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REVIEW ARTICLE

Human cytochrome b5 reductase: structure, function, and potential applications

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Abstract

Cytochrome b5 reductase is a flavoprotein that is produced as two different isoforms that have different localizations. The amphipathic microsomal isoform, found in all cell types with the exception of erythrocytes, consists of one hydrophobic membrane-anchoring domain and a larger hydrophilic flavin catalytic domain. The soluble cytochrome b5 reductase isoform, found in human erythrocytes, is a truncated protein that is encoded by an alternative transcript and consists of the larger domain only. Cytochrome b5 reductase is involved in the transfer of reducing equivalents from the physiological electron donor, NADH, via an FAD domain to the small molecules of cytochrome b5. This protein has received much attention from researchers due to its involvement in many oxidation and reduction reactions, such as the reduction of methemoglobin to hemoglobin. Autosomal cytochrome b5 reductase gene deficiency manifests with the accumulation of oxidized Fe^{+3} and recessive congenital methemoglobinemia in humans. In this article, we provide a comprehensive overview of the structure and function of cytochrome b5 reductase from different eukaryotic sources and its potential use in the food industry, biosensor, and diagnostic areas.

Keywords: FAD domain, FNR family, heterologous expression, mechanism of action, NADH domain, phylogenetics, purification, RCM

Introduction

Cytochrome b5 reductase (CyB5R, EC 1.6.2.2) is a member of the flavoprotein transhydrogenase family of oxidoreductase enzymes and is present as a relatively high concentration in liver tissues. This protein catalyzes the single electron reduction of ferricytochrome b5 to ferrocytochrome b5 using electrons from NADH, the preferred physiological pyridine-type electron donor, using its FAD domain. The three-dimensional structure of the protein reveals two distinct highly conserved regions called the FAD and NADH domains. The FAD domain has a large cleft in which the FAD prosthetic group is located, and the NADH domain provides a suitable position for the NADH coenzyme. The N-terminus of the NADH domain plays a hinge-connecting role between the two domains. CyB5R exists in two forms. The dominant isoform is an amphipathic microsomal membrane-bound variant consisting of a small hydrophobic membrane-anchoring domain of approximately 3 kD and one larger hydrophilic catalytic domain of approximately 31 kD. The other isoform is a truncated soluble cytoplasmic protein that consists of the flavin catalytic domain only. Previous studies have shown that the soluble and membrane-bound variants of CyB5R are both encoded by the same gene and are products of alternative splicing (Pietrini *et al.*, 1988). The amphipathic isoform is embedded in the plasma membrane and in the membranes of the endoplasmic reticulum, mitochondria, Golgi apparatus, peroxisomes, nucleus, sarcoplasmic reticulum, and neuronal synapses, and it has been shown to be a critical component of the microsomal electron transport system. This protein, together with the membrane-bound form of cytochrome b5 (CyB5), is involved in a variety of metabolic functions, including xenobiotics metabolism and detoxification (Rhoads *et al.*, 2011; Abouraya *et al.*, 2011); bioactivation of carcinogenic drugs (Sangeetha

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et al., 2012); bioactivation of anticancer drugs; fatty acid elongation and desaturation; cholesterol anabolism; metmyoglobin reduction; and redox signaling in neurons (Mirzaei, 2010). In contrast, the soluble isoform of CyB5R exists mainly in mammalian circulating erythrocytes and is important for the reduction of methemoglobin to hemoglobin, thereby effectively regulating the methemoglobin concentration in these cells. Functional deficiency in the protein isoforms of CyB5R is known as recessive congenital methemoglobinemia disease, the first disease to be directly associated with an enzyme deficiency. Patients are especially susceptible to toxic methemoglobinemia resulting from the ingestion of oxidant drugs (Percy *et al*., 2005b).

Cytochrome b5 reductase enzymes have been reported in a wide variety of eukaryotes including humans, mammals, plants, birds, fish, amphibians, worms, insects, fungi, yeasts, and protozoa, and many of these enzymes have been studied genetically, enzymatically, and structurally. In the literature, the specific activity (kcat) of purified functional native enzyme is reported to range from a minimum of 368 s^{-1} (human) to a maximum of 1060 s^{-1} (bovine variant) (Roma *et al*., 2006). To date, CyB5R has not been comprehensively reviewed. Here, we provide a summary of the enzymology, diversity, structure, kinetics, mechanism of action, possible roles, and potential for industrial applications of this enzyme.

Phylogenetics of CyB5R

CyB5R is a member of the NAD(P)H-ferredoxin reductase (FNR) super-family that was initially called NADH-diaphorase (Scott & Griffith, 1959); this protein was later determined to include dihydronicotinamide adenine dinucleotide-cytochrome b5 reductase, reduced nicotinamide adeninedinucleotide-cytochrome b5 reductase, NADH-ferricyanide reductase, NADH-ferricytochrome b5 oxidoreductase, NADH 5α-reductase, NADH-dehydrogenase, and NADH-methemoglobin reductase. Sequence alignment identified significantly similar molecular structures and strong homologies between CyB5R and the other enzymes in the FNR super-family, such as NADPH-cytochrome P450 reductase, NAD(P)H-ferredoxin reductase, NAD(P)H-nitrate reductase, nitric oxide synthase, methionine synthase reductase, NADPH-flavodoxin reductase, NADPH-sulfite reductase, and phthalate dioxygenase reductase. With regard to function, the FNR super-family contains highly conserved flavin- and nucleotide-binding domains. The conservation of the GxGxxP, CGxxxM, RxY(T/S)xx(S/N) and (G/R)xx(S/T) motifs, which are associated with the FNR flavoprotein family, demonstrates their importance in interactions with the flavin cofactor and pyridine coenzyme. The CyB5R motifs from a variety of eukaryotes are illustrated in Figure 1. The human diaphorase 1 (*DIA-1*; GenBank ID: NC000022.10) gene that codes CyB5R consists of nine exons and eight introns. This gene, with a total length of 31,560 nucleotides, is located on chromosome 22q13.2-q13.31, from –43013845 to –43045404 nts. Extensive homology studies on *DIA-1* revealed that two alternative mRNAs can be transcribed from this gene (Bulbarelli *et al*., 1998). The longer transcript consists of all nine exons and codes for 301 amino acids (^{1}MGAQLSTL … TERCFVF^{301}), including the initiator methionine, with a molecular mass of 34.1 kD. This N-myristoylated enzyme is found in almost all tissues; however, the second conserved AUG (M24) codon in exon 2 also initiates transcription efficiently but is used in only erythrocytes. This alternative erythrocyte-specific transcript (^{24}MKLFQRSTPA …TERCFVF^{301}) consists of exons 2–9. The first 23 N-terminal hydrophobic amino acids of the full protein are subsequently removed, and the remaining 278 amino acids give a total mass of 31.6 kD (Leroux *et al*., 2001).

Structure of CyB5R (NMR spectroscopy and crystallography)

The three-dimensional (3D) structure of CyB5R has been solved and refined using X-ray crystallography. This structure has provided important information regarding the function of the enzyme and its role in electron transport from NADH to the other enzyme complexes. The first successful X-ray structure was provided by Miki *et al*. (Miki *et al*., 1987). Currently, there are five 3D structures of CyB5R that have been submitted to the protein data bank (PDB: http://www.pdb.org/pdb). Crystals of the soluble isoform of CyB5R are usually grown using the sitting or hanging drop methods. A protein solution and a reservoir (containing a stabilizer such as glycerol and a precipitant such as ammonium sulfate or PEG in buffer) are mixed. Crystals grow as orthorhombic prisms over a few days (Bewley *et al*., 2003). The soluble CyB5R diffraction map (Figure 2) reveals two distinct domains: the N-terminal FAD binding domain (from I34 to R143), which contains a binding site for the FAD prosthetic group, and the NADH domain (residues K173 to F301). These domains are separated by a large interdomain cleft (G144-V172) known as a hinge region (Bando *et al*., 2004, Kim *et al*., 2007). The three anti-parallel β-sheets in the hinge region keep the two lobes in close proximity with the correct conformational orientation. This orientation appears to be critical for electron transfer from NADH to FAD. The FAD domain consists of six anti-parallel β-sheets and one α-helix with the order 5β/1α/1β. The NADH domain forms a α/β/α structure consisting of five β-strands and four α-helices (Nishida *et al*., 1995).

In contrast, needle-shaped crystals of membrane-CyB5R are normally too small for high-resolution X-ray diffraction studies (Miki *et al*., 1987). Membrane proteins comprise approximately 20–30% of the total proteome; however, only a few structures have been resolved. A large α-helix-type hydrophobic domain (of more than 15% of the total protein size) prohibits the application of standard structural biology techniques. Most X-ray diffraction patterns from membrane-CyB5R rely on truncated

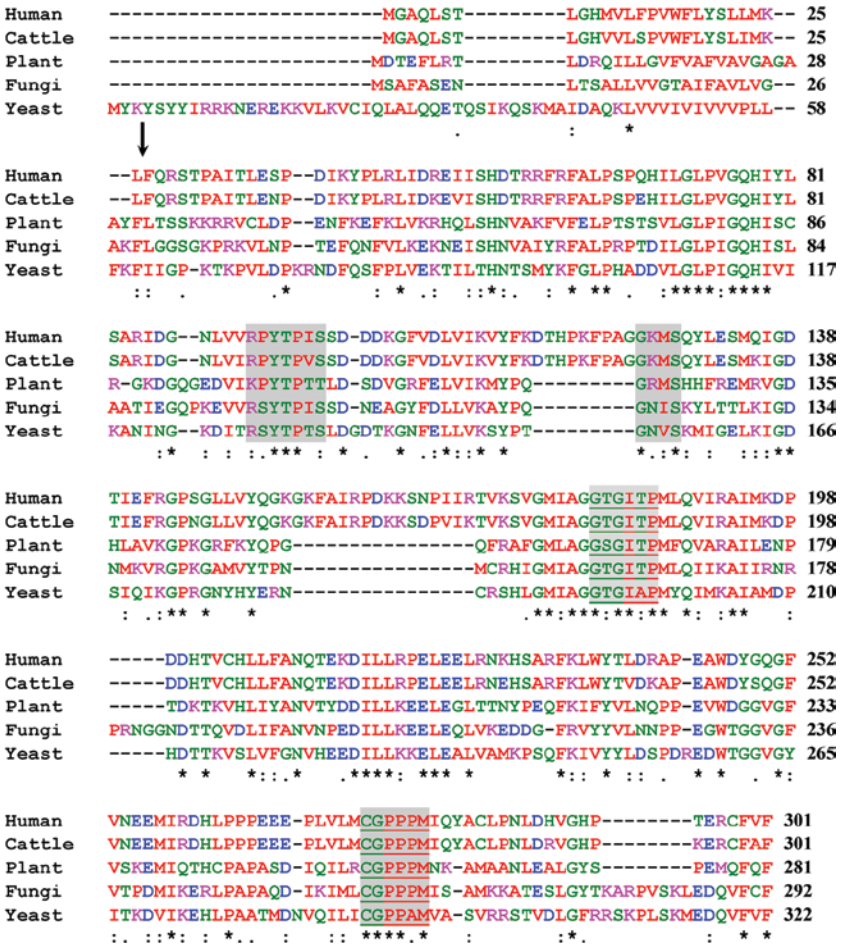

Figure 1. Multiple sequence alignment of CyB5R primary structure. Full-length, membrane-associated form of CyB5R retrieved from GenBank (*Homo sapiens*, GenBank ID: NP_000398.6, *Bos Taurus*, GenBank ID: NP_001096720.1, *Arabidopsis thaliana*, GenBank ID: NP_197279.1, *Aspergillus flavus*, GenBank ID: XP_002379810.1, and *Saccharomyces cerevisiae*, GenBank ID: Z28365). Sequence conservation comparisons were carried out by generating the ClustalW2 algorithm using the optimized EMBL-EBI model (EBI: http://www.ebi.ac.uk/Tools/web_clustalw2). Conserved sequences that show the FAD binding motifs "RxY(T/S)xx(S/N) and (G/R)xx(S/T)" are boxed. The conserved residues detected within NAD binding "GxGxxP and CGxxxM" motifs are boxed and underlined. The down arrow denotes the end of the hydrophobic anchoring sequence of the human protein. Consensus alignment symbols (*), (:), (.), and (-) represent identical, strong similar, weak similar, and gap, respectively. (See colour version of this figure online at www.informahealthcare.com/bty)

solubilized enzyme (the hydrophobic membrane anchor is cleaved off, and the soluble part is then crystallized). The highly flexibility, hydrophobicity, and conformational inhomogeneity of its transmembrane domain makes the enzyme prone to aggregation and misfolding and makes it very difficult to obtain stable crystals of good quality. The transmembrane domain influences the enzyme function, so this domain is essential for an accurate 3D-structure of the protein; therefore, a complete understanding of its structure and function can be achieved only by studying the complete molecule (Durr *et al.*, 2007). Although solid-state NMR spectroscopy of CyB5R has not yet been reported, this technique is now a well-established approach for achieving excellent spectral resolution for noncrystalline and amorphous molecules such as membrane-associated proteins (see http://blanco.biomol.uci.edu/mpstruc/listAll/list). Uniform isotopic labeling is the most critical stage in successful solid-state NMR spectroscopy. Common isotopic radionuclides used for the study of proteins include ^{15}N from labeled amino acids and ^{13}C from labeled glucose that are incorporated into the protein structure during heterologous protein expression (Opella & Marassi, 2004).

Mechanism of action

The general name "NADH-dependent oxidoreductase" applies to every enzyme that transfers electrons from the pyridine moiety to an acceptor. A great deal of our knowledge of the enzyme kinetics and mechanism of electron transfer comes from crystal structure studies and site-directed mutagenesis. Although CyB5R transfers electrons to a variety of electron acceptor molecules such as ferricyanide, dehydroascorbate, and quinones, the ferric atom of the heme cofactor within CyB5 is the most natural substrate for reduction. A functional 1:1 complex between CyB5R and CyB5 in humans has been verified. The electrostatic interactions between the lysyl residues (K42, K126, K163, and K164) in CyB5R and the carboxyl groups (E47, E48, E52, E60, and D64) of CyB5 keep these

proteins tightly complexed and are suitable for electron transfer (Shirabe *et al.*, 1998). Asada *et al.* proposed that R92 and R143 also participate in the CyB5/CyB5R interaction (Asada *et al.*, 2009). Electron transfer is conducted according to the experimentally proposed thermodynamic principles of energy levels. The redox potential differences between the FAD and NAD complexes are approximately –60 mV; therefore, CyB5R can participate in one or two electron transfers to a new electron acceptor and electrons can move efficiently through the electrochemical gradient (Iyanagi *et al.*, 1984). As shown in Figure 3, the sequence of this transfer is summarized as follows: 2e- from NADH → CyB5R → FAD → reduction of 2 CyB5 →electron transfer to desaturase, CyP450 or methemoglobin. The flavin reduction step has been shown to be the rate-limiting factor of this chain reaction (Kimura *et al.*, 2003). CyB5R domains consist of highly conserved motifs. The NADH domain motif, ^{181}GxGxxP^{186}, generates a flat surface in the polypeptide backbone in which NADH can rest in the correct orientation (Bewley *et al.*, 2003). G181 and G183 participate in hydrophobic interactions with the nicotinamide ring, while the isoalloxazine ring of FAD forms two hydrogen bonds with T185. T182 and P186 also contribute to hydrophobic interactions with the isoalloxazine ring by bringing the FAD prosthetic group sufficiently close to the nicotinamide ring of NADH (Roma *et al.*, 2005; Asada *et al.*, 2008). Although the ^{274}CGxxxM^{279} motif in the NADH domain has not been demonstrated to participate in any direct electrostatic or hydrogen bond contacts, it provides the correct orientation for NADH for efficient hydride transfer (Percy *et al.*, 2006a). Although NADH is the preferred electron donor for CyB5R, a D239T mutation will change this preference to one for NADPH. The reduction in negative charges due to this mutation appears to be the reason for this change (Marohnic *et al.*, 2003). The flavin binding ^{92}RxY(T/S)xx(S/N)^{98} motif and the FAD/FMN selectivity motif ^{125}(G/R)xx(S/T)^{128} are highly conserved among all of the flavoprotein family members and have been clearly shown to be structurally important for flavin cofactor interaction and electron transfer within CyB5R (Kimura *et al.*, 2001; Marohnic *et al.*, 2005).

Figure 2. The X-ray crystallographic structure of *Rattus norvegicus* cytochrome b5 reductase (PDB: 1IBO). The ribbon diagram of the three-dimensional rat CyB5R enzyme generated using the Swiss-PDB viewer 4.0.3 program (ExPASy tools: http://ExPASy.org/spdbv). The FAD-binding domain (left), the NADH-binding domain (right) and the connecting "hinge" region are depicted in red, blue, and black, respectively. Arrows represent β-stranded configurations, and α-helices are shown as coils. (See colour version of this figure online at www.informahealthcare.com/bty)

Purification

Regardless of its total protein (mitochondrial, lysosomal, etc.) origin, two main protocols can be used to purify native membrane-bound CyB5R. The detergent-solubilized method uses different detergents to solubilize the amphipathic enzyme intact. An aqueous solution of Triton X-100, Triton X-114, Emulgen 913, cholate, CHAPS, and n-dodecyl β-D-maltoside or an ethanol solution of digitonin has been reported for membrane CyB5R solubilization and extraction. Following adequate incubation periods at 4°C and high-speed centrifugation, the solubilized portion is removed for chromatography. Ion exchange chromatography with DEAE-cellulose,

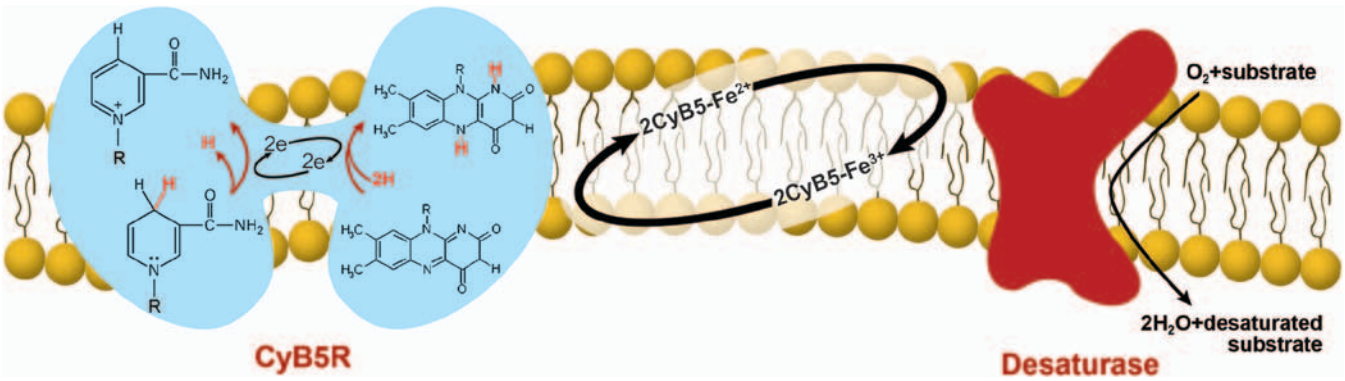

Figure 3. Schematic diagram of electron and proton transfer by the CyB5R complex. The isoalloxazine ring of FAD receives two electrons from NADH by a hydride transfer mechanism. Conformational changes in the flavin lobe of the enzyme make it ideal for reaching the heme group in CyB5. These electrons then reduce two equivalents of ferric atoms to ferro ions. (See colour version of this figure online at www.informahealthcare.com/bty)

DEAE-sepharose, or DEAE-Trisacryl is the most popular method designed to remove bulk impurities. DEAE-cellulose (Guray & Arinc, 1990), agarose-hexane-NAD (Nisimoto *et al.*, 1986), ADP-agarose (Zhang & Scott, 1996), 5′-AMP-Sepharose (Tauber *et al.*, 1985), blue-Ultrogel (Galle *et al.*, 1984), octylamino-Sepharose (Nisimoto *et al.*, 1986), or blue 2-Sepharose (Madyastha *et al.*, 1993) is used for intermediate purification steps. Agarose (Lee & Lee, 1994), Sephadex G-100 (Meldolesi *et al.*, 1980), 5′-ADP-hexane-agarose (Kitajima *et al.*, 1981) hydroxyapatite (Lee *et al.*, 1998; Nisimoto *et al.*, 1986), CM-Sephadex (Mihara & Sato, 1978), and NAD agarose (Meldolesi *et al.*, 1980) are used in polishing steps to produce pure and active protein. Lysosome-solubilized enzyme is obtained by the removing of the membrane-anchoring domain using cathepsin D (Iyanagi *et al.*, 1984; Borgese *et al.*, 1982), trypsin (Mihara & Sato, 1975), subtilisin (Kensil *et al.*, 1983) or lysosomal protease (Tamura *et al.*, 1983; Tamura *et al.*, 1987; Takesue & Omura, 1970). Following this step, similar chromatographic steps to those used for soluble CyB5R are used to purify the soluble domain.

The native soluble CyB5R purification protocol is much simpler: the total soluble protein fraction is isolated from target tissues or recombinant clones and followed up with chromatography. One or two DEAE-cellulose or Q-sepharose steps are usually used to remove bulk impurities and are followed by polishing with amberlite CG-50, 5′-ADP-agarose, or sephadex G-100 (Arinc *et al.*, 1992; Mirzaei *et al.*, 2010).

CyB5R assay

NADH-cytochrome b5 reductase is a multi-functional enzyme that catalyzes the redox reactions between NADH and CyB5. Several unique methods have been developed for measuring its activity.

Natural and artificial acceptors

NADH-cytochrome b5 reductase activity is usually measured usually by one of three different methods using potassium ferricyanide, cytochrome b5, or NADH-2,6-dichlorophenol-indophenol (DCPIP) as an electron acceptor. DCPIP reductase activity was the earliest method developed. A reaction mixture consisting of 0.1 mM DCPIP, 1 mM NADH, and an aliquot of enzyme fraction in a final volume of 2 ml of 100 mM potassium phosphate buffer (pH 7.5) is prepared. When the NADH is added, the change in absorption at 600 nm is recorded. The molar extinction coefficient of the oxidized dye at 600 nm is assumed to be 21.0 $mM^{-1}cm^{-1}$. Enzyme activity is calculated according to the Bear-Lambert equation ($A= \varepsilon bc$), and 1 unit of enzyme is defined as the amount required to reduce 1 µmol of DCPIP in 1 min (Miyake *et al.*, 1975). The NADH-dependent ferricyanide reduction is carried out using 100 mM potassium phosphate buffer (pH 7.5) containing 1.0 mM NADH and 1.0 mM $K_3Fe(CN)_6$ and adequate enzyme solution in a final volume of 1 ml. A kinetic enzyme assay is initiated following the addition of NADH, and 1 unit of CyB5R activity is defined as the amount of enzyme required to reduce 1 µmol of ferricyanide in 1 minute. The activity of CyB5R with ferricyanide is assayed by measuring absorbance reduction at 420 nm (associated with iron reduction) or increased absorbance at 340 nm (associated with NADH oxidation), taking 1.02 $mM^{-1}cm^{-1}$ or 6.25 $mM^{-1}cm^{-1}$ as the molar extinction coefficients, respectively. This is the simplest and most accurate assay for assessing CyB5R activity (Mirzaei *et al.*, 2010). CyB5-mediated reductase activity relies on an endogenous mechanism and is measured spectrophotometrically by the decrease in absorbance at 409 nm or the increased in absorbance at 423 nm. The reaction mixture contains 10 pM CyB5, 100 µM NADH, an aliquot of enzyme fraction, and potassium phosphate buffer (pH 7.2). For this assay, 1 unit of CyB5R is defined as the amount of enzyme that reduces 1 µmol of CyB5 in 1 min. The molar extinction coefficients of CyB5 at 423 nm and 409 nm are 100 $mM^{-1}cm^{-1}$ and 117 $mM^{-1}cm^{-1}$, respectively (Tamura *et al.*, 1983; Marohnic & Barber, 2001). According to the Lineweaver–Burk plot, the Km values for each substrate are determined from the intercept (–1/Km) of the plot of 1/ΔA versus [1/S] (Badwey *et al.*, 1983). The true Kcat values for CyB5 reduction are determined by calculating the l/ν axis intercept of the l/ν versus l/[NADH] replot obtained using different CyB5 concentrations. ENZFIT (Elsevier, Biosoft. Ferguson, MO) is the most widely used and sophisticated software for calculating apparent Km and Kcat values. Spectral binding constants, Ks, for the NADH are determined by differential spectroscopic titrations (Barber & Quinn, 2001). Absorbance changes versus nucleotide concentrations are plotted, and the resulting trend line is fitted to the following hyperbolic equation:

$$\Delta\text{Absorbance} = \Delta\text{Absorbancemax} \times[\text{nucleotide}] \div [\text{Ks} + (\text{nucleotide})]$$

The constant NAD(P)H specificity value is also calculated as the ratio of $(Kcat/Km^{NADH}) \div (Kcat/Km^{NADPH})$ that represents the magnitude of NADH specificity of the enzyme toward NADPH.

The electrophoretic assay

The electrophoretic assay of CyB5R in gels (zymographic technique) was described by Kaplan and Beutler in 1967. Cell-free lysate is subjected to vertical native starch gel electrophoresis at 4°C and 100 mM phosphate buffer is used as the running buffer. Following electrophoresis, enzyme activity is measured using 1.2 mM 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) together with 1.3 mM NADH and 0.06 mM DCPIP in a buffer of 250 mM Tris/HCl with a pH of 8.4 (Kaplan & Beutler, 1967).

Radioimmunoblotting

For radioimmunoblotting, a specific CyB5R monoclonal antibody is blotted onto a nitrocellulose membrane. A solution containing CyB5R or normal hemolysate is allowed to interact with the membrane. The enzyme activity is initiated and measured when MTT is added in the presence of DCPIP and NADH. The resulting purple-blue spots are then developed on each piece of nitrocellulose (Lan *et al.*, 1998).

Heterologous expression

Early studies on CyB5R relied on isolation of the native enzyme from either liver microsomes or erythrocytes. Many laborious purification steps must be carried out to extract the trace amounts of enzyme from the pool of proteins; however, most purification methods result in inadequate amounts and unacceptable purity. Once recombinant DNA and gene cloning technologies were developed and their use widened in the 1970s, all attention became focused on preparing proteins in heterologous hosts, and CyB5R was not an exception. Currently, 927 redundant CyB5R sequences, including 481 from animals, 87 from fungi, 261 from plants, 90 from Trypanosomatidae, and 8 miscellaneous, are deposited in the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov); only 96 of these sequences have been verified by RefSeq. More recently, a variety of recombinant expression systems have been developed for the production of soluble and membrane-bound variants following important advancements in protein expression and purification, for example, high level expression systems (Kimura *et al.*, 2005); systems with low degradation (Shirabe *et al.*, 1989); simple purification systems (Mirzaei *et al.*, 2010); and mutagenesis protocols (Marohnic *et al.*, 2003). These expression systems used to study the structure (Bewley *et al.*, 2001), mechanism of action (Percy *et al.*, 2005a), cancer (Belcourt *et al.*, 1998), gene silencing (Zhuang *et al.*, 2008), and gene therapy (Lostanlen *et al.*, 1981) of CyB5R. Recombinant *E. coli* has been the most widely used method for producing CyB5R and a wide array of suitable expression vectors have been generated including T7 (Barber & Quinn, 1996), pET3c (Hyde & Campbell, 1990), pET23b (Percy *et al.*, 2006a; Roma *et al.*, 2006; Davis *et al.*, 2002), pET16b (Setayesh *et al.*, 2009), pCP (Kimura *et al.*, 2005), λgt11 (Pietrini *et al.*, 1988), and pUC13 (Shirabe *et al.*, 1989). There are also reports of other methods used to express this protein, including yeast using pYES2 (Hatanaka *et al.*, 2004; Shockey *et al.*, 2005), pPICZ (Mirzaei *et al.*, 2010; Syed *et al.*, 2011), and pRC/CMV (Belcourt *et al.*, 1998); *Aspergillus oryzae* using pNGA142 (Sakuradani *et al.*, 1999); *Salmonella typhimurium* using pIN3 (Mokashi *et al.*, 2003); *Spodoptera frugiperda* (Sf9) insect cells using pFASTBAC baculovirus vectors (Fukuchi-Mizutani *et al.*, 1999); cell-free systems (Calza *et al.*, 1987); plant cells using pRTL (Shockey *et al.*, 2005); lymphoid cells using EBV (Lostanlen *et al.*, 1981); and CHO cells using SV40 (Holtz *et al.*, 2003). This protein has been reported in only eukaryotic species, and there are some reports of heterologous protein expression difficulties when using *E. coli* to express eukaryote-specific proteins (Sakuradani *et al.*, 1999; Mirzaei *et al.*, 2010). It appears that differences in codon usage are the largest obstacles in the efficient expression of eukaryotic proteins in *E. coli* (Mirzaei, 2010).

Diseases related to CyB5R dysfunctions

Rapid oxidation of ferro-hemoglobins resulting from the rapid metabolism of radical forming agents, increased levels of oxidants due to the ingestion of toxic compounds, hemoglobin M disorder, or familial reduced ability to regenerate methemoglobins to hemoglobins all lead to the manifestation of methemoglobinemia disease (Beauvais, 2000; Da-Silva *et al.*, 2003). Familial methemoglobinemia arising from mutations in CyB5R was the first idiopathic disease to be directly associated with an enzyme lesion or malfunction and was later defined as recessive congenital methemoglobinemia (RCM) or recessive hereditary methemoglobinemia (RHM). For 100 years, the abnormalities relating to the disease were reported until in 1948, Gibson solved the puzzle of the first instance of a hereditary disease caused by specific enzyme deficiency (Gibson, 1948). Based on pathophysiologic manifestations of the disease, two distinct clinical types of RCM have been classified. Type I RCM is a benign form of methemoglobinemia, with oxidized hemoglobin levels ranging from 10% to 40%, and a normal life expectancy. The enzyme is produced at the normal rate but with a small number of amino acid substitutions. These substitutions cause protein instability such that the soluble erythrocyte enzyme level is primarily affected. The predominant symptoms include well-tolerated cyanosis, fatigue, headache, and shortness of breath (Percy *et al.*, 2005b; Percy & Lappin, 2008; Ewenczyk *et al.*, 2008). In contrast, type II RCM is a much more severe disorder with global loss of both membrane-bound and soluble enzyme in tissues and severe symptoms including lifelong cyanosis, progressive encephalopathy, mental retardation, microcephaly, dystonia, strabismus, failure to thrive, hypotrophy, profound cognitive impairment, and movement disorders, often leading to premature death (Leroux *et al.*, 1975; Lawson *et al.*, 1977). In this disease type, amino acid substitutions often occur in the catalytic domains, and the mutations can cause premature stop sites, frame-shift mutations, the omission of large protein segments or missplicing. To date, three known single nucleotide polymorphisms resulting in Arg59His, The117Ser, or Arg297His substitution (Jenkins & Prchal, 1997; Sacco & Trepanier, 2010) and 50 different mutations, from unrelated cases with RCM, have been published in the literature. Among the 43 exon mutations, 26 have been associated with type I, 14 with type II, and 3 mutations were common to both types; for these 3 mutations, the disease type depended on additional

Table 1. Naturally occurring mutations in the human CyB5R gene and the associated RCM type.

No	Mutations[a]	Amino acid exchange[b]	Mutation position[b,c]	RCM type[d]	References
1	CAG →TAG	Q28 →@	Exon 2 Nu. 82	I	(Fermo *et al.*, 2008)
2	TAC →TAA	Y43 →#	Exon 2 Nu. 129	II	(Manabe *et al.*, 1996)
3	CGG →TGG	R46 →W	Exon 2 Nu. 136	I	(Fermo *et al.*, 2008)
4	CGG →CAG	R50 →Q	Exon 2 Nu. 149	I	(Dekker *et al.*, 2001)
5	AGC →AGG	S54 →R	Exon 3 Nu. 162	I	(Percy & Lappin, 2008)
6	CGG →CAG	R58 →Q	Exon 3 Nu. 173	I	(Shirabe *et al.*, 1992)
7	CCG →CTG	P65 →L	Exon 3 Nu. 194	I	(Dekker *et al.*, 2001)
8	GGC →GC; Frame shifted; 130 amino acids fewer than the native protein	G72 →A	Exon 3 Nu. 215	II	(Hudspeth *et al.*, 2010)
9	CTC →CCC	L73 →P	Exon 3 Nu. 218	I	(Wu *et al.*, 1998)
10	GGC →AGC	G76 →S	Exon 3 Nu. 226	I	(Percy *et al.*, 2006b)
11	CAG →TAG	Q77 →@	Exon 4 Nu. 229	II	(Aalfs *et al.*, 2000)
12	CGA →TGA	R84 →&	Exon 4 Nu. 250	II	(Higasa *et al.*, 1998)
13	ATT →AGT	I85 →S	Exon 4 Nu. 254	I	(Percy & Aslan, 2008)
14	CCC →CAC	P96 →H	Exon 4 Nu. 287	II	(Manabe *et al.*, 1996)
15	GTG →ATG	V106 →M	Exon 4 Nu. 316	I	(Shirabe *et al.*, 1992)
16	ATG →GTG	M127 →V	Exon 5 Nu. 379	II	(Kugler *et al.*, 2001)
17	TCT →CCT	S128 →P	Exon 5 Nu. 382	II	(Kobayashi *et al.*, 1990)
18	GGC →GAC	G144 →D	Exon 5 Nu. 431	I	(Fermo *et al.*, 2008)
19	CCC →TCC	P145 →S	Exon 5 Nu. 433	I	(Fermo *et al.*, 2008)
20	CCC →CTC	P145 →L	Exon 5 Nu. 434	I	(Dekker *et al.*, 2001)
21	CTG →CCG	L149 →P	Exon 5 Nu. 446	II	(Davis *et al.*, 2004)
22	TTT →TGT	F157 →C	Exon 6 Nu. 470	I	(Lorenzo *et al.*, 2011)
23	CGA →TGA	R160 →&	Exon 6 Nu. 478	I, II	(Davis *et al.*, 2004)
24	GCG →ACG	A179 →T	Exon 6 Nu. 535	I	(Kugler *et al.*, 2001)
25	GCG →GTG	A179 →V	Exon 6 Nu. 536	I	(Dekker *et al.*, 2001)
26	TGC →CGC	C204 →R	Exon 7 Nu. 610	II	(Vieira *et al.*, 1995)
27	TGC →TAC	C204 →Y	Exon 7 Nu. 611	I	(Wang *et al.*, 2000)
28	GAG →AAG	E213 →K	Exon 8 Nu. 637	I	(Jenkins & Prchal, 1996)
29	ATC →ACC	I216 →T	Exon 8 Nu. 647	I	(Grabowska *et al.*, 2003)
30	CTG →CCG	L217 →P	Exon 8 Nu. 650	I	(Nussenzveig *et al.*, 2006)
31	CGA →TGA	R219 →&	Exon 8 Nu. 655	I, II	(Vieira *et al.*, 1995)
32	TGG →TGA	W236 →&	Exon 8 Nu. 708	II	(Kedar *et al.*, 2011)
33	CTG →CGG	L239 →R	Exon 8 Nu. 716	I	(Dekker *et al.*, 2001)
34	GAC →GGC	D240 →G	Exon 8 Nu. 719	I	(Percy *et al.*, 2005a)
35	AGA →GGA	R241 →G	Exon 8 Nu. 721	II	(Toelle *et al.*, 2004)
36	GTG →ATG	V253 →M	Exon 9 Nu. 757	I, II	(Dekker *et al.*, 2001)
37	GAG →Del	E256 →Del	Exon 9 Nu. 766-768	I	(Percy *et al.*, 2002)
38	CGG →TGG	R259 →W	Exon 9 Nu. 775	I	(Maran *et al.*, 2005)
39	ATG →Del	M273 →Del	Exon 9 Nu. 817-819	II	(Vieira *et al.*, 1995)
40	CCC →CTC	P276 →L	Exon 9 Nu. 827	I	(Percy *et al.*, 2006a)
41	GGC →GAC	G292 →D	Exon 9 Nu. 875	I	(Percy *et al.*, 2002)
42	CAC →AA; Frame shifted; 46 amino acids longer than the native protein	T295 →Del	Exon 9 Nu. 883-885	II	(Leroux *et al.*, 2005)
43	TTC →Del	F299 →Del	Exon 9 Nu. 895-897	II	(Shirabe *et al.*, 1994)
44	A →G	Exon 5 loss	Intron 4 Nu. 418-2	II	(Kugler *et al.*, 2001)
45	G →A	Exon 5 loss	Intron 4 Nu. 418-1	I	(Dekker *et al.*, 2001)
46	A →C	Exon 6 loss (28 amino acids lost)	Intron 5 Nu. 548-2	II	(Maran *et al.*, 2005)
47	T →C	Exon 5 missplicing	Intron 5 Nu. 547+2	II	(Fermo *et al.*, 2008)
48	G →C	Exon 5 loss	Intron 5 Nu. 547+8	II	(Vieira *et al.*, 1995)
49	G →A	Exon 6 loss	Intron 6 Nu. 631+1	I	(Maran *et al.*, 2005)
50	G →T	Exon 9 missplicing	Intron 8 Nu. 818-1	II	(Shirabe *et al.*, 1995)

[a]Associated mutations in some cases are ignored to reduce table complexity.
[b]@, #, and & are standard stop codon symbols and "Nu" stands for "nucleotide".
[c]For convenience and homogeneity, some codons and amino acid numbers are revised. The last accepted membrane variant CyB5R3 reference sequence, NM_000398.6, comprises the 906 bp and 301 amino acids, including the initiator methionine.
[d]RCM phenotype depends on homozygosity/heterozygosity or other additional mutations.

mutations or whether the mutation was homozygous. Most of the seven intronic mutations are associated with type II (Table 1).

Possible applications of CyB5R in biotechnology

The catalytic and electron transfer properties of CyB5R present possibilities for its application in the food industry, electrochemistry, and medicine. Industrially important lipids such as hydroxy, epoxy, conjugated, acetylenic and poly-unsaturated fatty acids are collected from plant (Shockey *et al.*, 2005; Kumar *et al.*, 2006) and fungal (Shimizu *et al.*, 1997) species. Unfortunately, most of these species produce these oils in an inadequate supply. Recently, to increase essential fatty acids production and accumulation, many attempts have been made to increase levels of CyB5R by mutation or over-expression (Shockey *et al.*, 2005; Kumar *et al.*, 2006). Enzyme-based biosensors are rapid and robust methods for monitoring the chemical potential of oxidation and reduction reactions, and these sensors could be used to diagnose methemoglobinemia levels in real-time (Bartlett, 2008). Additional research needs to be conducted to engineer and express CyB5R with the desired physicochemical properties for these industrial and clinical purposes. Studies of CyB5R will likely continue to increase quickly, particularly in efforts to identify new applications for this enzyme.

Future outlook and conclusion

This review was designed to provide a valuable synopsis of the current structural, functional, and mechanistic properties of CyB5R, in addition to describing the known mutations associated with the human disease recessive congenital methemoglobinemia. These data also support the use of the enzyme kinetics and potential applications. Investigation of the primary structures of different CyB5R variants has revealed strong sequence similarities, the domains involved in electron transport, and the motifs involved in FAD-cofactor and NADH-coenzyme binding. Despite the tremendous amount of work already conducted, mainly involving the soluble or truncated soluble enzyme, several fundamental issues remain to be addressed, including the structure, interactions, and mechanism of function of the entire membrane variant of CyB5R. Recent progress in solid-state NMR for molecular biology, biochemical and structural investigations may aid in answering most of these remaining questions. The major constraint on this project is the development of an effective, robust, inexpensive, and potent expression system for labeled membrane protein production. Our preliminary research and many other investigations have shown that *Pichia pastoris* can be used to express and secrete sufficient amount of heterologous membrane CyB5R; however, the most important obstacle for membrane protein NMR is the production of protein with correct folding and uniform labeling. Thus, efforts have to be made to achieve these goals, and our research group is currently focused on this field of research.

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Declaration of interest

The authors report that there is no conflict of interest with any financial organization.

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