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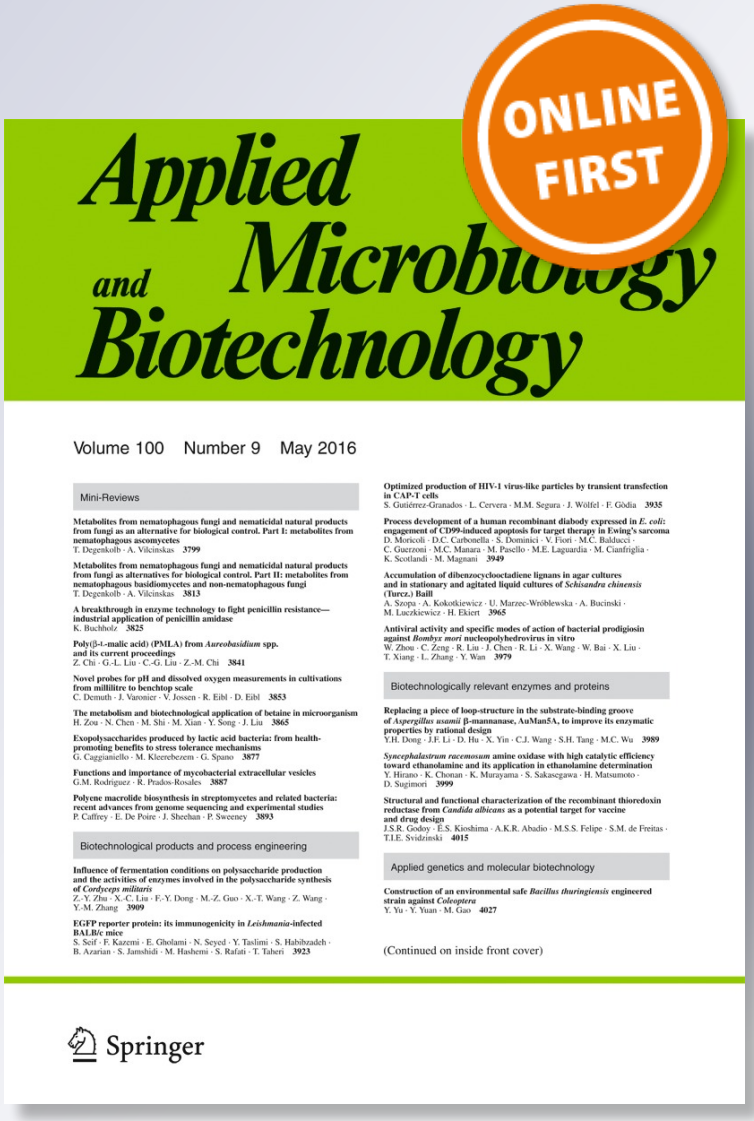

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MINI-REVIEW

Protein engineering of microbial cholesterol oxidases: a molecular approach toward development of new enzymes with new properties

Zahra Moradpour[1] · **Abdollah Ghasemian**[1]

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Abstract Cholesterol oxidase, a flavoenzyme, catalyzes two reactions in one active site: oxidation and isomerization. This enzyme has been isolated from a variety of microorganisms, mostly from actinomycetes. This enzyme has been widely used in clinical laboratories for cholesterol assays and was subsequently determined to have other potential applications. Engineering of cholesterol oxidase have enabled the identification of critical residues, and the information derived could lead to the rational development of improved types of the enzyme with increased stability and better functional properties. This review is the first that exclusively summarizes the reported results on the engineering of bacterial cholesterol oxidases aimed at improving their thermal and chemical stability, catalytic activity, and substrate specificity.

Keywords Cholesterol oxidase · Flavoenzyme · Protein engineering · Site-directed mutagenesis · Thermostable enzyme

Introduction

Cholesterol oxidase (3β-hydroxysteroid oxidase; EC 1.1.3.6, cholesterol oxidase) is a member of a large family of flavin-specific oxidoreductases that catalyzes the dehydrogenation of the C(3)-OH of cholesterol. Interestingly, cholesterol oxidase is a bifunctional enzyme and catalyzes the isomerization of cholest-5-en-3-one (temporary intermediate product) to cholest-4-en-3-one in the ensuing oxidative reaction. In 1974, Allain and coworkers described the first fully enzymatic method for the determination of total serum cholesterol (Allain et al. 1974). Equimolar amounts of cholest-4-en-3-one and hydrogen peroxide are the final products of the reaction shown in Fig. 1. The enzymatic reaction is performed in the presence of nonionic detergents or higher alcohols that form micelles, which is required to dissolve cholesterol. Cholesterol oxidase must derive substrate from these micelles using hydrophobic interactions (Nishiya et al. 1998). This enzyme is a bacterial flavoenzyme and is found in two different forms depending on the nature of the bond between the flavin adenine dinucleotide (FAD) cofactor and the enzyme: in cholesterol oxidase type I, the FAD cofactor is noncovalently linked to the protein, whereas in type II, the cofactor is covalently bound to the enzyme (Sampson and Vrielink 2003). Cholesterol oxidases belong to the flavin-dependent oxidoreductase superfamily. This superfamily is divided into two main families: glucose-methanol-choline (GMC) oxidoreductase and vanillyl alcohol oxidase (VAO). The type I cholesterol oxidases belongs to the GMC oxidoreductase family, and the type II cholesterol oxidases belongs to the VAO family. Glucose oxidase (EC 1.1.3.4), pyranose oxidase (EC 1.1.3.10), methanol oxidase (EC 1.1.3.13), aryl alcohol oxidase (EC 1.1.3.7), and choline oxidase (EC 1.1.3.17) are the other members of the GMC oxidoreductase family, and vanillyl alcohol oxidase (EC 1.1.3.38), reticuline oxidase (EC 1.21.3.3), alditol oxidase (EC 1.1.3.41), and prosolanapyrone-II oxidase (EC1.1.3.42) are the other members of the VAO oxidoreductase family (Dijkman et al. 2013). Comparison of the three-dimensional (3-D) structure of cholesterol oxidase with other oxidoreductases, such as aryl alcohol oxidase, glucose oxidase, and choline

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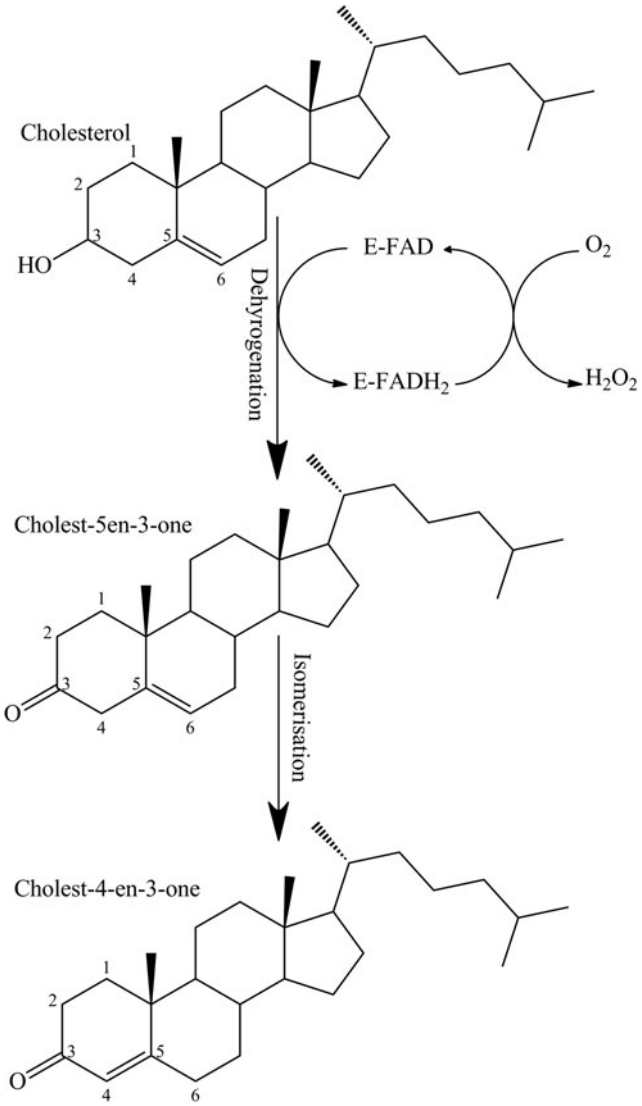

Fig. 1 Mechanism of cholesterol oxidase action

oxidase, reveal a highly conserved active site cavity, which is indicative of a similar reaction mechanism. All of these enzymes possess a conserved amino acid (GXGXXG/A) sequence known as the FAD-binding motif at the N-terminus (Goswami et al. 2013).

Although most bacterial strains secrete cholesterol oxidase into the culture broth, certain strains synthesize the enzyme as a membrane-bound protein. The *Rhodococcus erythropolis* produces both intracellular and extracellular cholesterol oxidases (Doukyu 2009; Lashkarian et al. 2010; Sojo et al. 1997). Cholesterol oxidase has been mostly isolated from actinomycetes. Therefore, the structural and functional analyses have been limited to the enzymes isolated from these microorganisms. However, recent studies have reported a cholesterol oxidase from a gram-negative bacterium, and this enzyme possesses thermal stability and detergent and organic solvent tolerance (Doukyu and Aono 2001; Doukyu et al. 2008). The enzymes have been reviewed in the recent years. These reviews addressed general aspects (Pollegioni 2009), biochemistry and structural features (Vrielink and Ghisla 2009), physiological functions (Kreit and Sampson 2009), biotechnological applications (Doukyu 2009), and their use as bioconversion enzymes or signal proteins (Aparicio and Martin 2008). The cholesterol oxidase type I from *Streptomyces* sp. SA-COO and *Rhodococcus equi* and the cholesterol oxidase type II from *Brevibacterium sterolicum* were subjected to gene manipulation procedures. In this manuscript, we provide an exclusive update of reports that focused on bacterial cholesterol oxidase protein engineering aimed at heterologous expression, identification of molecular determinants, improving the thermal and chemical stability, and altering the catalytic activity and substrate specificity of the enzyme. We also discuss the method for the constructing a catalytically inactive version of cholesterol oxidase that can be used as a negative control in the studies of the physiological role and biotechnological applications of the enzyme.

Biotechnological and physiological importance of cholesterol oxidases

Analytical applications

There is considerable commercial interest in the production of cholesterol oxidase because it is widely used in the enzymatic assay of the total and free cholesterol in clinical samples, serum, and food (Khan et al. 2009; Molaei et al. 2014). The enzyme is also employed in the microanalysis of steroids in food samples and for distinguishing the steric configurations of 3-ketosteroids from the corresponding 3β-hydroxysteroids (Toyama et al. 2002).

Larvicidal activity

The enzyme has been shown to have larvicidal activity and was developed in the agricultural biotechnology industry as a pest control. The mechanism of its larvicidal action is the lysis of the gut endothelial cells upon ingestion. These cells have a high concentration of 3β-hydroxysterols (Purcell et al. 1993).

Microbial pathogenesis

The enzyme has been found to play an important role in bacterial pathogenesis (Vrielnik 2010). Navas et al. (2001) reported that cholesterol oxidase is a major membrane-damaging factor produced by *R. equi*, a primary pathogen of horse and an opportunistic pathogen in humans. Moreover, several pathogenic bacteria, including *Rhodococcus* (de las Heras et al. 2014), *Bordetella* (Lin et al. 2010), and the fast-growing *Mycobacteria* (Yao et al. 2013) need cholesterol oxidase to

invade the host cells, perhaps because of the ability of cholesterol oxidase to alter the physical structure of the cell membrane by converting cholesterol to cholesten-4-en-3-one. As these enzymes are exclusive to the bacteria and fungi, they represent a possible target of a new class of antibiotics.

It has also been determined that cholesterol oxidase is involved in the manifestation of certain viral (HIV) and nonviral prion origin (Alzheimer's) diseases (Kumari and Kanwar 2012). There is no mammalian homolog of cholesterol oxidase. However, the beta-amyloids in Alzheimer's disease oxidize cholesterol at various carbon groups, including the C(3)-OH group, and catalytically produces 4-cholesten-3-one, thereby mimicking the activity of cholesterol oxidase (Gamba et al. 2011; Puglielli et al. 2005; Sottero et al. 2009). Additionally, the enzyme has been shown to be involved in the transformation of sterols and nonsteroidal compounds (Ahire et al. 2012), analysis of the membrane structure, production of steroid hormones precursors, and biosynthesis of the polyene macrolide pimaricin (Doukyu 2009; Mendes et al. 2007).

Protein engineering of cholesterol oxidases

Harnessing cholesterol oxidases for applications in harsh industrial operation conditions usually require their engineering to improve the activity or stability. Protein engineering requires a good knowledge of the protein features, including the structural conformation, activity, stability, and substrate specificity. A good understanding of these features serves as a solid foundation for protein design. Most cholesterol oxidases contain approximately 550 amino acids with a signal peptide of 40 to 50 amino acids (Yazdi et al. 2008). The molecular weight range of the enzymes is 47–60 kDa (MacLachlan et al. 2000). Cholesterol oxidase enzymes work best at a pH of 6.5–8.0 and have an optimum temperature in the range of 40 to 60 °C (Doukyu 2009). The crystal structure of cholesterol oxidases from different sources at high resolution has been determined (Lario et al. 2003; Li et al. 1993). Based on function, these enzymes are composed of two domains, the FAD-binding domain and the substrate-binding domain. Structural and kinetic studies have shown that His447 and Glu361 act as general base catalysts together with the conserved water molecule H_2O and Asn485 in type I enzyme (*R. equi* previously identified as *B. sterolicum* accession no. P22637, PDB 1COY) (Lim et al. 2006). The His447, Glu361, H_2O, and Asn485 tetrad of *R. equi* and His441, Glu356, H_2O, and Asn480 tetrad of *Streptomyces* sp. is conserved among other oxidoreductases (Fig. 2). The hydroxyl group of the steroid substrate is linked to both the flavin ring of the FAD cofactor and a bound water molecule via hydrogen bonding. The critical residues composing the active site of type II cholesterol oxidase from *B. sterolicum* include Arg447, Glu475, Glu311, and Asn516. In this type of enzyme, His121 (corresponding to His69 in mature form of enzyme) is covalently bound to the 8-methyl group of the isoalloxazine ring of FAD (Fig. 2) (Coulombe et al. 2001). The types I and II of enzyme show no significant sequence identity, possess different tertiary structure, and have different kinetics mechanism and redox potentials (Vrielink and Ghisla 2009; Piubelli et al. 2008).

In 1995, Nomura and colleagues performed the first alteration of cholesterol oxidase gene. These investigators overexpressed the genetically modified type I enzyme from *Streptomyces* in *E. coli*. The heterologous expression of the native enzyme failed because the codon bias has not been considered during expression process (Nomura et al. 1995). Increased thermostability, solvent tolerance, and alteration of the substrate specificity of this enzyme are important for its biotechnological applications (Pollegioni et al. 2009). High-level production of the enzyme and the identification of amino acid residues critical for enzyme function are other fields of interest. Although cholesterol oxidases from various sources have been subjected to mutational analysis, considerable attention has been devoted to cholesterol oxidase type I from *Streptomyces*.

Investigation of the role of amino acid residues in oxidation and isomerization activity of cholesterol oxidases

Site-directed mutagenesis has been widely used to identify the key amino acid residues of cholesterol oxidase. Identification of the role of histidine bound to FAD and the role of asparagine and glutamate residues in the active site of the enzyme have been the primary foci of cholesterol oxidase protein engineering.

To differentiate the key residues contributing to oxidation and isomerization, eight mutants of the *Streptomyces* sp. enzyme, N318A, N318H, E356A, E356D, H441A, H441N, N480A, and N480Q, were constructed according to the predicted role of the active site residues identified by the X-ray structural analysis (PDB 1MXT) (Yamashita et al. 1998). Of these, the E356D mutant retained only the oxidation activity and the mutants N480A and N480Q retained only the isomerization activity. Both the oxidase and the isomerase activities were completely lost in the H441A and the H441N mutants. The 3-D structure of cholesterol oxidase (PDB 1MXT) indicates that several amino acid residues, a water molecule, and FAD are present in the active site of the enzyme. The W541 has a key role in the oxidation activity of cholesterol oxidase, and the H441 modulates the reactivity of W541 by forming the tautomeric form of the imidazole ring of histidine. Thus, the H441A and H441N mutations lead to the total loss of the enzyme activity. The W541 also forms a hydrogen bond with Asn480. Substitution of Asn480 by

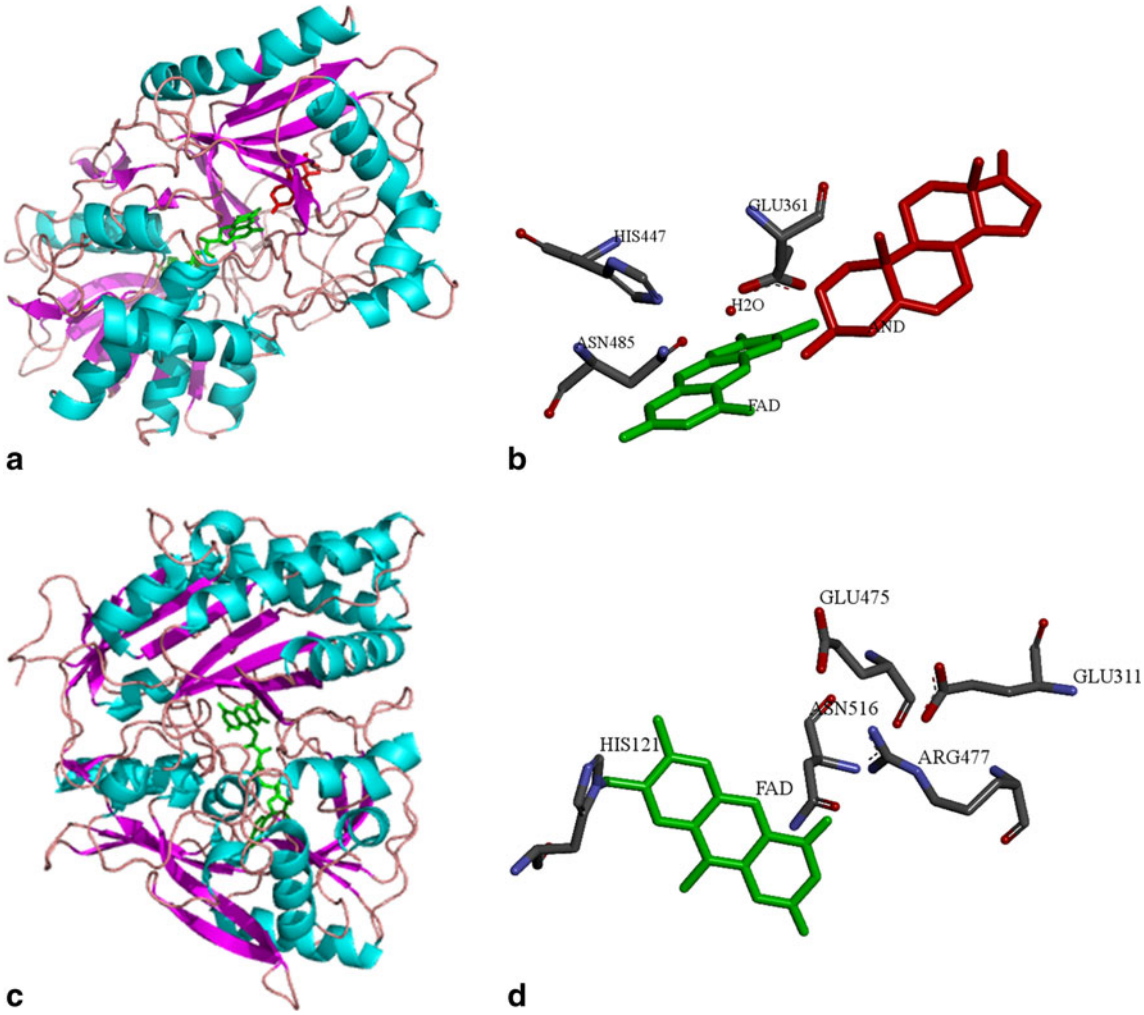

Fig. 2 a An overall 3-D view of the secondary structure of type I cholesterol oxidase from *Rhodococcus equi* previously identified as *Brevibacterium sterolicum* (accession no. P22637, PDB 1COY). The FAD (*green*) and 3-beta-hydroxy-5-androsten-17-one (AND, *red*) are shown in stick representation. b The tetrad residues of the type I cholesterol oxidases active site. The isoalloxazine ring of FAD is shown instead of the whole molecule. c An overall 3-D view of the secondary structure of type II cholesterol oxidase from *B. sterolicum* (accession no. Q7SID9, PDB 1I19). d The residues of the type II cholesterol oxidases active site. The isoalloxazine ring of FAD is shown instead of the whole molecule (*green*) (color figure online)

alanine disrupts this bond, and replacement of Asn480 by glutamine increases the distance between the amino acid and the water molecule (W541). Both of these mutants lost the oxidation activity. Furthermore, the N318H substitution increases the distance between Asn318 and His441 due to the bulkiness of His318, disrupting the hydrogen bond linking these amino acids and leading to reduced oxidation activity of the cholesterol oxidase.

The carboxyl group of Glu356 is involved in electrostatic interaction with the 3-OH group of the steroid and cooperates with W541 to catalyze the oxidation of the substrate. The distance between the catalytic base of cholesterol oxidase, Glu356, and the carbon-4 of the steroid controls the isomerization reaction in *Streptomyces* cholesterol oxidase. Mutation of the Glu356 to aspartic acid has no effect on the rate of deprotonation of cholcst-5-en-3-one, but the protonation of the dienolic intermediate became rate-limiting, possibly because of the large distance (Kass and Sampson 1998). Yamashita et al. (1998) concluded that Asn318, Glu356, and Asn480 must function cooperatively for both reactions to proceed efficiently.

The active site of type II cholesterol oxidase from *B. sterolicum* contains Glu311, Arg477, Glu475, and a FAD covalently linked to His69 of the protein backbone (PDB 1I19). Catalytic activity of E311L and R477A mutants generated by Piubelli and coworkers was substantially disrupted possibly because of steric and charge balance alterations, respectively. Glu311 is an essential residue involved in dehydrogenation activity of enzyme,

but considerable activity of E311Q mutant indicates that the carboxyl group of this amino acid is not critical for catalysis and possibly assists appropriate arranging of Arg477 (Piubelli et al. 2008). In an effort to study the role of His69, an H69A mutant (PDB 2I0K) of this enzyme was constructed. This mutation resulted in a 35-fold reduction in the turnover rate of the enzyme while maintaining the isomerization of the 3-ketosteroid intermediate. The redox potential of the mutant enzyme (−204 mV) was substantially lower than that of the native enzyme (−101 mV). In the mutant enzyme, replacement of the FAD with 8-chloroFAD, leading to the formation of (8Cl-FAD)-H69A, increased the redox potential of the mutant enzyme (−160 mV) and enhanced the catalytic activity 4-fold. These findings revealed that the flavin-histidine linkage plays an essential role in the modulation of the redox properties of the enzyme to enhance its oxidative activity but is not required for the catalysis (Motteran et al. 2001). This covalent bond appears to stabilize the tertiary structure of cholesterol oxidase because the mutant enzyme is more susceptible to denaturation (Caldinelli et al. 2005). Furthermore, the kinetic parameters of H121A mutant constructed by Lim et al. showed that the decreased turnover number of mutant is due to decease in the rate constant of flavin reduction (Lim et al. 2006).

Investigation of substrate specificity and affinity

There is extensive amino acid sequence homology between the cholesterol oxidases of type I from different sources, which lead to similar structures and functions of cholesterol oxidase variants (Fig. 3). However, the enzymes from different sources show variations in the substrate affinity and specificity (Yue et al. 1999). The substrate affinity of cholesterol oxidase is very important in the endpoint or rate assay of cholesterol present in clinical samples. To identify the role of specific amino acids in the substrate selectivity and structure-function relationships, mutant enzymes with amino acid substitutions have been constructed and evaluated. Most of these studies have been performed on *Streptomyces* cholesterol oxidase (Nishiya and Hirayama 1999; Toyama et al. 2002; Xiang and Sampson 2004).

Both the V145E and the G405S substitutions have been determined to exert significant influence on the K_m value of the *Streptomyces* cholesterol oxidase. Modification of the substrate affinity has been achieved by the screening of mutated *Streptomyces* cholesterol oxidase gene subjected to in vivo random mutagenesis (Nishiya and Hirayama 1999). The mutant cholesterol oxidase has been successfully adopted for the rate assay of serum cholesterol, whereas owing to its low K_m value for cholesterol, the wild-type enzyme could not be used for this assay. However, the wild-type enzyme has been employed in the endpoint assay (Nishiya and Hirayama 1999).

Using site-directed mutagenesis and based on the structural comparisons of the cholesterol oxidases, Toyama and co-workers introduced amino acid substitutions, including L117P, L119A, L119F, V145Q, Q286R, P357N, and S379T, into the *Streptomyces* cholesterol oxidase (Toyama et al. 2002). These investigators generated and evaluated 13 mutant enzymes and reported that the L117P mutation caused the disruption of the loop structure, rendering the cholesterol oxidase inactive. The V145Q mutant showed low catalytic activity for cholesterol and the S379T substitution led to altered substrate specificity. Further, the S379T mutant showed slightly larger k_{cat} value for pregnenolone than the wild-type enzyme (Toyama et al. 2002). The natural cholesterol oxidase shows high specificity for cholesterol over other 3β-hydroxysterols (Uwajima et al. 1974; Xiang and Sampson 2004). However, when the sterols are dissolved in liquid-disordered lipid bilayers instead of detergent micelles, variations in the activity of the sterols tended to reduce the cholesterol preference of the enzyme. Using liquid-disordered lipid bilayers, a library screening study uncovered the important role of water molecules in determining the binding affinity of cholesterol oxidase to similarly sized sterols. The active site of cholesterol oxidase contains 14 water molecules. Xiang and Sampson found that desolvation of the enzyme's active site contributes to the binding activity and specificity of cholesterol oxidase. These authors concluded that in order to alter the substrate specificity, it would be necessary to engineer the active site such that there is one more water molecule that can only be displaced by the desired substrate (Xiang and Sampson 2004).

Improvement of thermal stability

Harsh industrial operational conditions require highly stable and active enzymes. The thermal stability of native cholesterol oxidase from different sources differs and is not optimal for its use as an industrial enzyme. Following incubation at 60–80 °C for 30 min, all commercial enzymes lose most of their activity (Doukyu and Aono 2001; Pollegioni et al. 2009). Several attempts have been made to develop thermostable cholesterol oxidases via random and site-directed mutagenesis methods (Ghasemian et al. 2008; Nishiya et al. 1997). Such alterations are often accompanied by additional effects on other enzyme properties such as the pH range of the enzyme activity and enzyme kinetics (Nishiya et al. 1997).

Exploiting the power of random mutagenesis, Nishiya et al. developed thermostable variants of cholesterol oxidase

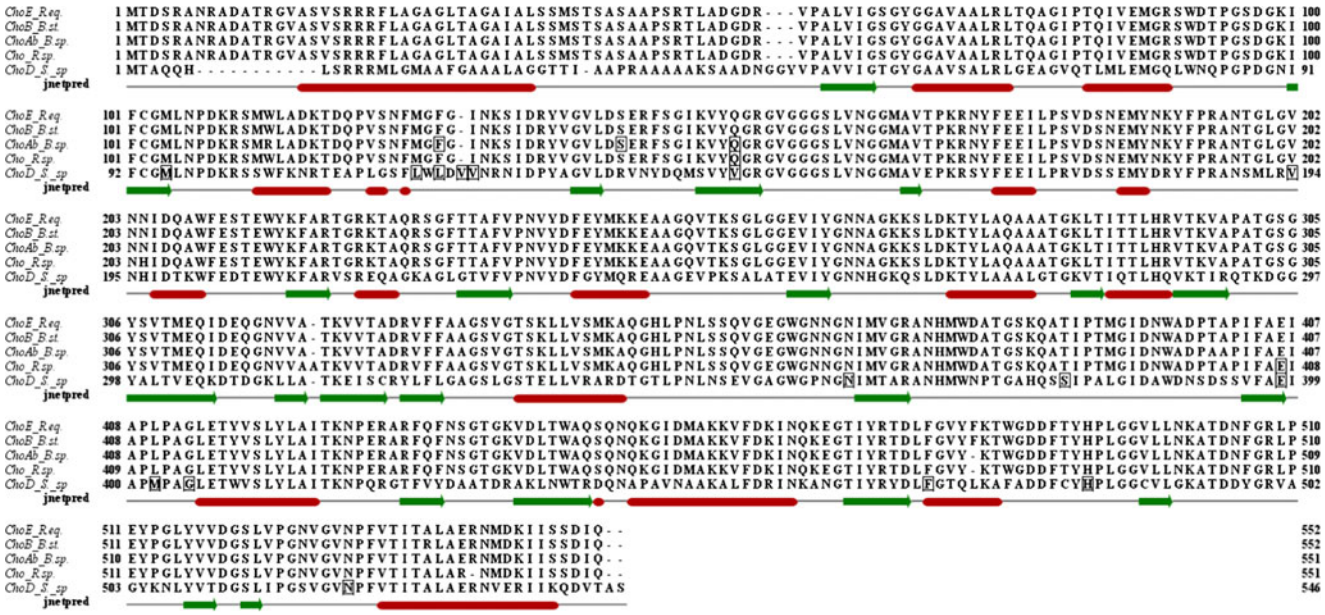

Fig. 3 Multiple sequence alignment of cholesterol oxidases from different microorganisms. The sequences were aligned using ClustalW2. The alignment includes choE from *Rhodococcus equi* (*R. eq.*; accession no. Q93JS8), choB from *R. equi* formerly identified as *Brevibacterium sterolicum* (*B. st.*; accession no. P22637), choAb *Brevibacterium* sp. (*B. sp.*; accession no. Q212N2), cho from *Rhodococcus* sp. (*R. sp.*; accession no. Q157H4), and choD from *Streptomyces* sp. (*S. sp.*; accession no. P12676). Mutated amino acid residues are *boxed*. *Red bars* indicate alpha helices, and the *green arrows* indicate beta strands in the secondary structure for cholesterol oxidase (color figure online)

(Nishiya et al. 1997). Amino acid substitutions, including S103T, V121A, R135H, and V145E, have been identified in *Streptomyces* sp. SA-COO cholesterol oxidase. All of these mutant enzymes showed greater thermal stability than the wild-type enzyme. It is assumed that the residue at position 145 is of great importance in the thermal stability of the enzyme because the half-life of the corresponding mutant cholesterol oxidase at 50, 55, and 60 °C was markedly higher. The enhanced thermal stability of the enzyme was not attributed to the expansion of hydrophobicity or the addition of disulfide bridges. However, the substitution of valine with glutamic acid led to the creation of two distinct interactions, namely, a hydrogen bond between the replaced glutamic acid and Asp134 and a salt bridge between this glutamic acid and Arg147 (Nishiya et al. 1997).

Multiple point mutants of cholesterol oxidases, including M2 (S103T and V145E), M3 (S103T, V121A, and V145E), and M4 (S103T, V121A, R135H, and V145E), were also created in this study. The half-lives of M2 and M3 were higher than that of V145E, and the half-life of M4 was less than that of V145E, M2, and M3. These results indicated that the thermal stabilization effects of the site-specific amino acid modifications at positions 103, 121, and 145 are additive and that the R135H mutation had a negative impact on other modifications (Nishiya et al. 1997). This variant also showed a boarder range of optimal pH compared with other mutants. The newly created interactions modified the deprotonation and protonation of the acidic and basic side chains of aspartic acid and arginine and therefore altered the optimal pH of the V145E mutant (Nishiya et al. 1997).

Site-directed mutagenesis has also been employed to increase the thermal stability of type I cholesterol oxidase from *R. equi*. The sites selected to alter should not be within the FAD-binding domain or the active site hollow. A mutant *R. equi* enzyme with Q145E amino acid substitution, which was created based on previous random mutation studies (Ghasemian et al. 2008; Sun et al. 2011), showed improved thermal stability, while other properties of this enzyme were similar to those of the wild-type enzyme. Because the protein sequences of the cholesterol oxidase from *R. equi* and *Streptomyces* sp. are homologous, a similar interpretation can explain the improvement in the thermal stability of the enzyme (Ghasemian et al. 2008; Nishiya et al. 1997; Sun et al. 2011).

The *Brevibacterium* sp. cholesterol oxidase has also been engineered to improve the thermal stability and activity. Different single (Q153E, F128L, and S143H), double (Q153E/F128L), and triple (Q153E/F128L/S143H) mutant enzymes have been constructed based on the structural analysis (Sun et al. 2011). The double mutant Q153E/F128L showed superior thermal stability and enzymatic activity than others with an 11.6 % increase in the specific activity and a 47 % increase in the relative activity compared to the wild-type enzyme when it was incubated for 2 h at 50 °C. Therefore, this mutant enzyme may be attractive for industrial applications (Sun et al. 2011). The Q153E substitution

resulted in the creation of a salt bridge between this glutamic acid and Arg155. This salt bridge reduced the distance between these two amino acids, and the side chain of glutamic acid shifted toward Arg155. This replacement also likely favored the creation a new hydrogen bond between Asp142 and Arg155. These changes stabilized the overall structure and improved the thermal stability of cholesterol oxidase (Sun et al. 2011).

Modification of enzyme kinetics

In *Streptomyces* cholesterol oxidase, the Val121 is placed in a hydrophobic loop, which is in the vicinity of the active site of the enzyme. The V121A mutation changed enzyme kinetics, perhaps due to the alteration of hydrophobic interactions of the enzyme with the nonionic detergents used to dissolve cholesterol. In other words, the mutant enzyme showed less affinity to the detergent (Nishiya et al. 1998). While the wild-type enzyme follows a simple Michaelis-Menten kinetics, the V121A follows a sigmoidal kinetics. The authors concluded that the interaction between the mutant cholesterol oxidase and cholesterol is highly dependent on the concentration of the detergents. The reduced hydrophobicity of the mutant cholesterol oxidase is responsible for the abnormal enzyme kinetics because it would be more difficult for such a mutant enzyme to deprive cholesterol from the micelle formed from nonionic detergents. The mutants containing V121A with a sigmoidal reaction rate-substrate concentration relationship are not suitable for enzymatic assay application. Additionally, this mutant enzyme is more sensitive to detergents.

To identify the factors affecting the redox potential of the *B. sterolicum* cholesterol oxidase, a mutant enzyme (H121A) was designed, and its kinetic and structural changes were studied. This histidine residue in the *B. sterolicum* cholesterol oxidase type II is covalently linked to the FAD cofactor. The H121A mutant showed approximately 40-fold lower turnover number compared to the wild-type enzyme (Lim et al. 2006). Kinetic analysis of this mutant confirmed that the decrease in the turnover number is primarily observed because of a corresponding decrease in the rate constant of the flavin reduction. Only slight changes in the 3-D structure of the protein were reported, which include nonplanar to a planar geometry change in the isoalloxazine ring of the FAD group (Lim et al. 2006).

Construction of catalytically inactive mutants

Catalytically inactive cholesterol oxidase variants are generally useful for studying the role of this enzyme in some bacterial pathogenesis, for investigating the effect of membrane structure in signal transduction pathways, and for the confirmation of the catalytic function of the active site amino acid residues (Javid Khalili et al. 2009; Navas et al. 2001; Yin et al. 2002).

As mentioned earlier, some active site amino acid residues function cooperatively for efficient cholesterol oxidation. The interactions between the active site amino acid residues of cholesterol oxidase have been evaluated by constructing the H447E, H447Q, H447E/E361Q, and H447Q/E361Q mutants using site-directed mutagenesis (Yin et al. 2002). The comparison of the catalytic efficiency (k_{cat}) of these mutants showed that the double mutant H447Q/E361Q had lower (3-fold) k_{cat} than H447Q and the H447E/E361Q mutant had much lower (10-fold) k_{cat} than the H447E mutant for oxidation. Further, for oxidation, the H447E/E361Q mutant showed much lower (31,000-fold) k_{cat} than the wild-type enzyme. These findings showed that the mutation of His447 to an acidic residue (glutamic acid) slows down the oxidation activity of the enzyme. Furthermore, the H447E/E361Q and H447Q/E361Q mutants failed to convert the cholest-5-en-3-one intermediate to cholest-4-en-3-one due to the missing carboxyl group at position 361 (glutamine). The authors interpreted that Glu361 could act as a general base and compensate for the missing of His447. Therefore, to obtain an inactive cholesterol oxidase mutant, it may necessary to mutate both His447 and Glu361. This double mutant (H447E/E361Q)'s 3-D structure and lipid membrane-binding affinity are identical to those of the wild-type enzyme and may be used to examine the effects of cholesterol-containing membranes in signal transduction (Yin et al. 2002). To evaluate this utility of the catalytically inactive cholesterol oxidase (H447E/E361Q), caveolae membrane (concentrated by platelet-derived growth factor receptor β) fractions were incubated with either the wild-type or the catalytic inactive enzyme and were stimulated with PDGF. Similar to the untreated sample, the H447E/E361Q mutant did not alter the tyrosine phosphorylation of the neighboring proteins, but the wild-type enzyme reduced the tyrosine phosphorylation (Yin et al. 2002).

A number of pathogenic bacteria exploit cholesterol oxidase as a virulence factor to invade their host by altering the integrity of the macrophage cell membrane (de las Heras et al. 2014). Navas et al. (2001) constructed an inactive mutant and investigated cholesterol oxidase as a putative membrane-active virulence factor of *R. equi*. The cholesterol oxidase gene was inactivated by the introduction of the *aacC4* gene (apramycin resistance gene). The construct was cloned into a plasmid vector and transformed into *R. equi*. Homologous recombination between the wild-type chromosome and the plasmid harboring the disrupted cholesterol oxidase gene resulted in the complete inactivation of the chromosomal gene. The mutational inactivation of cholesterol oxidase abolished the cooperative (CAMP-like) hemolysis activity of *R. equi*. Functional complementation via expression of the cholesterol oxidase restored the hemolytic activity of the bacterium (Navas et al. 2001).

To identify the role of the active site amino acids in *R. equi* cholesterol oxidase, Javid Khalili and coworkers created E361N, E361Q, and E361D mutants by site-directed mutagenesis. Compared with the wild-type enzyme, the first two mutants were 28 and 35 times respectively less active, and the latter was inactive (Javid Khalili et al. 2009). These findings pointed toward the critical role of E361 in the activity of *R. equi* cholesterol oxidase and the catalytically inactive enzyme can be used as a null control while probing the signal transduction pathways activated through membrane binding (Yin et al. 2002).

Modification of dehydrogenase activity by altering oxygen binding of enzyme

Enzyme-based biosensors, an alternative to instrumental analytical methods used in experimental diagnostics, are a major tool in biotechnology. Cholesterol oxidase biosensor has great potential as a simple and economical sensor system for the analysis of clinical samples. However, an electrochemical biosensor based on electron mediator for oxidases is essentially influenced by dissolved oxygen. Therefore, the development of enzyme biosensors using electron acceptors with less oxygen-sensitive oxidases is a major goal of industries. The amino acid residues responsible for the oxidase activity of cholesterol oxidase have been identified by site-directed mutagenesis of the oxygen-binding residues. A mutant enzyme with V191A substitution showed 400-fold higher dehydrogenase/oxidase activity compared with the wild-type enzyme. This mutant offers an oxygen-insensitive enzyme for electrochemical cholesterol oxidase biosensors (Kojima et al. 2013). Piubelli and coworkers constructed several mutant type II cholesterol oxidases from *B. sterolicum* to compare the reactivity of wild-type enzyme toward dioxygen with that of mutants. The results showed that Glu311 plays a major role in the dehydrogenation activity as its substitution reduced the activity by approximately 1000-fold. Substitution of Glu311 caused a basic change in the kinetic mechanism of the reaction of reduced enzyme with dioxygen indicating that the residue adjusts the Glu475-Arg477 pair open and closed conformations in a gate functioning in the control of oxygen access to the active center of enzyme (Piubelli et al. 2008).

Genetic modification of the cholesterol oxidase gene for heterologous expression

Large-scale production of individual enzymes in wild-type microorganisms for commercial applications, even in the partially purified form, is a costly process. One approach to cut the enzyme production cost is to construct recombinant strains overexpressing the target enzyme. Lower cost of recombinant cholesterol oxidase production and increased productivity level allows manufacturers to consider the preferential use of this technique. *E. coli* is the most commonly used expression host in protein production because of the viability of genetic manipulation and availability of abundant molecular biology tools. However, *E. coli* fails to actively express some heterologous proteins because of the different codon usage, scheme for protein folding, and posttranslational protein processing such as excision of the signal peptide. Heterologous overexpression is especially difficult in the case of some cholesterol oxidases. Therefore, improving the expression level is a goal of genetic modification.

To date, cholesterol oxidases from *Stereptomyces* sp. (Murooka et al. 1986; Nomura et al. 1995), *Brevibacterium* sp. (Fujishiro et al. 2002; Ohta et al. 1992; Sampson and Chen 1998; Volonte et al. 2010; Wang and Wang 2007), *Burkholderia cepacia* (Doukyu and Aono 2001), *Chromobacterium* sp. (Doukyu et al. 2009), and *Rhodococcus* sp. (Ghasemian et al. 2009) have been successfully cloned and overexpressed. Both types I and II enzymes (respectively containing noncovalently and covalently bound FAD) have been produced in *E. coli* cells.

The first heterologous expression of cholesterol oxidase was reported in 1991. Although the *Streptomyces* sp. SA-COO cholesterol oxidase gene was cloned and expressed extracellularly in *Streptomyces lividans* (Molnár et al. 1991), the activity of the enzyme was low. Furthermore, the wild-type sequence of the gene failed to express the protein in *E. coli*. To overcome this problem, the native codons for the ribosome-binding site (RBS) and the signal peptide were replaced with favorable codons of *E. coli* (Nomura et al. 1995). Although this permitted the expression of the recombinant enzyme, the signal peptide was cleaved closer to the N-terminus (Ala20 and Ala21) in *E. coli* than in *Streptomyces* (excised between Ala42 and Asp43).

In this regard, the expression of cholesterol oxidase from *B. sterolicum* in *E. coli* was improved up to 60-fold through genetic modification and culture condition optimization (Sampson and Chen 1998). With the help of a long (99 nucleotides) forward primer and a normal reverse primer, the investigators synthesized a 681-bp DNA fragment of the N-terminal sequence of the *B. sterolicum* cholesterol oxidase gene using PCR. To adapt the codon usage for improved heterologous expression in *E. coli*, in the design of the long primer, they replaced the codons of the first 21 amino acids with high-expression *E. coli* codons. The N-terminal sequence of the wild-type cholesterol oxidase gene was substituted with synthetic fragment using restriction enzymes. The mutant gene expressed under the *T7lac* promoter and the culture condition optimized for overexpression. This strategy improved the heterologous expression of the *B. sterolicum* cholesterol oxidase up to 60-fold (Sampson and Chen 1998).

To overexpress the type II cholesterol oxidase from *B. sterolicum*, the same strategy was also implemented (Volonte et al. 2010). Full-length cDNA of mature

Table 1 Examples of studies on engineered cholesterol oxidases type I

Aim of study	Mutations	Method	Gene origin	Result(s)	Reference(s)
Significant increase in the dehydrogenase/oxidase ratio	V191A	Site-directed mutagenesis	*Streptomyces* sp.	400-fold increase in the dehydrogenase/oxidase activity	Kojima et al. (2013)
Enhancement of the thermostability and activity of the enzyme	Q153E, F128L, and S143H	Site-directed mutagenesis	*Brevibacterium* sp.	11.6 % increase in the specific activity and 47 % increase in the relative activity when it was incubated for 2 h at 50 °C	Sun et al. (2011)
Investigation of the vital role of Glu361 in cholesterol oxidase active site	E361N, E361Q, and E361D	Site-directed mutagenesis	*Rhodococcus* sp. PTCC 1633	E361N and E361Q mutants were 28 and 35 times respectively less active and E361D was inactive compared to the wild-type enzyme	Javid Khalili et al. (2009)
Construction of a thermally stable cholesterol oxidase mutant by site-directed mutagenesis	Q145E	Site-directed mutagenesis	*Rhodococcus* sp. PTCC 1633	Retained more than 80 and 71 % of the activity of the mutant enzyme at 45 and 55 °C, respectively after 1 h, while only 54 and 55 % of the activity were retained by the wild-type enzyme	Ghasemian et al. (2008)
Construction and investigation of a mutant library of cholesterol oxidase to study the substrate specificity for similarly sized sterols dissolved in vesicles	Libraries containing multiple mutations leading to the identification of five critical residues, M58, L82, V85, M365, and F433, that are in direct contact with the steroid tail of the substrate	Cassette mutagenesis	*Streptomyces* sp.	Refer to text	Xiang and Sampson (2004)
Examination of the interplay between the active site-residues in catalysis, Construction of a catalytically inactive cholesterol oxidase mutant	H447E/E361Q	Construction of double mutant using cut-past and subcloning mutant fragments	*Streptomyces* sp.	31,000-fold slower activity than wild type	Yin et al. (2002)
Alteration of substrate specificity of the *Streptomyces* cholesterol oxidase using site-directed mutagenesis based on differences between the enzymes from *Streptomyces* and *Brevibacterium*	L117P, V145Q, S379T	Site-directed mutagenesis	*Streptomyces* sp.	1.8- and 6.0-fold increase in the catalytic efficiencies of the S379T mutant enzyme for cholesterol and pregnenolone, respectively	Toyama et al. (2002)
Investigating cholesterol oxidase as a virulence factor of *Rhodococcus equi* by targeted mutagenesis	disrupted allele of the gene	Allelic exchange	*Rhodococcus equi*	N/A	Navas et al. (2001)
Application of in vivo mutagenesis techniques to alter the K_m value of cholesterol oxidase for the purpose of rate assay	V145E, G405S	In vivo random mutagenesis	*Streptomyces* sp.	Approximately 10-fold larger K_m value than that of the wild type	Nishiya and Hirayama (1999)
Site-directed mutagenesis was used to identify key amino acid residues that catalyze the oxidation of cholesterol and the isomerization of 5-cholesten-3-one	N318A, N318H, E356A, E356D, H441A, H441N, N480A, and N480Q	Site-directed mutagenesis	*Streptomyces* sp.	Mutant E356D only retained oxidation activity and the mutants N480A and N480Q only retained their isomerization activities. Both the oxidase and isomerase activities were completely lost in the H441A and H441N mutants	Yamashita et al. (1998)
Kinetic analysis of V121A	V121A	Random mutagenesis	*Streptomyces* sp.	V121A follows a sigmoidal kinetics and the wild type followed a simple Michaelis–Menten kinetics	Nishiya et al. (1998), Nishiya et al. (1997)

Table 1 (continued)

Aim of study	Mutations	Method	Gene origin	Result(s)	Reference(s)
Importance of Glu361 position in the reaction	E361Q	PCR cassette mutagenesis	*Streptomyces* sp.	The study obviously revealed that Glu361 has the role of base for isomerization	Kass and Sampson (1998)
Improvement of thermal stability	S103T, V121A, R135H, V145E	Random mutagenesis	*Streptomyces* sp.	The half-lives of wild type, S103T, V121A, R135H, and V145E were estimated to be 7.8, 11.3, 12.2, 11.0, and 24.1 min, respectively	Nishiya et al. (1997)
Modification of cholesterol oxidase gene for expression in *E. coli*	Replacement of codons for the precursor NH_2-terminal region and the ribosome binding site	Refer to the text	*Streptomyces* sp.	N/A	Nomura et al. (1995)

N/A not applicable

Table 2 Examples of studies on engineered cholesterol oxidases type II

Aim of study	Mutations	Method	Gene origin	Result(s)	Reference(s)
Overexpression of cholesterol oxidase in *E. coli* to achieve fully active type II cholesterol oxidase production at valuable expression levels	Optimized Codons for *E. coli*	DNA synthesis	*Brevibacterium sterolicum*	N/A	Volonte et al. (2010)
Investigation on the oxygen access to the active site of cholesterol oxidase	E311D/Q, E475D/Q, R477A/K	Site-directed mutagenesis	*B. sterolicum*	Glu311 finely adjusts the tunnel gate for access/reactivity of dioxygen forming by Glu475-Arg477 pair	Piubelli et al. (2008)
Investigation of the structural and kinetic role of the histidyl-FAD binding	H121A	Purchased	*B. sterolicum*	≥80-fold decrease in the rate of flavin reduction/cholesterol dehydrogenation	Lim et al. (2006)
Investigation of the functional role of the histidyl-FAD bound in cholesterol oxidase	H69A	Purchased	*B. sterolicum*	Approximately 10–15 °C decrease in melting temperature of mutant protein	Caldinelli et al. (2005)
The relationship between covalent flavinylation and redox properties	H69A	Purchased	*B. sterolicum*	≈35-fold decrease in the turnover rate of enzyme	Motteran et al. (2001)

N/A not applicable

B. sterolicum cholesterol oxidase was synthesized according to the 3-D structure, with an extra 27-nucleotide sequence at the 5′ end of the cDNA encoding a tail peptide (MSNHHHGHA) for crystallization of the protein. Codons of the synthetic cDNA encoding the mature protein and the production conditions, including the medium composition and the best culture/induction conditions, were optimized for expression in *E. coli*. Due to improved thermostability and covalently bound FAD, the enzyme overexpressed in this work offers a superior platform for the immobilization of cholesterol oxidase for use in industrial and clinical biosensors (Volonte et al. 2010).

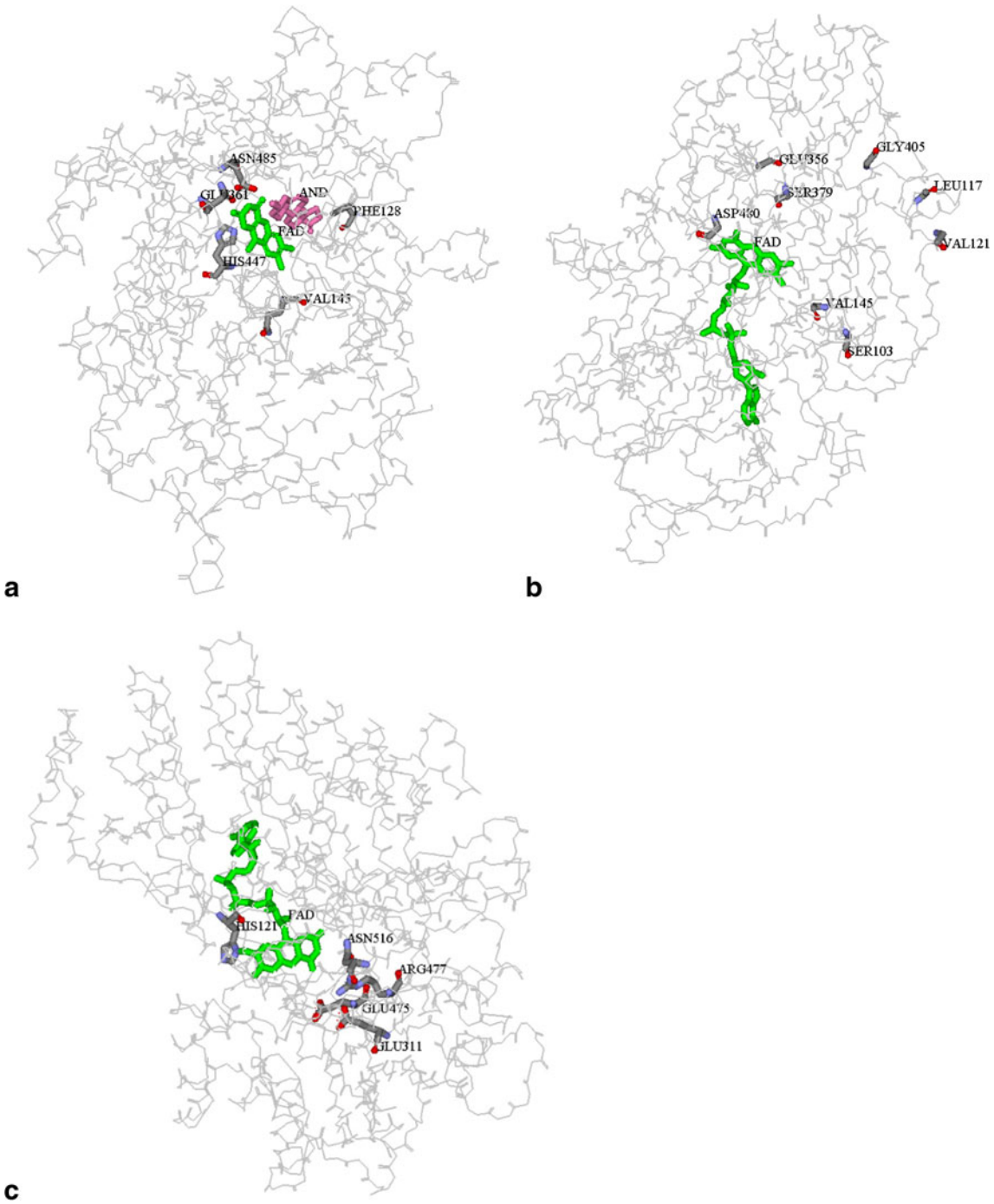

Fig. 4 Some of the mutated residues in cholesterol oxidases were shown in 3-D structure of the enzyme. a Type I cholesterol oxidase from *Streptomyces* sp. (*S. sp.*; accession no. P12676, PDB 1MXT). In a, the isoalloxazine ring of FAD is shown instead of the whole molecule. b Type I cholesterol oxidase from *Rhodococcus equi* formerly classified as *Brevibacterium sterolicum* (*B. st.*; accession no. P22637, PDB 1COY). c Type II cholesterol oxidase from *Brevibacterium sterolicum* (*B. st.*; accession no. Q7SID9, PDB 1I19)

Conclusions and perspectives

Protein engineering of microbial cholesterol oxidases shed light on some critical points. Tables 1 and 2 respectively summarize all residue changes of type I and II enzymes reported in the literature. Some of these residues are shown in the corresponding 3-D structure of the protein in Fig. 4. The most important active site residues of the type I enzyme from *Streptomyces* sp. are Glu631, His447, and Asn485. These residues have key role in the oxidase and isomerase activity of cholesterol oxidase. Substitution of His441, which controls the reactivity of active site water, resulted in complete loss of enzyme activity. The carboxyl group of Glu356 is also essential for oxidation. Although the oxidation activity was enhanced in E356D mutant due to noninterference with the function of water, the isomerization activity was lost owing to short side chain of the aspartic acid. Asn480 is not involved in isomerization reaction and is hydrogen bonded to W541 in active site (Yamashita et al. 1998). The V145E, V121A, and S103T mutations improved the thermal stability of the *Streptomyces* sp. enzyme. These mutations had a synergistic effect in combination and also yielded a broader optimal pH range. Additionally, the V121A mutation altered the enzyme kinetics (Nishiya et al. 1997). The V191A mutant had very low oxidase/dehydrogenase activity ratio and minimal oxygen sensitivity (Kojima et al. 2013). Therefore, this enzyme is an ideal candidate for use in electrochemical biosensors. The putative active site residues of the type I enzyme from *R. equi* are Glu631, His447, and Asn485, which can be altered for the construction of catalytic inactive variants (Yin et al. 2002). The Q153E/F128L mutation of the *Brevibacterium* sp. type I enzyme improved the catalytic activity at 50 °C when compared with the wild-type enzyme (Sun et al. 2011). The His69 residue is covalently linked to FAD in type II cholesterol oxidases, stabilizing the 3-D structure of the enzyme (Motteran et al. 2001; Caldinelli et al. 2005). The largest decrease in k_{cat} values of some mutated forms of this enzyme were observed for mutants at positions Glu311 and Arg477 particularly when the charge was removed (Piubelli et al. 2008).

In conclusion, as revealed by the analysis of engineered cholesterol oxidases, the combined use of several different directed evolution approaches is likely to be necessary for improving multiple target properties of an enzyme. Furthermore, the highly conserved 3-D structure of flavoprotein oxidases suggests that the structure-based rational design and protein engineering strategies used for cholesterol oxidases could also be applied for the improving the properties of other industrial oxidoreductase enzymes. It is expected that in the upcoming years, the use of molecular dynamics and quantum mechanics simulations along with rational experimental design will lead to the development of cholesterol oxidases with desired properties and advance our knowledge of the molecular mechanisms underlying the behavior of this group of oxidoreductases.

Compliance with ethical standards The authors have contributed sufficiently to the work and therefore share collective responsibility for the manuscript. This article does not contain any studies with human participants or animals. All authors have approved the manuscript.

Conflict of interest The authors declare that they have no conflicts of interest.

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