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Review

Human choline dehydrogenase: Medical promises and biochemical challenges ☆

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ABSTRACT

Human choline dehydrogenase (CHD) is located in the inner membrane of mitochondria primarily in liver and kidney and catalyzes the oxidation of choline to glycine betaine. Its physiological role is to regulate the concentrations of choline and glycine betaine in the blood and cells. Choline is important for regulation of gene expression, the biosynthesis of lipoproteins and membrane phospholipids and for the biosynthesis of the neurotransmitter acetylcholine; glycine betaine plays important roles as a primary intracellular osmoprotectant and as methyl donor for the biosynthesis of methionine from homocysteine, a required step for the synthesis of the ubiquitous methyl donor S-adenosyl methionine. Recently, CHD has generated considerable medical attention due to its association with various human pathologies, including male infertility, homocysteinuria, breast cancer and metabolic syndrome. Despite the renewed interest, the biochemical characterization of the enzyme has lagged behind due to difficulties in the obtainment of purified, active and stable enzyme. This review article summarizes the medical relevance and the physiological roles of human CHD, highlights the biochemical knowledge on the enzyme, and provides an analysis based on the comparison of the protein sequence with that of bacterial choline oxidase, for which structural and biochemical information is available.

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Introduction

Human choline dehydrogenase (CHD[1]; E.C. 1.1.99.1) is a nuclear-encoded, mitochondrial enzyme involved in choline metabolism. According to the NCBI gene database [1], the gene coding for CHD has been identified on chromosome 3 (gene location 3p21.1). From a study of Haubrich et al. on human tissues, the enzymatic activity of CHD has been detected mainly in kidney, with 6-times lower levels in liver [2]. Other tissues like blood, spleen and heart displayed very low CHD activity, whereas no detectable activity was reported for muscle and fat tissue [2]. CHD is also present in bacteria, fungi and other mammals (Fig. 1), but it is notably absent in plants where an iron-sulfur choline monooxygenase (CMO; E.C. 1.14.15.7) has been identified instead [3]. CHD from eukaryotic organisms is associated with the inner mitochondrial membrane on the matrix side [4–6]. The homologous protein from prokaryotic sources is associated with the cytosolic side of the cell membrane [7].

From a medical point of view, human CHD is of interest due to its association with various pathologies, including male infertility [8], homocysteinuria [9], and cancer [10,11]. The enzyme also readily metabolizes choline when the latter is administered as a pharmacological agent, thereby limiting its potential therapeutic use [2]. Great attention has also been directed to bacterial CHD for medical, biotechnological and fundamental reasons. The medical interest is primarily due to the fact that the prokaryotic enzyme plays an important role for the ability of bacteria to grow in environments with high salinity, such as human infection sites, and thus represents a potential pharmaceutical target for combination therapy [12]. Biotechnological applications primarily focus on the genetic engineering of glycine betaine biosynthesis from choline to provide osmotic stress resistance in economically relevant plants and the detection of choline and its derivatives in biological fluids [13,14]. Biochemical interest stems mainly from the comparison of the catalytic strategies utilized in the oxidation of choline by the three types of enzymes that carry out the reaction: CHD, CMO, and choline oxidase (CHO; E.C. 1.1.3.17) (Scheme 1) [15]. In organisms that oxidize choline using CHD or CMO, a second enzyme, i.e., betaine aldehyde dehydrogenase (BADH; E.C. 1.2.1.8), is responsible for further oxidation of betaine aldehyde to glycine

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[1] *Abbreviations used:* CHD, choline dehydrogenase; CHO, choline oxidase; CMO, choline monooxygenase; BADH, betaine aldehyde dehydrogenase; PMS, phenazine methosulfate; GMC, glucose–methanol–choline; PQQ, pyrroloquinoline quinone.

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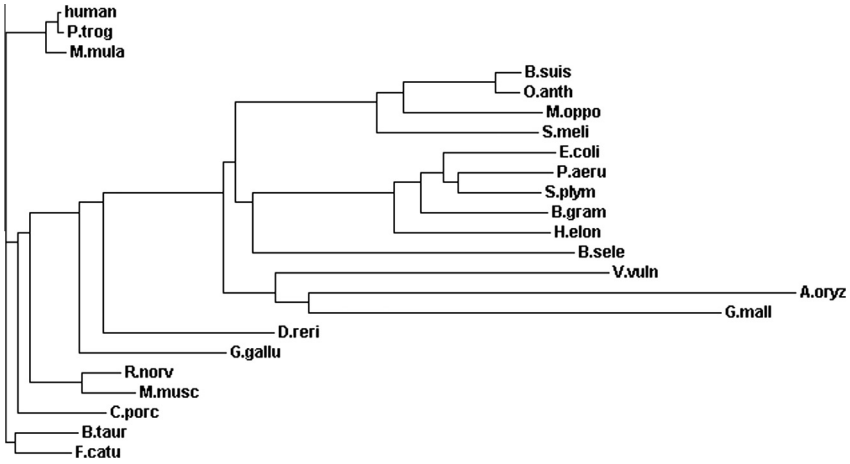
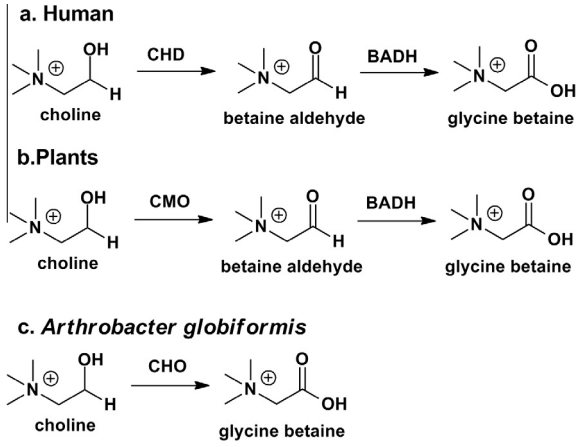


Fig. 1. Phylogenetic tree of CHD from *Homo sapiens* (NP_060867.2), *Brucella suis* (YP_005154241.1), *Vibrio vulnificus* (ADV86038.1), *Ochrobactrum anthropic* (ABS15421.1), *Bos Taurus* (NP_001192493.1), *Rattus norvegicus* (NP_942026.1), *Mus musculus* (NP_001129712.1), *Gallus gallus* (XP_414335.3), *Escherichia coli* (BAE76094.1), *Pseudomonas aeruginosa* (NP_254059.1), *Pan troglodytes* (XP_001173164.1), *Macaca mulatta* (NP_001244738.1), *Bacillus selenitireducens* (YP_003698280.1), *Serratia plymutica* (YP_004504883.1), *Halomonas elongata* (YP_00389692 YP_003896928.18.1), *Aspergillus oryzae* (EIT82252.1), *Felix catus* (XP_003982381.1), *Cavia porcellus* (XP_003480015.1), *Sinorhizobium meliloti* (YP_004548030.1), *Mesorhizobium opportunistum* (YP_004613943.1), *Burkholderia graminis* (ZP_02886806.1), *Granulicella mallensis* (YP_005059092.1), *Danio rerio* (XP_002663301.1).



Scheme 1. The three biosynthetic pathways for glycine betaine from choline.

betaine [16]. In contrast, CHO catalyzes both steps of oxidation of choline to betaine aldehyde and betaine aldehyde to glycine betaine [17]. Despite considerable interest in both human and prokaryotic CHD, the biochemical characterization of the enzyme has significantly lagged behind its medical and biotechnological applications due to high instability of the enzyme once it is removed from the inner mitochondrial membrane. This has prevented the obtainment of active and stable purified enzyme for structural-functional studies.

In this review article, we present the physiological roles, medical relevance, and cellular localization of human CHD; we provide a summary of the attempts and difficulties of purifying CHD, summarize the available biochemical knowledge on the enzyme, and introduce a comparison of the protein sequence of CHD with that of bacterial CHO, for which structural and biochemical information is available. A model of the 3D structure of CHD built on the known 3D structure of CHO previously obtained by X-ray crystallography is also presented.

Physiological roles of human CHD

Human CHD catalyzes the oxidation of choline to betaine aldehyde, which is further oxidized to glycine betaine by a second enzyme, BADH [16]. This enzymatic reaction achieves the dual physiological role of regulating the concentration of free choline in cells and bodily fluids and synthesizing a metabolite that is relevant to both osmoprotective and methylating processes (Fig. 2).

Since 1983 [18], the osmoprotectant role of glycine betaine has been studied in regard to the regulation of cell volume and the increase in stability of intracellular macromolecules [19]. In humans and mammals glycine betaine plays an important osmoprotectant role in the kidney, where it was shown to increase in concentration during dehydration [20], and in the liver, where it regulates hepatocellular hydration [21]. Fatty liver is often observed as a consequence of a low intake of choline with the diet due most likely to a decrease in the concentration of phosphatydilcholine, which is necessary for the synthesis of the very-low-density lipoprotein (VLDL) [22]. In rodents, it has been established that fatty liver is developed when they are fed on a low choline diet [23]. It is interesting to note that humans, animals, plants and many microorganisms share the use of glycine betaine as the primary tool for protection from various forms of stress besides osmotic pressure due to swift variations in the concentrations of osmolites in cellular environments, including high and low temperatures, reactive oxygen species and other forms of cellular insults [24]. In humans and mammals, glycine betaine plays also an important role as a methyl group donor involved, for example, in the methylation of homocysteine to methionine (Scheme 2) [19]. The concentration of glycine betaine is thus considered important for metabolic syndrome, lipid disorders, diabetes, vascular diseases and the development of the embryo [25].

The regulation of the concentration of choline in tissues and blood is very important as choline plays key roles in different pathways. Choline is involved in the epigenetic regulation of gene expression through DNA methylation [26], as shown for example in the global hypomethylation of hepatic DNA of rats fed a low choline diet [27], in the biosynthesis of lipoproteins and membrane phospholipids and in the biosynthesis of the neurotransmitter acetylcholine (Scheme 2) [28]. It is therefore important for the integrity of cell membranes, lipid metabolism and nerve function. Choline is considered an important nutrient for fetal and brain development [29–31], as shown for example by the different rate of development of the hippocampus in the fetal brains of rodent models in the case of low and high maternal choline intake [32]. Choline is a constituent of phospholipids involved in signal

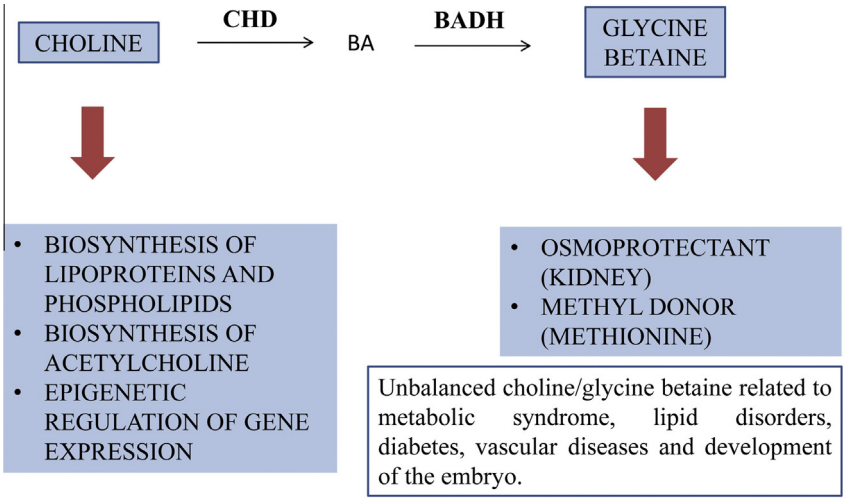
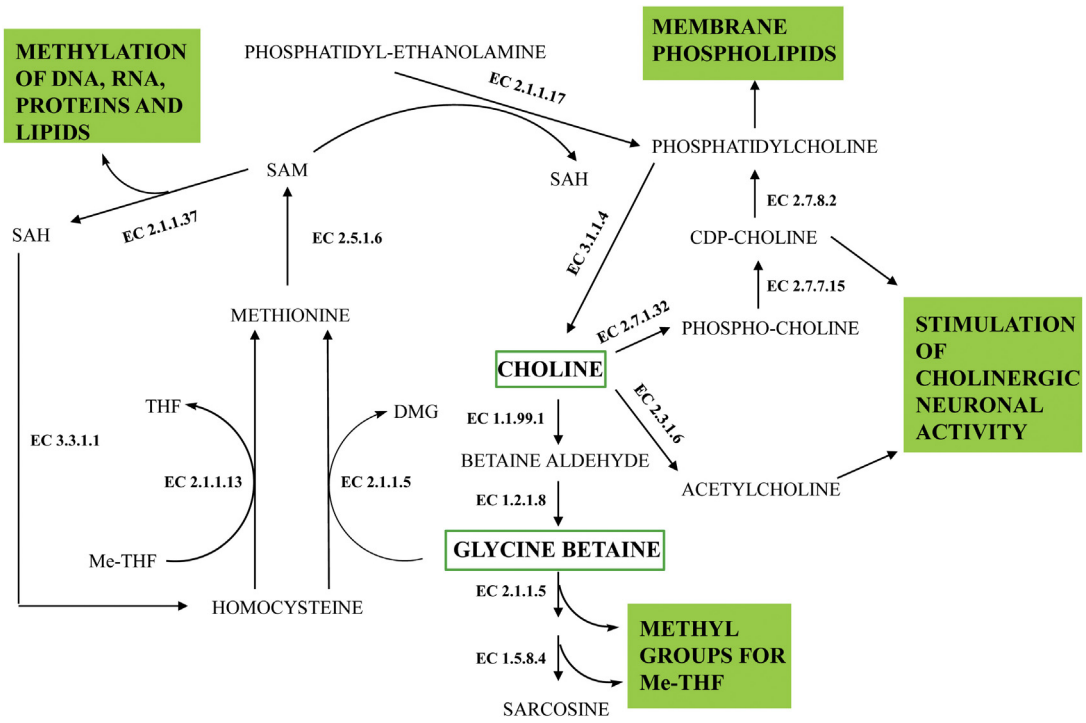


Fig. 2. Physiological roles of human CHD in catabolism of choline and synthesis of glycine betaine. CHD: choline dehydrogenase, BA: betaine aldehyde, BADH: betaine aldehyde dehydrogenase.



EC 1.1.99.1, CHD; EC 1.2.1.8, BADH; EC 1.5.8.4, dimethylglycine dehydrogenase; EC 2.1.1.5, betaine-homocysteine methyltransferase; EC 2.1.1.13, methyltetrahydrofolate-homocysteine methyltransferase; EC 2.1.1.17, phosphatidylethanolamine N-methyltransferase; EC 2.1.1.37, DNA (cytosine-5-)-methyltransferase; EC 2.3.1.6, choline acetyltransferase; EC 2.5.1.6, S-adenosylmethionine synthetase; EC 2.7.1.32, choline kinase; EC 2.7.7.15, choline-phosphate cytidylyltransferase; EC 2.7.8.2, diacylglycerol choline phosphotransferase; EC 3.1.1.4, phospholypase A2; EC 3.3.1.1, adenosylhomocysteinase; DMG, dimethylglycine, Me-THF, methyl tetrahydrofolate, SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine, THF, tetrahydrofolate.

Scheme 2. Choline metabolism.

transduction, such as phosphatidylcholine and plasmalogen, and of the phospholipid platelet activating factor [33]. The metabolism of choline is also interrelated with the metabolism of folate and it has been shown that the folate content in the liver of choline deficient rats decreased by 31% compared to control rats (Scheme 2) [34]. To the best of our knowledge the utilization of choline by other

Table 1
Medical relevance of human CHD.

Medical relevance	References
Male infertility	[8,35,36]
Homocysteinuria	[9,19]
Metabolic syndrome	[28]
Choline as pharmacological agent	[2,39]
Breast cancer	[10,11]

xenobiotic oxygenases present in the liver, such as for example cytochrome P450 or flavin monooxygenase, has not been described in the literature. It would be interesting to evaluate whether these enzymes, besides CHD, can oxidize choline.

Medical relevance of CHD

In the past 5 years, attention on human CHD and its involvement in various pathologies has grown considerably (Table 1). The enzyme has been associated with male infertility in multiple independent studies [8,35,36]. In 2010, Johnson et al. established a correlation between the activity of CHD and male fertility by deleting the corresponding gene in mice [35]. The absence of CHD activity resulted in diminished sperm motility that greatly affected the reproductive ability of mice, with only one out of eleven CHD(−/−) mice being able to reproduce. Mitochondrial alterations were described in testis as well as liver, kidney and heart [35]. Polymorphisms in the human gene have recently been identified and associated with decreased activity of human CHD and alterations in human sperm (e.g., rs12676, Leu-78 → Arg) [8,36].

Impairments in human CHD activity have been associated with homocysteinuria, an accumulation of homocysteine that represents an independent risk factor for cardiovascular diseases [9,19]. This observation is consistent with glycine betaine being the main methyl donor in the conversion of homocysteine to methionine catalyzed by the enzyme betaine-homocysteine methyltransferase (Scheme 2) [37]. The biosynthesis of methionine is important for the metabolism of methyl groups, because it is necessary for the synthesis of the widely used methyl donor S-adenosylmethionine (SAM) (Scheme 2) [38].

A population-based study in 2008 monitored the blood level of choline and its metabolite glycine betaine in relation to components of metabolic syndrome, such as percent body fat, blood pressure, serum lipids, etc. [28]. A correlation was found between high concentrations of choline, low concentrations of glycine betaine in blood and a high-risk profile for cardiovascular disease. It was shown that the amount of choline and glycine betaine supplied in the diet did not have a significant effect on the blood concentrations of these compounds. Therefore, the authors proposed that altered concentrations of choline and glycine betaine in blood should arise from a malfunction of the mitochondrial biosynthesis of glycine betaine rather than dietary patterns.

Human CHD is important for the catabolic utilization of choline when the latter is administered as a pharmacological agent, because choline is involved in the stimulation of cholinergic neuronal activity (Scheme 2) [2] and in restoring phosphatidylcholine levels in the neuronal membrane, thus displaying a neuroprotective action relevant for diseases such as memory and cognitive deficits [39]. Some studies have suggested a mechanism of neuroprotection associated with the supply of exogenous choline to be based on the fact that in case of choline deficiency the brain may degrade the membrane phospholipids of the neurons in order to recycle choline for the production of acetylcholine (Scheme 2) [39]. CHD, predominantly active in the two main detoxifying organs liver and kidney, determines the half-life of choline in blood. In a study in which [^{3}H]-methyl-choline was administered intravenously to guinea pig the main metabolite of choline detected in blood was shown to be glycine betaine, with kidney and liver removing about 50% of the administered dose within 3 min following injection [2,40]. The rapid turnover of choline when administered as a drug is clearly not desirable, since it limits the therapeutic action and requires the administration of higher doses. More recently, the administration of choline as pharmacological agent has evolved into the use of cytidine 5′-diphosphocholine as choline donor [39]. This compound is readily hydrolyzed in the intestine to yield choline and cytidine, which are rapidly absorbed yielding increased plasmatic levels of the compounds. Both compounds can cross the blood-brain barrier and be utilized for the re-synthesis of cytidine 5′-diphosphocholine (Scheme 2) in the brain. Cytidine 5′-diphosphocholine showed promising results in clinical trials for stroke therapy, memory impairment in the elderly, recovery from brain injury, Alzheimer's disease, glaucoma and gave improvements in bradykinesia and muscular rigidity [39]. Cytidine 5′-diphosphocholine is sold as dietary supplement in the United States and as a prescription drug in Japan and Europe [39].

A recent population-based study showed that the metabolic oxidation of choline is related to the risk of developing breast cancer [10]. It was concluded that the dietary intake of higher doses of choline in women is related to a lower risk of developing breast cancer. In that study, increased risk factor for breast cancer was also associated with the polymorphism +432G>T (rs12676) in the human gene coding for CHD [10]. Curiously, this is the same polymorphism that has been linked to altered sperm mobility patterns and altered mitochondrial morphology in human sperm associated with sterility [8,36]. A study of prognostic biomarkers for breast cancer identified the expression of CHD among three human genes controlled by estrogens, and showed that this is a strong predictor of the outcome of treatment with tamoxifen in early-stage (ER)-positive breast cancer patients (CHD originally reported as Genbank accession number AI240933) [11].

Subcellular localization of mammalian CHD

The subcellular localization of mammalian CHD has been studied in rat. Experiments of gradient centrifugation have shown the association of the enzyme with the inner membrane of mitochondria [4,41]. The N-terminal sequencing of CHD extracted from rat liver mitochondria demonstrated that mature CHD begins with amino acid 35. In that study it was suggested that CHD contains an N-terminal cleavable mitochondrial targeting presequence of 34 amino acids and it was hypothesized that two cleavage sites may be present for recognition and processing by Mitochondrial Processing Protease and Inner Membrane Protease [41]. These data are in agreement with the notion that mitochondrial proteins encoded by nuclear genes are synthesized on cytosolic ribosomes and include in their primary structure specific leader sequences for import to the mitochondria and localization in either the outer or inner membranes, intermembrane space or matrix [42]. Based on these studies, it is presumed that human CHD is similarly localized on the inner mitochondrial membrane.

Glucose–methanol–choline enzyme oxidoreductase superfamily

CHD is one of the enzymes that were originally grouped in the glucose–methanol–choline (GMC) enzyme oxidoreductase superfamily based on primary structure alignment [43]. This superfamily of enzymes was established in 1992 when the analysis of the protein sequences of *Drosophila melanogaster* glucose dehydrogenase, *Escherichia coli* CHD, *Aspergillus niger* glucose oxidase and

Hansenula polymorpha methanol oxidase highlighted the fact that these proteins are homologs [44]. Members of this family are flavoenzymes and catalyze the oxidation of a variety of alcohols with different chemical structures. Over the years several enzymes have been added to the superfamily, and the crystal structures of eight members are currently available, including *A.globiformis* CHO [45], *A.niger* glucose oxidase [46], *Brevibacterium sterolicum* cholesterol oxidase [47], *Phanerochaete chrysosporium* cellobiose dehydrogenase [48], *Trametes ochracea* pyranose-2-oxidase [49], *Pleurotus eryngii* aryl-alcohol oxidase [50], *Aspergillus oryzae* formate oxidase [51], and *Mesorhizobium loti* pyridoxine 4-oxidase [52]. Despite sharing limited sequence similarity, these enzymes all share similar overall 3D structures and highly conserved catalytic sites (for a recent review see [53]) (Fig. 3). Not surprisingly, major differences are present in the substrate domains of these enzymes, primarily because the alcohol substrates are structurally unrelated [54]. Given the difficulty in the obtainment of purified, active and stable enzyme, structural information derived from X-ray crystallography is not available for CHD. However, based on the similar active site architectures and the available biochemical data on other members of the superfamily that have been characterized in depth, including CHO, it is reasonable to expect that CHD utilizes a catalytic mechanism for the oxidation of choline similar to that of CHO, in which alcohol oxidation occurs by hydride transfer [17]. As illustrated in a later section, several amino acids whose role has been elucidated in CHO by site-directed mutagenesis are conserved in human CHD, allowing proposing similar roles for the residues in the active site of the membrane-bound dehydrogenase.

Purification of CHD

The characterization of CHD from a variety of cellular sources has so far been limited by the fact that it is difficult to obtain stable, active and highly purified enzyme once it is removed from its cellular location. The association of the enzyme with the membrane represents a further challenge for the purification and *in vitro* stability of the enzyme. We are aware of only one report on the partial

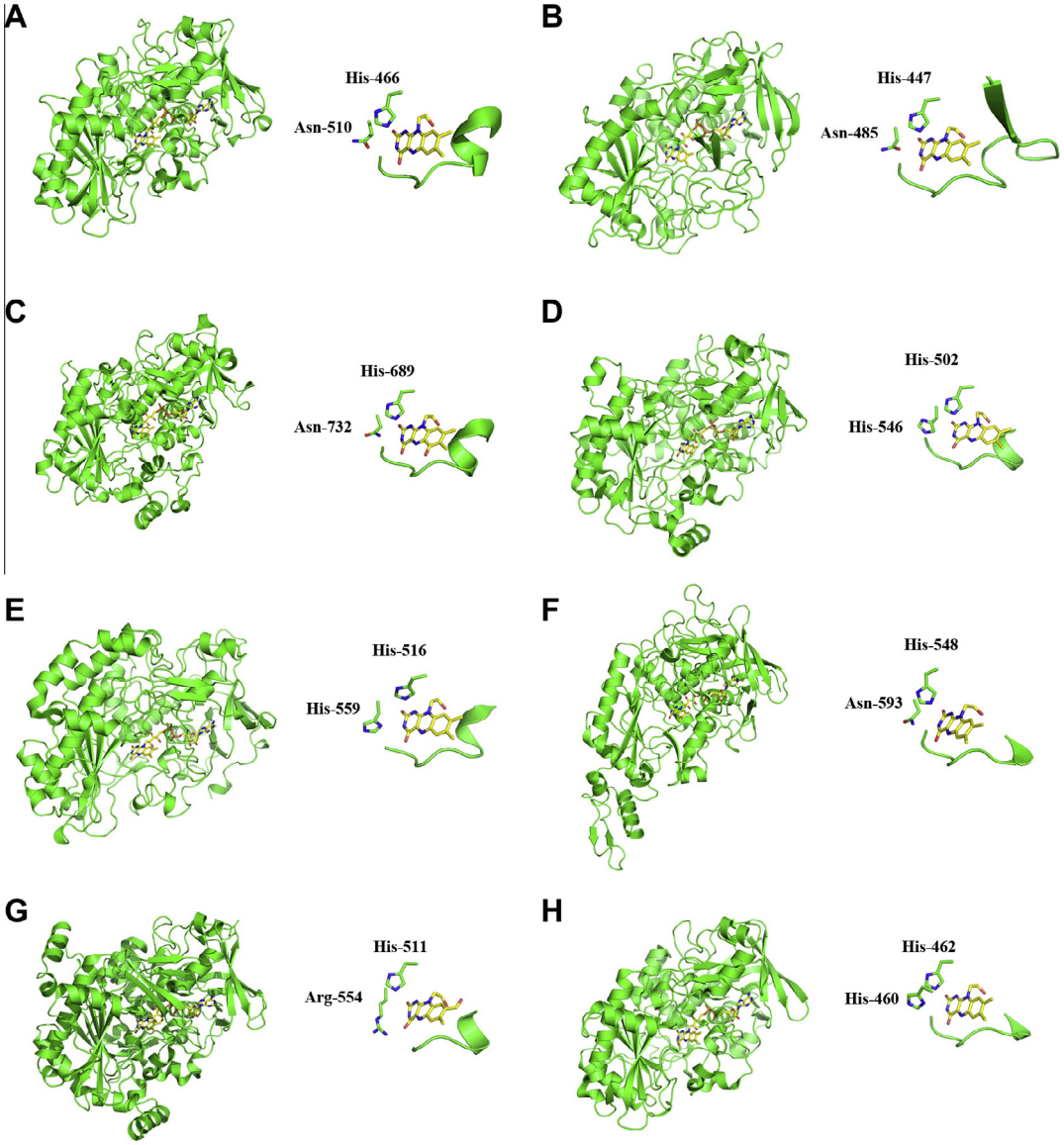


Fig. 3. Overall structures and active sites of members of the GMC oxidoreductase enzyme superfamily: *Arthrobacter globiformis* CHO (S101A) PDB 3NNE (A), *Brevibacterium sterolicum* cholesterol oxidase PDB 1COY (B), *Phanerochaete chrysosporium* cellobiose dehydrogenase PDB 1KDG (C), *Plerotus eryngii* aryl-alcohol oxidase PDB 3FIM (D), *Aspergillus niger* glucose oxidase PDB 1CF3 (E), *Trametes ochracea* pyranose 2-oxidase PDB 2IGK (F), *Aspergillus oryzae* formate oxidase PDB 3Q9T (G) and *Mesorhizobium loti* pyridoxine 4-oxidase PDB 3T37 (H).

purification of recombinant human CHD expressed in *E.coli*, described in a Ph.D. Thesis [55]. In that study, the final enzyme yield was low, with 2 mg of purified CHD out of 24 l of bacterial cell culture, and the limited stability of the purified enzyme prevented further characterization of human CHD other than partial characterization of the cofactor and generation of antibodies [55].

Most of the biochemical and kinetic properties of CHD have been determined in experiments on crude mitochondrial fractions and not purified enzyme, primarily from rat liver. Other studies have been carried out on oysters' mitochondrial fractions without further purification of the enzyme [56]. The first attempt at obtaining purified enzyme was conducted by Williams et al. in 1953, who succeeded to isolate CHD from rat liver upon extraction with choleate [57]. It was later recognized, however, that this protocol did not yield a soluble form of the enzyme, but rather a dispersion of insoluble particles [58]. In 1959, Rendina and Singer developed a new extraction method for CHD from rat liver that was based on the use of *Naja naja* venom as source of phospholipase A to solubilize CHD from the mitochondrial membrane, but without further purification of the protein [59]. In 1980, a partial purification from rat liver mitochondria was reported by Tsuge et al. upon extraction with the detergent Triton X-100 followed by DEAE-Sepharose and Choline-Sepharose affinity column chromatography [60]. However, the preparation of the enzyme analyzed by SDS–PAGE showed three bands and demonstrated poor *in vitro* stability [60]. In 1985, Ameyama et al. partially purified the enzyme from dog liver with less than 1% yield [61]. Despite multiple efforts to purify CHD from various eukaryotic sources the problems associated with the scarce solubility and *in vitro* stability of the mammalian enzyme are still a major challenge (Table 2).

The purification of CHD from prokaryotic sources has revealed challenges similar to those encountered with the mammalian enzyme. A protocol for the partial purification of CHD from *P.aeruginosa* has been reported, but the authors stated limited stability of the enzyme and the co-purification of the second enzyme of the biosynthetic pathway, BADH, as byproduct of this protocol [62]. An attempt to obtain a pure preparation of CHD was performed by our group in 2003 [63]. In that study, recombinant protein from *Halomonas elongata* was expressed in *E.coli* and partially purified to ~70% homogeneity by treatment with ammonium sulfate followed by DEAE-Sepharose column chromatography. A subsequent partial purification of recombinant CHD from *E.coli* was carried out in 2010 by Rajan et al. [64] by applying the purification protocol developed for CHD from *H.elongata* [63].

Biochemical properties of CHD

In 1980, Tsuge et al. noted in their study on the purification of rat liver CHD that "all of the characteristics reported thus far have been obtained using a relatively crude preparation and a highly purified preparation is an urgent priority in the field" [60]. As illustrated in the previous section, this remains true in 2013 and little is known on the biochemical properties of CHD, with no reports on the human enzyme other than genetic studies with only an isolated determination of the enzymatic activity from tissue homogenates [2]. Moreover, the limited biochemical knowledge on CHD has been acquired on either mitochondrial fractions enriched with the enzyme or partially purified and unstable preparations of enzyme.

The cofactor content of CHD has been investigated, but unequivocal evidence on its identity is not available. Based on the presence of a glycine box GXGXXG at the N-terminus of the protein, it has been proposed that FAD is the cofactor of CHD. This would agree well with what is known on other GMC members, each containing FAD as cofactor (Table 3). However, direct evidence for the presence of the flavin has not been reported in the partially purified preparations of CHD, with the enzymes from *P.aeruginosa* [62] and dog [61], which contain the glycine box, proposed to use pyrroloquinoline quinone (PQQ) instead of a flavin. We are aware of two Ph.D. Theses showing UV-visible absorbance spectra of partially purified CHD from *E.coli* with a peak in the 450 nm region consistent with the presence of a flavin cofactor but further characterization of the cofactor has not been reported [55,65]. Based on the high number of cysteine residues, on the location of the eukaryotic enzyme in the mitochondrial inner membrane next to the respiratory chain and on early studies of the presence of labile sulfur groups [60] it is often assumed in the introduction of many studies that the enzyme contains iron sulfur clusters [41]. However, no evidence is available to conclude unequivocally that iron-sulfur clusters are present in CHD, with the notion that the enzyme is only partially purified contributing further to raise questions of whether contaminant proteins may instead harbor some of the reported cofactors.

The electron acceptor in the reaction of choline oxidation catalyzed by CHD is not known. It has been reported that oxygen is not the preferred electron acceptor even though the enzyme is able to utilize it [63]. The most commonly used electron acceptor in the enzymatic assays on CHD from various cellular sources [56,59,60,62,63] is phenazine methosulphate (PMS). Other electron acceptors, such as cytochrome c and ferricyanide, have been tested by Rendina and Singer [59] and by Barrett et al [66] on rat CHD extracted from mitochondria, but the highest enzymatic activity was measured in the presence of PMS. It has been proposed that the enzyme can utilize coenzyme Q as electron acceptor based on the cellular localization of the enzyme close to Complex II on the inner mitochondrial membrane, but further investigation is necessary to validate this hypothesis [60,67].

The substrate specificity of partially purified rat liver CHD was investigated by Tsuge et al. and enzymatic activity was determined only with choline or betaine aldehyde as substrate [60]. In agreement with these data CHD from *H.elongata* can also use betaine aldehyde as substrate besides choline [63]. *In vitro* inhibition of partially purified rat liver CHD was observed with 2-dimethylaminoethanol, monoethanolamine, semicarbazide and to lesser extents L-malate and glycine betaine [60].

Table 2
Purification attempts of CHD from various cellular sources.

Source	Years	References
Rat liver	1980	[60]
Dog liver	1985	[61]
P.aeruginosa	1994	[62]
Recombinant *H.elongata*	2003	[63]
Recombinant *E.coli*	2010	[64]

Table 3
GMC enzyme superfamily cofactor content.

GMC member	Genbank code	Glycine box	Cofactor
Choline oxidase	AAP68832.1	GGGSAG	FAD
Glucose oxidase	1CF3_A	GGGLTG	FAD
Cholesterol oxidase	1COY_A	GSGYGG	FAD
Cellobiose dehydrogenase	1KDG_A	GAGPGG	FAD
Aryl-alcohol oxidase	3FIM_B	GGGNAG	FAD
Pyranose 2-oxidase	2IGK	GSGPIG	FAD
Formate oxidase	3Q9T_A	GGGTAG	FAD
Pyridoxine 4-oxidase	3T37_A	GGGSAG	FAD
PEG[a] dehydrogenase	BAE96591.1	GAGSAG	FAD
Choline dehydrogenase	AAH34502.1	GAGSAG	n.d.[b]

[a] PEG, polyethylene glycol.
[b] n.d., not determined.

Preliminary kinetic studies on partially purified CHD from rat liver demonstrated an increase in the specific activity of the enzyme with choline at alkaline pH [60]. A steady state kinetic characterization with choline and PMS as electron acceptor yielded a K_m value of 7 mM for choline and allowed to propose a Ping–Pong Bi–Bi steady state kinetic mechanism for partially purified rat CHD [60]. For the enzyme from *H.elongata* the kinetic parameters were determined at pH 7.0 at fixed concentrations of either PMS or oxygen as electron acceptor, thus no information on the steady state kinetic mechanism for bacterial CHD is available [63].

Homology model of human CHD

We have generated an homology model of human CHD with the SWISS-MODEL server [68] using CHO from *A.globiformis* as template. The two enzymes share 30% sequence identity (Fig. 4) and catalyze the same oxidation reaction of choline. The active site mutant of CHO with Ser-101 replaced with alanine (PDB 3NNE) was used instead of the wild type enzyme, since the flavin in the latter crystallographic structure is present in an unusual C(4a)-adduct. The N-terminal and internal extra peptides present in the sequence of CHD were deleted *in silico* for the construction of the homology model, because they are absent in CHO. Two other homology models of human CHD were generated using pyridoxine 4-oxidase (PDB 3T37) or aryl-alcohol oxidase (PDB 3FIM) as templates, yielding structures that were practically superimposable with that generated using CHO (data not shown). Fig. 5 illustrates the overall structure and the active site of the homology model of human CHD, superimposed with the tridimensional structure of another active site mutant of CHO, i.e., Val-464-Ala.

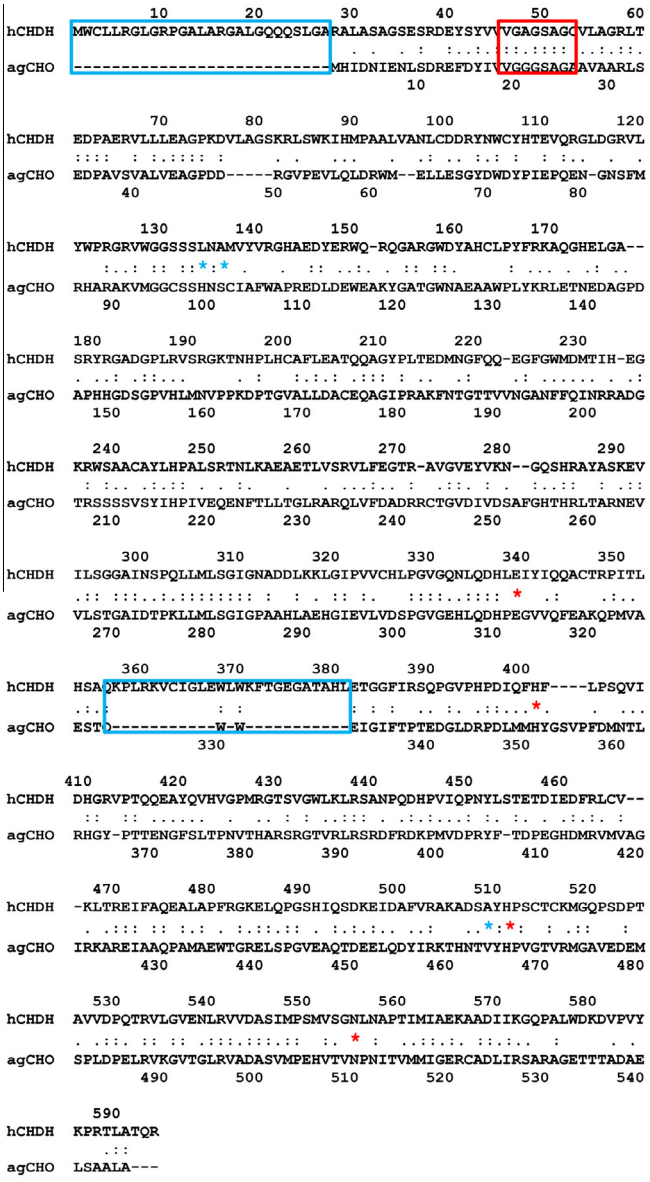


Fig. 4. Alignment of the amino acid sequences of human CHD and *A.globiformis* CHO. Main gaps are boxed in blue, the glycine box is contoured in red, active site residues of CHO conserved in CHD are highlighted by a red star and active site residues of CHO not conserved in CHD are highlighted by a blue star.

The overall structure of the model of human CHD appears to be similar to the conserved overall folding of the GMC superfamily members (Fig. 3) and it does not highlight any transmembrane helix domain or other well-defined membrane-binding domain. Therefore, we hypothesize that human CHD is not an integral membrane protein. Other possible ways of association of CHD with the inner mitochondrial membrane can be considered. For example, an insertion of a short hydrophobic anchor of the N terminal region of the protein in the membrane, similar to the C terminal anchor described for human monoamine oxidase B [69]. Alternatively, hydrophobic interactions between exposed hydrophobic regions of the protein and the membrane or ionic interactions between positively charged residues and the polar heads of membrane phospholipids. Given the difficulties encountered to extract CHD from the membrane it is also possible that there is a covalent attachment of the protein to a phospholipid, as in the case of rodent neural cell adhesion molecule (NCAM) [70].

The homology model of human CHD allows us to propose the localization of Leu-78, which is relevant to the polymorphism rs12676 associated with male infertility and increased risk factor for breast cancer, on the surface of the enzyme (Fig. 5). Such a replacement of a hydrophobic residue with a positively charge one would locally alter the polarity of the enzyme surface, perhaps decreasing the stability of the enzyme.

Comparison of CHD with CHO

In contrast to CHD, CHO from *A.globiformis* has been extensively investigated in its structural, biochemical, kinetic and mechanistic properties [71–82]. This primarily stems from the fact that the oxidase is a soluble protein, which can be purified to high yields in stable and active form [12]. This offers the opportunity to compare and contrast the amino acid sequences of human CHD and bacterial CHO and, because the two enzymes catalyze the oxidation of the same substrate, hypothesize that the residues conserved in the active site of the two enzymes have similar roles in catalysis. Alignment of the amino acid sequences of human CHD and *A.globiformis* CHO returns 30% identical and 28% similar residues, as shown in Fig. 4. Several of the active site residues previously investigated in CHO by using site-directed mutagenesis and biochemical, structural and mechanistic tools are conserved in CHD, including Glu-312 [45], His-351 [83], His-466 [84] and Asn-510 [77] (Table 4 and Fig. 5). Thus, we propose that Glu-339 and His-401 of CHD participate in substrate binding and positioning for the subsequent hydride transfer, His-511 modulates the polarity of the active site and stabilizes the transition state for the oxidation of choline to betaine aldehyde, and Asn-555 is important for choline and FAD oxidation.

Three residues investigated in the active site of CHO are notably different in CHD, i.e. His-99, Ser-101, and Val-464, which are replaced in CHD by Leu-134, Ala-136 and Ala-509, respectively. His-99 is the site of covalent attachment of the flavin to CHO

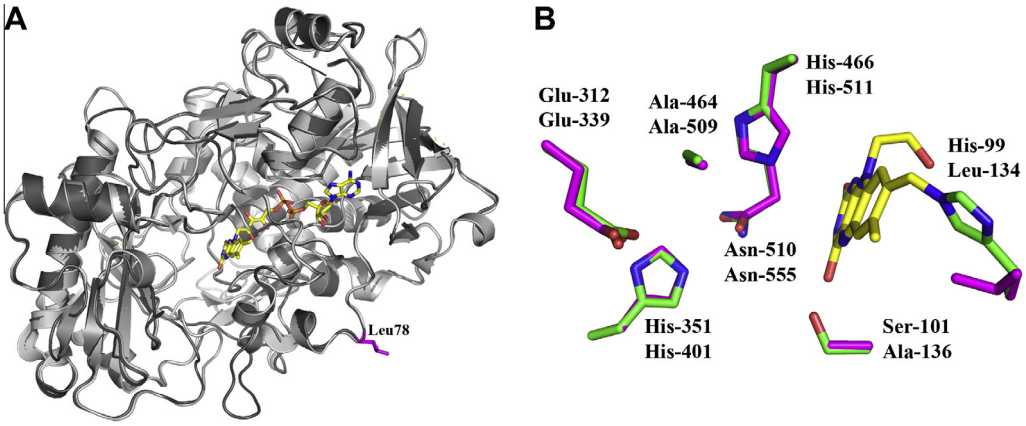


Fig. 5. Homology model of human CHD and comparison of its active site with CHO. Panel A: superimposition of human CHD in dark grey and CHO variant Val-464-Ala (PDB 3LJP) in light grey. The CHD residue Leu-78, associated with the polymorphism rs12676, Leu-78 → Arg, is shown in magenta. Panel B: superimposition of the active site of human CHD (magenta) with the active site of CHO variant Val-464-Ala (green); labels are for CHD (top line) and CHO (bottom line). The FAD cofactor of CHO is shown in yellow.

Table 4
Proposed roles for putative active site residues of human CHD.

Residue in CHD	Corresponding residue in CHO	Proposed role in CHO	References
Leu-134	His-99	Covalent link of the flavin cofactor	[74]
Ala-136	Ser-101	Activation of the alcohol substrate for the hydride transfer reaction	[80,81]
Glu-339	Glu-312	Substrate binding	[45]
His-401	His-351	Substrate binding and positioning	[83]
Ala-509	Val-464	Oxidation of the flavin cofactor	[78]
His-511	His-466	Modulation of active site polarity and stabilization of the transition state for choline oxidation	[84]
Asn-555	Asn-510	Oxidation of choline and FAD	[77]

[74], allowing proposing that a flavin, if present, in CHD would not be covalently linked to the protein. Ser-101 contributes to the optimization of the overall turnover of CHO, which requires the fine-tuning of four consecutive half-reactions for the oxidations of choline to betaine aldehyde and betaine aldehyde to glycine betaine, each followed by the oxidation of the flavin by oxygen [81]. Since a second enzyme, i.e., BADH, catalyzes the oxidation of betaine aldehyde to glycine betaine, such a fine-tuning of multiple half-reactions is not required in CHD. Val464 in CHO provides a nonpolar side chain that is required to guide oxygen in proximity of the C(4a) atom of the flavin, where it will subsequently react with the reduced flavin [78]. This conclusion derived from site-directed mutagenesis studies in which Val-464 was replaced with either threonine or alanine [78]. Interestingly, an alanine is present in lieu of Val-464 in CHD (Fig. 4, Table 4), allowing proposing that lack of a valine may be partly responsible for the low reactivity of CHD toward oxygen.

Another difference that emerges from the comparison of the amino acid sequences of the oxidase and the dehydrogenase is the presence in CHD of extra peptides at the N-terminus and at position 357–381 that are not present in CHO (Fig. 4). The extra peptide at the N-terminus of CHD may be relevant to the import and the targeting of the enzyme to the matrix of mitochondria. This would agree well with the observation that mature rat liver CHD in mitochondria lacks the first 34 amino acids at the N-terminal end [41]. The second extra peptide, with a length of 25 residues, may be responsible for the membrane association of CHD, although no evidence is available to back up this hypothesis at this stage.

Conclusions

Fig. 6 provides a timeline of the major developments on CHD since the first report in 1937 on the oxidation of choline by rat liver [85]. The mammalian enzyme responsible for the oxidation of choline and its connection to the mitochondrial respiratory chain were recognized in the 50 s, and the first partial purifications of CHD were carried out in the 80 s. The difficulties encountered in the purification of the mammalian and subsequently of the prokaryotic

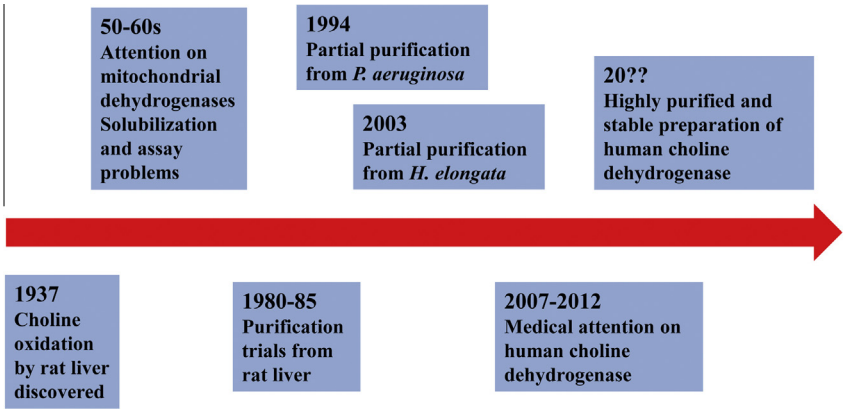


Fig. 6. Timeline of biochemical and medical interest on CHD.

enzyme led only to preliminary biochemical characterizations of CHD, with a progressive fading in interest by biochemists toward the enzyme. Since 2007, however, human CHD has received considerable interest by the medical community due to the involvement of the enzyme in a number of human pathologies, including male infertility, homocysteinuria, breast cancer and metabolic syndrome. A biochemical characterization of the enzyme is thereby required to gain knowledge on the molecular bases linking CHD to these pathologies. For example, *in vitro* structural and kinetic characterizations of the CHD variants associated with polymorphisms would be fundamental to provide a biochemical rationale to explain the malfunction of the enzyme, which may be due to several factors such as protein aggregation, increased protein instability, decreased enzymatic activity, more limited access of the substrates to the enzyme active site, etc.

To date, a biochemical characterization of CHD has been severely hampered by the lack of obtainment of stable, active and highly purified enzyme from either mammalian or prokaryotic sources. Several strategies have been developed with other enzymes to express difficult proteins associated with membranes. A choice for CHD could be to use the yeast *Pichia pastoris*, which can allow import and localization of the recombinant enzyme in the mitochondria. This protocol has been successfully employed for human liver monoamine oxidase A [86]. Other approaches to overexpress recombinant human CHD in *E. coli* could exploit the use of the C43 strain, which was selected for its high tolerance towards membrane proteins [87]. Alternatively, a fusion protein of CHD with maltose-binding protein or other types, such as SUMO (small ubiquitin-related modifier) protein, could be engineered to increase the solubility of human CHD. This latter approach was successful for the bacterial expression of the 5-lipoxygenase-activating protein (FLAP) or the severe acute respiratory syndrome coronavirus (SARS-CoV) membrane protein [88]. Thus, developing a successful protocol for the purification of stable and active enzyme is an absolute requirement to advance our knowledge on this old and important enzyme.

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