far from being completed and the model depicted here (fig. 3) will require further research. Several methods must be applied to finish the description of a model for CoQ biosynthesis. It will require obtaining crystallographic models for Coq proteins and also the application of massive proteomic analysis, both promising strategies in order to analyze Coq protein relationships and possible interactions with other mitochondrial complexes or proteins.

The studies performed on regulation of CoQ₆ biosynthesis in yeast can be transferred to the human model in which most of the Coq proteins are homologous to yeast proteins and can complement successfully null mutant strains [López-Martín et al., 2007; Casarin et al., 2008; Heeringa et al., 2011]. A better knowledge of this mechanism will be useful to find potential targets for new therapeutic approaches to improve the level of endogenous CoQ₁₀ in patients affected by primary or secondary deficiency.

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