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Production and characterization of recombinant perdeuterated cholesterol oxidase

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ABSTRACT

Cholesterol oxidase (CO) is a FAD (flavin adenine dinucleotide) containing enzyme that catalyzes the oxidization and isomerization of cholesterol. Studies directed toward elucidating the catalytic mechanism of CO will provide an important general understanding of Flavin-assisted redox catalysis. Hydrogen atoms play an important role in enzyme catalysis; however, they are not readily visualized in protein X-ray diffraction structures. Neutron crystallography is an ideal method for directly visualizing hydrogen positions at moderate resolutions because hydrogen and deuterium have comparable neutron scattering lengths to other heavy atoms present in proteins. The negative coherent and large incoherent scattering lengths of hydrogen atoms in neutron diffraction experiments can be circumvented by replacing hydrogen atoms with its isotope, deuterium. The perdeuterated form of CO was successfully expressed from minimal medium, purified, and crystallized. X-ray crystallographic structures of the enzyme in the perdeuterated and hydrogenated states confirm that there are no apparent structural differences between the two enzyme forms. Kinetic assays demonstrate that perdeuterated and hydrogenated enzymes are functionally identical. Together, structural and functional studies indicate that the perdeuterated protein is suitable for structural studies by neutron crystallography directed at understanding the role of hydrogen atoms in enzyme catalysis.

The Protein Data Bank [1] contains atomic coordinates for more than 100,000 structures (as of 2015), the vast majority of which have been determined using X-ray crystallography. Although X-ray diffraction is an excellent technique for visualization of the more electron-rich atoms commonly found in proteins (C, O, N, P, S), hydrogen atoms are visible only at very high resolutions (<1.0 Å). Despite their small size, hydrogen atoms play a dominant role in the structure and function of macromolecules. They are critical in forming hydrogen bonds, which dictate the stability and local conformations of the structure, drive the pK_a of amino acid side chains within the protein microenvironment, and so affect the chemistry of these complex molecules in different ways. Furthermore, enzyme chemistry often involves movements of hydrogen atoms between amino acid side chains of the protein, cofactors, and bound ligands during the catalytic cycle. Thus, detailed knowledge of the positions of hydrogen atoms is critical to fully understand the chemistry of macromolecules and especially enzymes.

Often, however, even when ultra-high-resolution X-ray crystallographic data are available, the hydrogen positions are still only very weakly visible in electron density maps [2–9]. This may be due to intrinsic flexibility of the atoms to which they are bonded, which would result in a diffuseness of the position of the single electron of the hydrogen atom. In addition, regions of the structure may adopt discrete alternate conformations, often providing important insights into the mobility and flexibility of the molecule correlated with function. However, these multiple states, or occupancies, further challenge the ability to visualize hydrogen atoms, which are already difficult to see at the highest resolutions obtained by X-ray crystallography. Furthermore, the physical nature of the X-ray beam during data collection can result in photoinduced reduction events [10–12]. Particularly in the case of redox-specific enzymes, these beam-induced events can confound interpretation of the electron density because the beam causes mixed conformational states due to the accumulation of mixed redox states of the molecule during data collection [13].

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Neutron protein crystallography is a technique that can be used to more readily visualize hydrogen within protein molecules because these atoms scatter neutrons comparably to heavier atoms such as carbon and nitrogen and so can be visualized at moderate resolutions (1.5–2.5 Å). However, hydrogen exhibits a negative coherent scattering length, which often results in cancellation of density for the positively scattering atom to which it is bonded. In addition, hydrogen has a large incoherent scattering cross section, which decreases the signal-to-noise ratio of data collected from hydrogen-containing samples. Deuterium, in contrast, has a small incoherent scattering cross section and has a positive coherent scattering length, nearly twice the magnitude of the hydrogen scattering length and comparable to that of carbon, nitrogen, and oxygen atoms. Exchanging the hydrogen atoms for deuterium atoms, therefore, improves the visibility of hydrogen (deuterium) atom positions in neutron structures. Solvent hydrogen atoms and labile protein hydrogen atoms can be exchanged by incubating protein or protein crystals in deuterated buffers. However, partial hydrogen–deuterium exchange can occur at buried or protected labile sites, resulting in cancellation of density at these positions. In addition, nonlabile carbon-bound hydrogen atoms—approximately 75% of the protein hydrogen atoms—will not be exchanged by this technique and will significantly contribute to diffraction background. Full protein deuteration can alleviate the nonlabile hydrogen contribution to background. Perdeuterated protein (all hydrogen atoms are exchanged for deuterium atoms) is produced by expressing proteins in fully deuterated medium [14]. Shu and coworkers [15] presented the first perdeuterated neutron crystal structure of myoglobin and demonstrated the power of perdeuteration in combination with neutron diffraction for visualizing hydrogen atom positions. More recently, a neutron diffraction structure at 1.65 Å of perdeuterated rubredoxin was able to identify 8-fold more deuterium atom positions than hydrogen atom positions that could be identified in an X-ray structure at 1.1 Å resolution [4].

Another advantage of neutron diffraction in the structural characterization of redox proteins is that neutrons are nonionizing. Detailed and unambiguous analysis of the protonation and redox states of enzymes at defined steps along catalytic pathways can be performed by neutron crystallography. We are using a combination of high-resolution X-ray and neutron crystallography to study the redox mechanism of cholesterol oxidase (CO).[1] CO (EC 1.1.3.6) is a flavin-containing redox enzyme catalyzing the oxidation and isomerization of cholesterol to cholest-4-en-3-one with the concomitant production of hydrogen peroxide. The oxidation reaction is catalyzed by the tightly bound FAD (flavin adenine dinucleotide) cofactor, which is usually noncovalently bound (type I enzyme) but may also have a covalent attachment to the protein (type II enzyme). CO is produced by a wide variety of bacterial species; however, only the three-dimensional structures of the type I CO from *Streptomyces SA-COO* [16] and *Brevibacterium sterolicum* [17,18] and the type II COs from *B. sterolicum* [19] and *Chromobacterium* sp. *DS-1* [20] have been determined by X-ray crystallography. Of these, the CO from *S. SA-COO* has been most extensively characterized by X-ray crystallography [3,8,9,16,21–23] in combination with kinetic analysis of active site mutants [3,16,22,24]. Crystals of the enzyme diffract to high resolution with several atomic resolution structures obtained [3,8,21–23]. Atomic resolution X-ray structures of CO at various pH values [9] revealed pH-dependent changes in the positions of some hydrogen atoms that were involved in strong hydrogen bonding interactions between the flavin N3 and O4. In addition, these structures showed that His447 is protonated and, therefore, cannot be the base for deprotonation of the hydroxyl of the substrate.

Despite the very high-resolution structures obtained from diffraction studies of crystals of CO, questions regarding the role of hydrogen atoms in the redox chemistry remain to be answered. Because the reaction mechanism of CO involves abstraction of a hydroxyl proton and transfer of a hydride to FAD, it is of considerable interest to identify the location of hydrogen atoms of CO in various redox states. Neutron diffraction studies provide an excellent technique for elucidating the positions of hydrogen atoms through the catalytic cycle of the enzyme and will be highly complementary to X-ray diffraction studies of the enzyme.

Perdeuteration may affect the expression and purification of proteins because replacing all ^{1}H atoms for the heavier ^{2}H isotope in the growth medium can affect the level of expression of proteins in the cell [25,26]. Expression and purification procedures, therefore, must be optimized for perdeuterated proteins to ensure adequate production of highly pure protein. Perdeuteration also affects protein solubility, and crystallization conditions need to be reoptimized. Prior to analysis of a fully perdeuterated neutron structure, it is important to establish that the hydrogenated and perdeuterated forms of the protein are structural isomorphs and behave functionally in a comparable manner. For CO, this involves a kinetic comparison of the hydrogenated and perdeuterated forms of the enzyme as well as a structural comparison by X-ray crystallographic analysis. Toward this aim, we have successfully expressed and purified perdeuterated CO. Kinetic characterizations were undertaken for the two protein forms. In addition, the stability of both protein forms was assessed by differential scanning fluorimetry. Finally X-ray structure analysis was undertaken on single crystals of each protein form, and a structural comparison was carried out to establish whether perdeuteration has a significant effect on the protein structure.

Materials and methods

Cloning

The wild-type *S. SA-COO* cholesterol oxidase gene was amplified from pCO117 [27] and cloned into the *Nco*1 and *Hin*dIII restriction sites of pET28a-His6-MBP-TEV-AEW. The forward and reverse primers are 5′-gactccatggccactgcacaacagcatctg-3′ and 3′-gcagtgccgca gcgtggtggtggtggtggtgattcgaactga-5′, respectively. The reverse primer included codons for six histidine residues. The vector contains the lac repressor (LacI) and the kanamycin resistance gene.

Expression and purification

A 1-L culture of perdeuterated *E. coli* expressing CO (^{2}H-CO) was produced using the bioreactors at the National Deuteration Facility (NDF) located at the Australian Nuclear Science Technology Organisation (ANSTO) using a procedure similar to that of Chen and coworkers [28]. In brief, 50 µl of OneShot BL21*(DE3) *E. coli* (Invitrogen) was transformed with the pCO_P1 plasmid (90 ng) and rested in 250 µl of SOC medium for 2 h at 37 °C before being used to inoculate a 50% (v/v) D_2O ModC1 preculture [29] containing deuterated glycerol (Sigma, 98%) as the sole carbon source. The bacteria were adapted to D_2O at 37 °C, shaking at 220 rpm in sealed flasks filled to approximately 5% of capacity, by preparing successive precultures with increasing concentrations of D_2O (50, 90, and 100%), the first overnight and the latter for at least two generations (>2 h). The 100% D_2O preculture was obtained by gently pelleting the ice-chilled cells from the 90% D_2O preculture, followed by resuspension in 100% D_2O medium. The 100% D_2O adapted preculture was subsequently used to inoculate a bioreactor containing a 1-L final volume of perdeuterated ModC1 medium

[1] *Abbreviations used:* CO, cholesterol oxidase; FAD, flavin adenine dinucleotide; IPTG, isopropyl β-D-1-thiogalactopyranoside; DSF, differential scanning fluorimetry; PEG, polyethylene glycol; rmsd, root mean squared deviation.