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Cholesterol Oxidase: Role in Pathogenesis

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ABSTRACT

Cholesterol oxidase (COx) catalyzes the oxidation of cholesterol into 4-cholesten-3-one. COx has various clinical and industrial applications. Cholesterol oxidase a bifunctional FAD-containing microbial enzyme belongs to the family oxidoreductases. In recent time, cholesterol oxidase has received great attention due to its wide use in clinical (determination of serum cholesterol) and laboratory practices. The COx has also been implicated in the manifestation of some of the diseases of bacterial (tuberculosis), viral (HIV) and non-viral prion origin (Alzheimer's). This review summarize the important pathogenic features of COx enzyme, its protein structure, pathogenic bacteria requiring COx for their virulence in host, and some of its clinical applications.

Keywords: Cholesterol oxidase, applications, classification, properties, structure
Abbreviations: COx, cholesterol oxidase; **C**, carbon; **QTL**, quantitative trait locus; **COP**, cholesterol oxidation product; **FAD**, adenine dinucleotide; **VAO**, vanillyl alcohol oxidase

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INTRODUCTION

Cholesterol (cholest-5-en-3β-ol) as it is now known as a soft waxy compound belongs to the steroid family of molecules and is found among the lipids (fats) in the blood stream as well as in all cells of the body. Cholesterol is not a life threatening toxin, but a medium sized molecule that is really a building block for important body parts. In particular it is an essential component of mammalian cell membranes. Cholesterol also stabilizes a cell against temperature changes. It is a major part of the nervous system, the brain, the spinal cord and the peripheral nerves. Most of the cholesterol is an animal sterol. However, plants also make trace amount of cholesterol (Nelson 2005), but make other sterols in larger amounts. Cholesterol is formed from squalene via lanosterol (Nelson 2000; Christie 2003). Healthy individual is capable of synthesizing all of the cholesterol one needs, without a dietary source. The human body contains about 100 g of cholesterol. Most of this incorporates in the membrane from which cells are constructed, and is an indispensable component of them. The insulating layers of myelin wound around neurons are especially rich in cholesterol. All the cholesterol required for biological functions are produced endogenously (by body) that constitutes approximately 80% of the blood cholesterol level.

Cholesterol biosynthesis is a highly regulated process that occurs in almost all animal tissues. In mammals cholesterol is more abundant in tissues which either synthesize or have more abundant densely-packed membranes, for example, the liver, spinal cord, brain and atheromata (arterial plaques). Cholesterol plays a central role in many biochemical processes, but is best known for the association of cardiovascular disease with various lipoprotein cholesterol transport patterns and high levels of cholesterol in the blood. It is insoluble in blood, but is transported in the circulatory system bound to one of the varieties of lipoprotein, the spherical particles which have an exterior composed mainly of water-soluble proteins. Some new biosensor based sensitive techniques have been reported for the detection of serum cholesterol (Ahmadalinezhad and Chen 2011; Ruecha *et al*. 2011; Yang *et al*. 2011), whole blood-cholesterol (Fang *et al*. 2011) and cholesterol in the vegetable derived foodstuffs (Jove *et al*. 2010).

Foods that contain cholesterol

The accumulation of excess cholesterol contributes to the causes of hyperlipemia, which increases the risk of arteriosclerosis (Silbernagel *et al*. 2009). An excessive intake of fatty compounds may also increase cholesterol absorption

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in vivo (Matsuoka *et al*. 2012). The cholesterol in diet comes mainly from the saturated fats found in animal products. All foods of animal origin contain some-cholesterol content e.g. chicken liver, chicken giblet, eggs, beef liver, butter, shrimp, veal, pork shoulder, salmon, lard, crab etc. as the data suggested by USDA database. Foods from plants do not contain cholesterol. Other sources of dietary cholesterol are full-fat dairy foods, eggs and some seafood. Often the mixtures of cholesterol with selected foodstuffs (like cocoa and/or green tea) and bile, cause strong interference in widely used biochemical method of cholesterol assay and high end detection method like mass-spectrometry based chromatography allows accurate detection of cholesterol in the foodstuff (Jove *et al*. 2010).

CHEMISTRY OF CHOLESTEROL

Cholesterol consists of four fused rings; an aliphatic side chain branched to the D ring at C-17, a hydroxyl group attached to the A-ring at C-3, and a double bond between C-5 and C-6 of B ring. This double bond makes both C-4 of A-ring and C-7 of B ring on the same plane. One may expect that both the C-4 and C-7 position should have an equal opportunity for an oxidative attack to occur. However, C-7 is indeed a common position for oxidants to react. In contrast, the attack rarely occurs at C-4 because of the possible shielding effect provided by the neighboring hydroxyl group at C-3 and the trialkyl substituted C-5 (Smith 1981). Both the 20- and 25-C of the aliphatic side chain are at a tertiary position, and are, therefore, more susceptible to oxidative attack than the other carbons (Maerker 1987). The oxidation of cholesterol produces a variety of cholesterol oxidation products (COPs).

CHOLESTEROL OXIDASE

Cholesterol oxidase (COx) is a flavo-protein that catalyzes the oxidation and isomerization of steroids containing a 3β-hydroxyl group and a double bond at C-5 of the steroid ring system. The enzyme has been used in the determination of serum cholesterol and in the clinical diagnosis of arteriosclerosis and other lipid disorders. In addition, it has been shown to be a potent parricide (Purcell *et al.* 1993; Corbin *et al*. 1994) and is currently being developed in the agricultural industry as a pest control (Corbin *et al.* 1998). Furthermore, COx is an example of a soluble enzyme that interacts with a lipid bilayer to bind an insoluble substrate. Structural and biochemical studies on the enzyme containing the flavin adenine dinucleotide (FAD)-1 cofactor non-covalently bound to the protein have revealed the region of the enzyme involved in interaction with the lipid bilayer and had led to a possible mechanism for membrane interaction (Li *et al.* 1993; Sampson *et al.* 1998). COx in *Brevibacterium sterolicum* was found to exist in two forms, one in which the FAD cofactor was non-covalently bound to the enzyme (BCO1) and the other in which the cofactor was covalently linked (BCO2).

Furthermore, some pathogenic bacteria require COx to infect their host macrophage, probably because of the ability of the COx to convert cholesterol to cholesterol-4-en-3-one in the membrane (Brozstek *et al.* 2007). As these enzymes are unique to bacteria, they represent a potential target for a new class of antibiotics. It has been demonstrated that Alzheimer's disease β-amyloid selectivity oxidized cholesterol at the C-3 hydroxyl group and catalytically produced 4-cholesten-3-one; therefore it mimics the activity of COx (Puglielli *et al.* 2005). COx have been isolated from several sources other than *Streptomyces*, including members of the genera *Brevibacterium* (Uwajima *et al.* 1974), *Schizophyllum* (Fukuyama and Miyake 1979), *Burkholderia* (Doukyu and Aono 2001), *Rhodococcus* (Aihara *et al.* 1986; Navas *et al.* 2001) and *Micrococcus* sp. (Kanchana *et al*. 2011). The crystal structures of the enzymes from *Brevibacterium sterolicum* and *Streptomyces* sp. SACOO have been determined at 1.8 and 1.5Å resolutions, respectively (Vrielink *et al.* 1991; Yue *et al.* 1999; Lario *et al.* 2003). Most of the COx are monomeric and contain flavin-adenine dinucleotide (FAD) as a prosthetic group (Uwajima *et al*. 1974).

COx is produced by two types of bacteria: (a) nonpathogenic bacteria, which utilize cholesterol as a carbon source; and (b) pathogenic bacteria, which require COx for infection of the host macrophage because of its ability to alter the physical structure of the lipid membrane by converting cholesterol into cholest-4-en-3-one. Both pathogenic and nonpathogenic bacteria up-regulate the expression of COx in the presence of cholesterol.

Classification of COx

The taxonomical classification of COx-containing microorganisms has developed all through the years since its discovery and some species with originally distinct names have been shown by such methods as DNA profiling to be identical/related. COx produced by several microorganisms that are found in quite different environments. Turfitt was the first to isolate the enzyme from the microbe *Nocardia erythropolis* and to show its effect as an oxidant of cholesterol (Turfitt 1944, 1946, 1948). A soil *Mycobacterium* produced COx that was involved in the production of 4-cholesten-3-one. The 4-cholesten-3-one was first isolated by incubation of cholesterol with a cell free extract of the enzyme from this *Mycobacterium* sp. (Stadtman *et al.* 1954). Since then, the enzyme has been found in many microorganisms that include *Arthrobacter* (Wilmanska and Sedlaczek 1949), *Arthrobacter rhodochrous* (Doukyu 2009) *Corynebacterium* (Shirokane *et al.* 1977), *Nocardia erythropolis* and *Rhodococcus erythropolis* (Richmond 1973; Buckland *et al.* 1976; Cheetham *et al*. 1979; Cheetham *et al.* 1980, 1982; Aihara *et al.* 1986; Atrat *et al.* 1992; Minuth *et al*. 1995) now regarded as the same species), *Nocardia rhodochrous* and *Rhodococcus rhodochrous*, *Mycobacterium* (Schatz *et al*. 1949; Smith *et al*. 1995), *Pseudomonas* spp. (Rhee *et al*. 1991; Doukyu and Aono 1998), *Schizopyllum commune* (Fukuyama and Miyake 1979), *Brevibacterium sterolicum* (Uwajima *et al*. 1973; Ohta *et al*. 1991, Croteau and Vrielink 1996: Mottern *et al*. 2001), *Streptoverticillium cholesterolicum* (Inouye *et al*. 1982), *Streptomyces violascens* (Kamei *et al*. 1978; Ishizaki *et al*. 1989; Lartillot *et al*. 1990), *Streptomyces* spp. (Nishiya *et al*. 1997; Ghoshroy *et al*. 1997; Lario *et al*. 2003), *Rhodococcus* spp. (Watanabe *et al*. 1986; Watanabe *et al*. 1989; Kreit *et al*. 1992), *Rhodococcus erythropolis* (Sojo *et al*. 1997), *Enterobacter* spp (Ye *et al*. 2008) and *Micrococcus* sp. (Kanchan *et al*. 2011).

Reaction catalyzed by COx

The enzyme COx has the ability to convert 3-hydroxy-sterols to their respective keto derivatives. The conversion of cholesterol (5-cholesten-3β-o1) to cholestenone (4-cholesten-3-one) by COx has been used to probe the localization and distribution of cholesterol in different biological structures (Gottlieb 1977; Moore *et al.* 1977; Patzer *et al.* 1978; Thurnhofer *et al.* 1986; Slotte *et al.* 1987, 1989). The interaction of cholesterol with sphingomyelins significantly retards the enzyme-catalyzed oxidation of cholesterol in biological as well as in monolayer membranes (Slotte *et al.* 1989; Gronberg *et al.* 1990). It was further observed that the surface pressure (i.e. the lipid packing) of the substrate membranes (Gronberg *et al.* 1990) markedly affected the catalytic activity of cholesterol oxidases at the water/ lipid interphase.

COx a FAD-dependent bifunctional enzyme catalyzes both the oxidation of cholesterol (5-cholesten-3β-ol) to the temporary intermediate 5-cholesten-3-one with the reduction of molecular oxygen to hydrogen peroxide and the isomerization of the steroid with a *trans* A: B ring junction to reduce 4-cholesten-3-one (Stadtman *et al.* 1954; Cholesterol (5-cholesten-3-ol) → 5-cholesten-3-one → 4-cholesten-3-one). Bacterial COx also exhibited ketosteroid mono-

oxygenase activity, which catalyzed the hydroxylation of cholesterol to 4-cholesten-6-ol-3-one (Molnar *et al.* 1993).

Types of cholesterol oxidases

COx is a monomeric flavoenzyme that catalyzes the oxidation and isomerization of cholesterol to cholest-4-en-3-one. Two forms of the enzyme are known, one containing the cofactor non-covalently (class I) bound to the protein and the other in which the cofactor is covalently linked (class II) to a histidine residue. The X-ray structure of the enzyme from *Brevibacterium sterolicum* containing covalently bound FAD has been determined and refined to 1.7 Å resolutions (Coulombe *et al*. 2001). The active site consists of a cavity sealed off from the exterior of the protein. A model for the steroid substrate, cholesterol, can be positioned in the pocket revealing the structural factors that result in different substrate binding affinities between the two known forms of the enzyme. The structure suggests that Glu475, located at the active site cavity, may act as the base for both the oxidation and the isomerization steps of the catalytic reaction. A water filled channel extending towards the flavin moiety inside the substrate-binding cavity, may act as the entry point for molecular oxygen for the oxidative half reaction. An arginine and a glutamate residue at the active site, found in two conformations are proposed to control oxygen access to the cavity from the channel. These concerted side-chain movements provide an explanation for the biphasic mode of reaction with dioxygen and the ping-pong kinetic mechanism exhibited by the enzyme.

The sequence and structure of a class II oxidase has been obtained from a different *Brevibacterium* strain and has been shown to be significantly different (Coulombe *et al*. 2001). This enzyme has also been studied by kinetic analysis of site-directed mutant targeting the His residue which is the FAD attachment site and the importance of the covalent bond in enzyme stability and redox power has been stressed (Motterran *et al*. 2001; Caldinelli *et al*. 2005; Lim *et al*. 2006). Although both classes of COx exhibit a broad range of steroid specificities and can oxidize a number of hydroxysterols including sterols, steroid hormones and bile acids (Maclachlan *et al*. 2000), the presence of a 3β-hydroxyl group in the substrate is an important requirement for activity (Smith and Brook 1976). The hydroxyl group at C3β of cholesterol has to be equatorial but the double bond is not necessary for the oxidation to proceed (Biellmann 2001). The side chain of cholesterol is also not essential for the oxidation, and various functionalities may be present at different positions of the steroid nucleus, thus making this enzyme a rather flexible one regarding substrates. Moreover, the enzyme has been described to oxidize substrates not belonging to the cholestan family such as allylic alcohols (Dieth *et al*. 1995).

COx binding

Both types class I and class II of COx catalyze the same reaction but show different kinetic mechanisms. This functional difference appears with the large difference in the structure of two forms of enzymes (Vrielink *et al*. 1991; Yue *et al*. 1999; Coulombe *et al*. 2001). The exact structural state (E.S) of either types of COx is not clear yet. The binding geometry of the substrate for type I enzyme from atomic resolution has been worked out (Lario *et al*. 2003) and that explained the structure of a steroid substrate bound to the reduced enzyme (Li *et al*. 1993). In 2000, a structural model for steroid placed C3 steroid atom within a hydrogen bond distance (3.0Å) of N5 of FAD and in a position similar to those of hydride donor in many other flavoenzyme oxidases (Fraagi and Mattevi 2000). To know the accuracy of E.S-Michaelis complex and to better understand the effect of the protein microenvironment on substrate binding and turnover, a structure of the E.S complex of oxidized COx was studied (Lyubimov *et al*. 2007). They took two high resolution structure of a double mutant of bacterial COx in the absence and presence of ligand, glycerol, showing trajectory of glycerol as it bind the complex in active site. Earlier it was reported that oxidation rate of cholesterol by COx is dependent on the enzyme concentration and is orders of magnitude faster than the rate of sterol desorption from the membrane (Phillips *et al*. 1987; Lund-Katz *et al*. 1988; Bar *et al*. 1989). This kinetically suggested the interaction of COx with lipid bi-layer in order to bind its substrate.

The X-ray crystal structure of COx given by Blow and co-workers (Vrielink *et al*. 1991; Li *et al*. 1993) revealed that the enzyme must undergo a conformational change involving 10-20 amino acid residues in order to bind substrate. Although the conformation of the open enzyme was not known, it was postulated that an active-site lid, composed of some of these 20 residues, opens to form a hydrophobic channel between the membrane and the active site in order to bind sterol iso-ergonically. Binding buried the four rings of the sterol completely in the active site. Later in 1995 it was demonstrated that COx does complete lysis of epithelial cells in boll weevil (*Anthonomus grandis grandis*) larvae. Dye leakage experiment was conducted (Ghoshry *et al*. 1997) to check the membrane disruption mechanism. Leakage was observed with cholesterol containing vesicles and wild type enzyme only; the rate of leakage was dependant on the rate of cholest-4-en-3-one production. The COx binding to membrane and COx partitioning to the active site did not perturb the bilayer to cause leakage of vesicle content. However, the formation of cholest-4-en-3-one did increase the membrane permeability. The activity of COx was directly and sensitivity-dependent on the physical properties of the membrane with which its substrate was bound (Ahn and Sampson 2004). COx was considered as an interfacial enzyme that transiently associates with lipid membrane to convert cholesterol to cholest-4-en-3-one (Sampson and Kwak 2007).

FAD binding

The FAD is deeply buried in the protein structure and is involved in extensive contacts mainly with the FAD binding domain. The pyrophosphate group involved in hydrogen bond contacts with main chain atoms of the loop between Gly118 and Gly122 and this situation can be easily understood with the its structure (Vrielink and Ghisla 2009). A similar type of interaction has also been observed in the structure of vanillyl alcohol oxidase (VAO) and this loop region has been defined as the PP loop (Mattevi *et al*. 1997). The cofactor is linked to the protein through a covalent bond between ND1 of the imidazole side chain of His121 (a residue in the PP loop) and the 8-methyl group of the isoalloxazine ring. The histidine side chain approaches the isoalloxazine ring from the *si* face of the flavin unlike VAO where the covalent histidine originates in the substrate binding domain and approaches the flavin moiety from the *re* face. Therefore, in BCO2, the PP loop appears to play a critical role in positioning the cofactor correctly within the structure of the enzyme through two factors: the covalent linkage to the isoalloxazine ring and the hydrogen bond interactions with the pyrophosphate moiety.

Active site of COx

The binding sites of the enzyme in both forms are packed by a number of loops from the external environment, which provide high mobility than other proteins. This nature of protein packing shows easy access of the steroid molecule to enter in the hydrophobic pocket. The structure comparisons of SCOx and ReCOx (recombinant COx) showed difference in the nature of loops. The rigidity of loops is more in SCOx as it contain amphipathic helical turn; while in case of ReCOx loops is more extendable, lack secondary structure element and exhibit higher temperature factors (Yue *et al*. 1999). The substrate mobility of SCOx and ReCOx was compared (Yue *et al.* 1999) by correlating the

elevated K_M values for both, cholesterol and dehydroisoandosterone. Finally it was reported that the increased rigidity of SCOx loops pre-orient the residue needed for binding the 8-carbon isoprenoid tail at C17 of the substrate, thus increases the efficiency of the enzyme for catalysis (for details see review by Vrielink and Ghisla, 2009). The active site of BCO2 consists of a cavity (with a volume of 450 $Å^3$) bounded on one side by the â-pleated sheet in the substrate-binding domain and, on the opposite side, by the isoalloxazine ring of the cofactor. Two loops in the substrate-binding domain (between â12 and á5 and between á7 and â14) exhibit higher than average temperature factors suggesting a possible entrance for cholesterol to the active site. The residues lining the cavity near the pyrimidine moiety of the cofactor are highly hydrophilic and include Arg477, Glu475, Glu551, Glu432, Glu311, Asn516 and Lys554. The charged nature of this region of the active site is in sharp contrast to that seen in the structure of BCO1 and it is precisely these differences that are likely to play an important role in the reactivity of the flavin cofactor. The region near to the dimethylbenzene ring of the cofactor consists mainly of hydrophobic residues (Coulombe *et al*. 2001).

Applications of COx

1. COx and pathogenicity

COx is produced by a number of microorganisms including life threatening pathogens such as *Rhodococcus equi*, *Mycobacterium tuberculosis* and *Mycobacterium leprae*. COx is an interesting clinical enzyme for the treatment of bacterial infections. Gene disruption studies indicated that COx is the main membrane damaging factor, contributing to the pathogenicity of the microbes *in vivo*. Mutational analysis indicated that COx membrane damaging factor imparts to heamolytic reaction elicited by *Rhodoccocus equi* in the presence of sphingomyelinase C-producing bacteria, such as *Listeria ivanivii*, *Bacillus cereus* and *Staphylococcus aureus* (Navas *et al*. 2001). As these enzymes are unique to bacteria, they represent a potential target for a new class of antibiotics. Intriguingly, it has been demonstrated that Alzheimer's disease β–amyloid selectively oxidizes cholesterol at the C-3 hydroxyl group and catalytically produces 4-cholesten-3-one (Puglielli *et al*. 2005). Therefore, it mimics the activity of COx (Puglielli *et al*. 2005).

Removal of cellular cholesterol rendered primary cells and cell lines highly resistant to HIV-1-mediated syncytium formation and to infection by both CXCR4- and CCR5-specific viruses. Thus it appears that cholesterol may be critical to the HIV-1 co-receptor function of chemokine receptors and is essentially required for infection of cells by HIV-1 (Liao *et al*. 2004).

2. Mycobacterium infections

Mycobacterium tuberculosis, the causative agent of tuberculosis, is a very successful pathogen that infects one-third of the human population (Maher and Raviglion 1999). Only 10% of primary infected individuals develop active disease during their lifetimes. Tubercle bacilli are able to persist in a dormant state, from which they may reactivate and induce the contagious disease state (Glickman and Jacobs 2001). In asymptomatic hosts, *M. tuberculosis* exists in reservoirs called granulomas, which are cellular aggregates that restrict bacterial spreading (Tufariello *et al*. 2003). Granulomas are organized collections of mature macrophages that exhibit a certain typical morphology and that arise in response to persistent intracellular pathogens (Adams 1976).

Pathogenic mycobacteria can induce the formation of foamy macrophages filled with lipid-containing bodies; these have been postulated to act as a secure, nutrient-rich reservoir for tubercle bacilli (Peyron *et al*. 2008). Moreover, *M. tuberculosis* DNA has been detected in fatty tissues surrounding the kidneys, as well as those of the stomach, lymph nodes, heart and skin. Tubercle bacilli are able to enter adipocytes, where they accumulate within intracytoplasmic lipid inclusions and survive in a nonreplicating state (Neyrolls *et al*. 2006). *In vivo*, it is expected that *M. tuberculosis* adapts metabolically to nutrient-poor conditions characterized by glucose deficiency and an abundance of fatty acids (Muñoz and Mckinney 2006; Neyrolls *et al*. 2006). The presence of a complex repertoire of lipid metabolism genes in the genome of *M. tuberculosis* suggests that lipids, including steroids, are important alternative carbon and energy sources for this pathogen (Cole *et al*. 1998). One attractive potential alternative nutrient that is readily available in the mammalian host is cholesterol, a major sterol of the plasma membrane. The presence of cholesterol in lipid rafts is required in order for microorganisms to enter the intracellular compartment (Goluszko and Nowicki 2005). Studies have shown that cholesterol is essential for the uptake of mycobacterium by macrophages, and it has been found to accumulate at the site of *M. tuberculosis* entry (Gatfield and Pieters 2000; Peyron *et al*. 2000; Astarie *et al*. 2009). Moreover, cholesterol depletion overcomes the phagosome maturation block experienced by *Mycobacterium avium*-infected macrophages (Chastellier and Thilo 2006). It is well known that cholesterol can be utilized by fast growing nonpathogenic mycobacterium (Martin 1977; Mahato and Garai 1997; Brzostek *et al*. 2007) but it was previously thought that pathogenic mycobacterium might not be able to use cholesterol as a carbon and energy source (Cole *et al*. 1998).

The bioinformatics analysis identified a cassette of cholesterol catabolism genes in *Actinomycetes*, including the *M. tuberculosis* complex (Gaize *et al*. 2007). Microarray analysis of *Rhodococcus* sp. grown in the presence of cholesterol revealed the up regulation of 572 genes, most of which fell within six clearly discernible clusters (Gaize *et al*. 2007). Most of the identified genes had significant homology to known steroid degradation genes from other organisms and were distributed within a single 51-gene cluster that appears to be very similar to a cluster present in the genome of *M. tuberculosis* (Gaize *et al*. 2007). Many of the cholesterol-induced genes had been previously selected by transposon site hybridization analysis of genes that are essential for survival of tubercle bacilli (Rengarajan *et al*. 2005) and/or are up regulated in interferon-activated macrophages (Schnappinger *et al*. 2003; Voskuil *et al*. 2003). It was also demonstrated that the *M. tuberculosis* complex can grow on mineral medium with cholesterol as a primary source of carbon (Gaize *et al*. 2007; Pandey and Sassetti 2008). Moreover, the growth of tubercle bacilli on cholesterol was significantly affected by knockout of the *mce4* gene, which encodes an ABC transporter responsible for cholesterol uptake (Mohn *et al*. 2008; Pandey and Sassetti 2008).

Earlier studies had shown that disruption of *mce4* attenuated bacterial growth in the spleens of infected animals that had developed adaptive immunity (Sassetti and Rubin 2003; Joshi *et al*. 2006). It was demonstrated for the first time that *M. tuberculosis* utilizes cholesterol via the 4-androstene-3, 17-dione/1, 4-androstadiene-3, 17-dione pathway (AD/ADD) and this process requires production of an intact KD enzyme. Moreover, the tubercle bacilli growing in medium containing an alternative carbon source can accumulate cholesterol in the free-lipid zone of their cell walls, and this accumulation affects cell wall permeability.

3. Rhodococcus equi infection

Rhodococcus equi is a well know pathogen able to induce lysis in the cell membrane with the help of COx enzyme (Linder and Bernheimer 1997). Human suffering from HIV is susceptible to *Rhodoccocus equi*. This is a Gram positive *cocobacillus* that reside within macrophage of the host. It is a common soil organism that frequently infects young horse. Bacteria can replicate in primary phagosomes within the macrophage. Activated macrophages produce a number of reactive oxygen intermediate (ROI) and nitric oxide with potent anti-microbial activity. A membrane bonded oxidase

catalyses the reduction of oxygen to superoxidase anion (SOA). This highly toxic oxygen intermediate can combine with nitric oxide and generate other anti-microbial agents, e.g. hydrogen peroxidae (H_2O_2) and peroxynitrite. It was shown that peroxynitrite mediates the intracellular killing of *Rhodococuss equi* (Darrah *et al*. 2000). The induction of enzymes by bactericidal agents H_2O_2 and SOA was studied in the presence of cholesterol in vivo for viewing the role of COx for cell invasion, and SOD as well as catalase for the intracellular survival of *R. equi*. The data suggested that the presence of cholesterol induces COx in bacteria grown on agar plates; moreover catalase, SOD and even membrane bound COx responded to reactive oxygen species. The hemolytic activity of *Rhodococuss equi* COx was confirmed through genetic experiments (Navas *et al*. 2001). Cholesterol oxidation was significantly increased when the strain was co-phagocytosed with *Corynebacterium pseudotuberculosis*, a sphingomyelinase-producing bacterium and a co-operative partner of *Rhodococuss equi* in the *in vitro* hemolysis of sheep erythrocytes (Linder and Berheimer 1982).

All these studies did not confirm whether it is the lytic function or a nutritional function which contributes to bacterial survival in macrophage (Kreit and Sampson 2009). This concept was further elaborated with the help of mutated strain of *Rhodococuss equi* that was originally isolated from foal (a young horse) infected with pneumonia, in which *Cho E* (COx) was disrupted by allelic exchange. This mutant strain was devoid of any COx activity. Further, this mutant was assessed for *in vivo* virulence in mice or foal and for *in vitro* cytotoxicity to macrophages (Pei *et al*. 2006, 2007). Based on the results obtained from mutated and parental strain, and their similar cytotoxicity to macrophages it was concluded that COx is not a virulence factor and its role may be limited to the catabolism of cholesterol as a carbon source and energy source of the infecting bacterium (Kreit and Sampson 2009).

4. Role of COx in agriculture

COx represents a novel type of insecticidal protein with potent activity against the cotton boll weevil (*Anthonomus grandis* Boheman). Genetically modified plants that produce insecticidal proteins (e.g. the *Bacillus thuringiensis* toxin) are now available to control insect pests of several major crops. Purified COx is active against boll weevil larvae at a 50% lethal concentration (LC_{50}) of 20.9 µg/mL), which is comparable to the bioactivity of *Bacillus thuringiensis* proteins against other insect pests. Upon ingestion, this protein causes developmental arrest and death of boll weevil larvae (Purcell *et al*. 1993) and a marked decrease in fecundity of female adult boll weevils (Greenplate *et al.* 1995). The enzyme also exhibits more moderate insecticidal effects when ingested by several species of lepidopteran cotton insect pests including tobacco budworm (*Heliothis virescen*s), corn earworm (*Helicoverpa zea*), and pink bollworm (*Pectinophora gossypiell*a; Greenplate *et al.* 1997). In addition to boll weevil, several lepidopterans were negatively affected by the presence of COx at a dietary concentration of 0.001%. The addition of cholesterol oxidation products by COx (i.e. cholest-en-3-one and hydrogen peroxide) to the diet, and re-treating the diet with the enzyme, excluded insecticidal effects caused by the ingestion of toxic compounds. However, the boll weevil larvae are acutely sensitive to ingested COx because it induces lysis at the mid-gut epithelium. Boll weevil adults are insensitive to ingested COx, although the fecundity of adult females was greatly reduced if 50 µg/mLof the enzyme was present in the diet (Greeplate *et al*. 1995). COx reduced subsequent oviposition (up to 83% in eggs laid) and larval survival (97% reduction as compared to controls) because of poorly developed ovaries and few developing oocytes. COx was expressed in transformed tobacco plants, and the synthesis levels in leaf tissues routinely ranged from approximately 5-50 µg of enzyme/g fresh weight.

In the absence of a chloroplast targeting sequence, COx production resulted in severe abnormalities in plant development and fertility. When produced as a fusion with a chloroplast-targeting peptide, synthesis of the mature and the full-length enzyme did not cause the deleterious phenotypic effect observed with untargeted COx (Corbin *et al*. 2001). Transgenic leaf tissues expressing COx exerted insecticidal activity against boll weevil larvae. When produced in the cytosol, or when targeted to chloroplasts, COx metabolizes phytosterols *in vivo*. Transgenic plants expressing COx in cytosol accumulated low levels of saturated sterols (stanols), while the transgenic plants expressing chloroplast-targeted COx maintained a greater accumulation of stanols and appeared phenotypically and developmentally normal. It was proposed that COx could modify sterol ratios, thus influencing cell division, or could affect brassino steroid biosynthesis in steroid hormones. Thus this enzyme is industrially important and is commonly used for the enzymatic transformation of cholesterol (Arima *et al.* 1969).

5. Clinical use

COx is also useful for the clinical determination of total or free serum cholesterol by coupling with a related enzyme for the assessment of arteriosclerosis and other lipid disorders and of the risk of thrombosis (Allain *et al.* 1974). It is also use to determine cholesterol from low-density lipoprotein to high-density lipoprotein, on the cell membrane of erythrocytes (and of other cells and cellular compartments), in gall stone and in human bile. The level of cholesterol in blood range from less than 50 mg/dL in infants to an average of 215 mg/dL in adults and to 1,200 mg/dL or more in individuals suffering from a rare, inherited disorder called familial hyper-cholesterolemia. Total cholesterol is the sum of HDL cholesterol, LDL cholesterol and 20% of the triglycerides values. Using the various values, one can calculate a cardiac risk ratio is equal to total cholesterol divided by HDL cholesterol. A cardiac risk ratio greater than 7 is considered a warning (Marian 2007).

The risk for Alzheimer disease is also related to hypercholesterolemia via mechanisms involving oxidative stress, this disease is characterized by the accumulation of amyloid β-peptide (a 39-43 amino acid peptide) in the neocortex, which is connected to peroxidative damage. The amyloid β-peptide forms complexes with Cu^{2+} ions, which oxidize cholesterol into cholest-4-en-3-one, thus mimicking the activity of COx. In fact, brain tissues from Alzheimer disease patients had a cholest-4-en-3-one content approximately 2-fold higher than brain tissues from controls. A different method for determining serum cholesterol has also been reported (Pollegioni *et al*. 2009). Another use of the COx enzyme is in the microanalysis of steroids in food specimens for determining the stearic configuration of 3-ketosteroids from their corresponding 3β-hydroxysteroids.

6. Cholesterol biosensor

COx with cholesterol esterase and peroxidase has been co-immobilized onto electrochemically prepared polyaniline films. These polyaniline-enzyme films characterized using spectroscopic techniques, have been used to fabricate a cholesterol biosensor (Singh *et al.* 2005). They have also co-immobilized COx and cholesterol esterase onto tetraethylorthosilicate (TEOS) sol-gel films for cholesterol biosensor (Singh *et al.* 2007). The cholesterol immobilized on a nylon membrane was used to build a fiberoptic biosensor based on the change in fluorescence of an oxygen-sensitive dye (Ishizaki *et al*. 1989). Cholesterol biosensor based on the electrochemical reduction of oxygen was subsequently developed by using bilayer-film coating; this sensor is less sensitive to organic interferences (Solaiman and Somkuti 1981). An amperometric biosensor was also obtained by reconstituting the apoprotein of *Pseudomonas flurorescence* COx with FAD monolayer (Solaiman and Somkuti 1997).

CONCLUSION

In recent times, COx has rapidly become an enzyme of great interest due to its different biological applications. The present review highlights different applications of cholesterol oxidase and its role in pathogenicity. The broad range of clinical and industrial applications of COx has generated a renewed interest in exploring various natural habitat/environments for discovering newer microbial sources as potential producers of this enzyme.

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