Solution structure of the reduced active site of a starch-active polysaccharide monooxygenase from *Neurospora crassa*

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Abstract:

X-ray absorption spectroscopy (XAS) was utilized to gain insights into the structure and electronic properties of the reduced copper active site in NCU08746, a polysaccharide monooxygenase (PMO) from *Neurospora crassa* that activates O_2 to cleave glycosidic linkages in starch. The reaction of NCU08746 likely starts with binding of O_2 to the copper(I) center. However, the solution structure of the reduced active site in NCU08746 has not been properly elucidated. In this study, we prepared Cu(I)-NCU08746 in solution, which was snap-frozen to preserve the solution structure of the copper(I) active site prior to XAS analysis. Results show that the copper(I) center in Cu(I)-NCU08746 exhibits a 4-coordinate geometry, which is different from the 3-coordinate geometry observed for some other PMOs. This difference likely arises from the coordination of the active site tyrosine residue and could contribute to the difference in activity between NCU08746 and other PMOs.

Keywords: **oxygen activation, polysaccharide monooxygenase, X-ray absorption spectroscopy.**

Classification number: **2.2**

Introduction

Enzymes that contain a copper active site capable of activating O_2 for C-H bond cleavage are of great fundamental and practical interests. Around 2010, a new superfamily of oxygen-activating mono-copper enzymes called polysaccharide monooxygenases (PMOs) were discovered [1]. It is generally accepted that PMOs degrade polysaccharide via hydroxylating either C-H bond of the glycosidic linkages (Fig. 1). PMOs can act directly on the surface of their polysaccharide substrates in an "endo" fashion, which enables them to work synergistically with currently available industrial hydrolytic enzymes in converting recalcitrant polysaccharides to fermentable sugars [1]. Currently there are 6 families of PMOs listed in the carbohydrate active enzymes (CAZy) database [2]. The starch-active PMO family was discovered in 2014, which is classified as AA13 family in the CAZy database [3]. While other PMOs act on β(1→4) glycosidic linkages found in chitin, cellulose, and xylans, AA13 PMOs only cleave α(1→4) linkages found in starch, which has expanded the perspectives in starch metabolism.

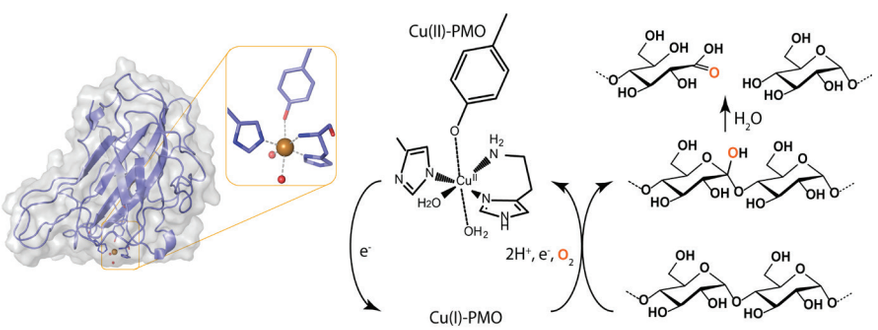

Fig. 1. Structure of Cu(II)-PMO (left) and the PMO reaction (right).

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The structures of PMOs have been characterized extensively with single crystal X-ray crystallography (XRD), which revealed an absolutely conserved Type 2 mono-copper active site coordinated by two histidine residues in all PMOs in a motif termed as *histidine brace* [4] (Fig. 1). The *N*-terminal histidine residue coordinates in a bidentate mode using the N atom of its amine group and the $N_{\delta 1}$ atom of the imidazole group (Fig. 1). The other histidine residue coordinates via its $N_{\varepsilon 2}$ atom. The reaction of PMOs likely starts from the reduced state, in which the copper(I) center readily binds O_2 to generate a reactive copper-oxygen species. The structure and electronic properties of the copper(I) center thus play an important role in the mechanism of PMOs. Nevertheless, the structure of PMOs in the copper(I) state (Cu(I)-PMO) have not been well characterized by single crystal XRD. The available structures of Cu(I)-PMO are obtained either from the photo-reduction of the copper(II) center during XRD data collection or from *in crystallo* chemical reduction, which may not represent the true structure of Cu(I)-PMO in solution [4].

In this work, we attempted to obtained insights into the structure and electronic properties of the copper(I) active site in the starch-active PMO NCU08746 of *Neurospora crassa* using X-ray absorption spectroscopy (XAS). The X-ray Absorption Near Edge Structure (XANES) of the XAS spectrum could provide insights into the geometry and electronic properties of the copper center. The extended X-ray absorption fine structure (EXAFS) region contains the important local structural information up to ca 4.5 Å from the copper center. The reduced sample for XAS analysis was prepared under inert gas atmosphere and snap-frozen in a sealed sample holder, which closely represents the solution state of Cu(I)-NCU08746.

Materials and methods

Cu(II)-NUC08746 was prepared as previously described [3]. The enzyme was buffer exchanged to 700 mM MES buffer pH 5.0 and degassed under a stream of argon for 30 minutes and stored in a refrigerator inside an anaerobic Mbraun glove box. Buffer solution and glycerol were degassed by bubbling with argon for 2 hours and left open in the glove box overnight. Ascorbic acid solution was prepared by mixing ascorbic acid powder with anaerobic MES buffer inside the glove box. Cu(I)-NCU08746 sample was prepared by incubating anaerobic Cu(II)-NCU08746 with 15 fold excess anaerobic ascorbic acid at room temperature in the glove box for 30 minutes. Anaerobic glycerol (20% final concentration) was subsequently added to the sample to prevent ice crystal formation when the sample was frozen. The concentration of the enzyme in the final reduced sample was 1.14 mM. The reduced sample was transferred to an XAS sample holder, which was put in a reaction vial sealed with septum screw cap. The vial was taken out of the glove box, immediately frozen in liquid isopentane, and stored in liquid nitrogen until the data was collected. Data collection was carried out at Beamline X3B of the National Synchrotron Radiation Light Source (NSLS) of Brookhaven National Laboratory in Long Island, New York, USA. Data reduction and processing were carried out using Athena [5]. Fitting of the EXAFS data was carried out using Artemis [5] and FEFF6.0 [6]. The FEFF model was built based on a PMO crystal structure (Fig. 2).

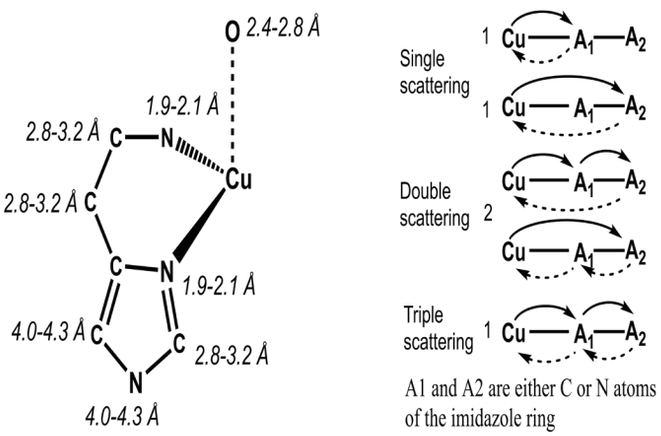

Fig. 2. FEFF input model used for EXAFS fitting and representation of single and multiple scattering paths of the imidazole ring.

Results and discussion

The XANES spectrum of Cu(I)-NCU08746 is shown in Fig. 3, which is significantly different from that of Cu(II)-NCU08746 previously reported [3]. The XANES spectrum of Cu(I)-NUC08746 exhibits a clear shoulder at 8983 eV, which is absent in the spectrum of Cu(II)-NCU08746. The featureless edge of Cu(II)-NCU08746 is consistent with a 5- or 6-coordinate copper center as previously described [3]. In contrast, the shoulder in Cu(I)-NCU08746 is indicative of a coordination number of 3 or 4, which arises from the 1s→4p electron transition of the copper(I) center [7]. This result indicates that the structure of the copper center is

significantly altered upon reduction from Cu(II) to Cu(I).

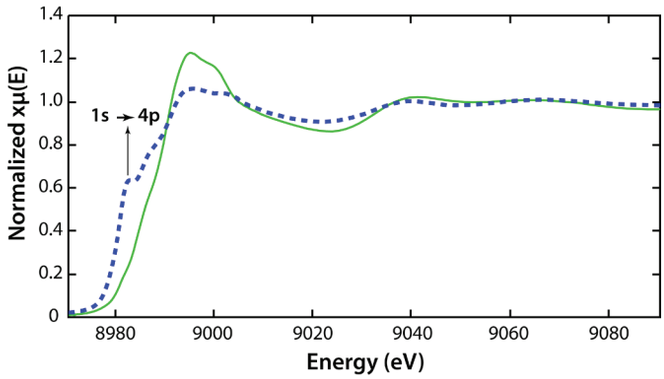

Fig. 3. XANES spectrum of Cu(I)-NCU08746 (dashed blue) obtained in this work in comparison with that of Cu(II)-NCU08746 (solid green) reproduced from ref. [3] with permission.

EXAFS data of Cu(I)-NCU08746 is shown in Fig. 4, which is exhibits a typical double-humped feature of histidine coordinated metal species near 4-4.5 $Å^{-1}$ [4, 8, 9]. The Fourier transform of Cu(I)-NCU08746 exhibits an inner shell at 1.5-2.0 Å, a second shell at 2.0-3.0 Å, and a third shell at 3.0-4.0 Å. The inner sphere can be fitted with several Cu-N/O paths at 1.9-2.5 Å, which is typical for copper enzymes including PMOs. The second shell can be fitted with several Cu•••C paths at ~ 2.9 Å and 3.2 Å, which can be attributed to the C atoms of the histidine ligands as depicted in Fig. 2.

The third shell corresponds to the double-humped feature in the EXAFS spectrum. As shown for many metal-imidazole species, the double-humped feature can be simulated with significant single and multiple scattering paths due to the imidazole ring (Fig. 2). By floating the coordination number of the imidazole ring in the fitting process, Vu, et al. were previously able to closely predict the number of histidine ligands in iron [9] and copper [3] enzymes. Here we used the same approach by fixing the number of histidine ligands at 2 according to the crystal structure of a starch-active PMO in *Aspergillus oryzae* [10]. The double-humped feature of Cu(I)-NCU08746 is reasonably well simulated with two imidazole rings, which were included in all the fits.

Table 1. Fitting results to unfiltered k^3-weighted EXAFS data of Cu(I)-NCU08746.

Fit #	First shell						Second shell			Third shell			R-factor
	Cu-N/O			*Cu-N/O*			*Cu•••C*			*Cu•••Im*			
	N	*R*	σ^2	*N*	*R*	σ^2	*N*	*R*	σ^2	*N*	*R*	σ^2	
1	2	1.90	6.6	1	2.52	3.53	3	2.91	6.0	2	n/a	n/a	0.04835
	1	2.25	5.7				1	3.18	2.8				
2	2	1.88	6.0				3	2.89	0.4	2	n/a	n/a	0.13689
	2	2.60	12.4				2	3.13	6.0				
3	2	1.99	5.1				3	2.94	1.9	2	n/a	n/a	0.18967
	1	2.19	8.0				2	3.19	2.8				

k range = 2-11 $Å^1$; number of independent points is 17; resolution = 0.174 Å; scale factor S_0^2 = 1.0; N = coordination number; R = distance (Å); σ^2 = respective Debye-Waller factor (10^{-3} $Å^{-2}$). Cu•••Im represents the significant single and multiple scattering paths of an imidazole ring.

Notably, the best fit requires a Cu-N/O path at ~ 2.5 Å (Fit #1, Table 1). Removing this path severely lowers the fit quality (Fits # 2 and 3). This path likely arises from the O atom of the active site tyrosine residue (Fig. 1), which is also observed in the crystal structure of photoreduced starch-active PMO from *Aspergillus oryzae* [10]. We thus propose the structure of Cu(I)-NCU08746 as shown in Fig. 5. The coordination of tyrosine to the reduced copper active site has only observed in starch-active PMOs but not on other PMO families [4]. Thus, this difference may contribute to the difference in activity between the starch-active PMO family and other PMO families.

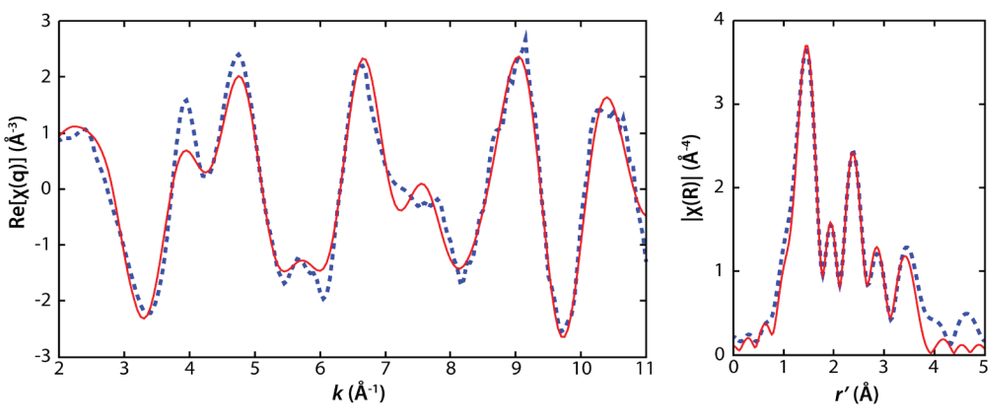

Fig. 4. k^3-weighted EXAFS spectrum (left) and its Fourier transform (right) of Cu(I)-NCU08746. Data is shown as dashed blue line and fit as solid red line. The best fit parameters are provided in Table 1 (Fit # 1).

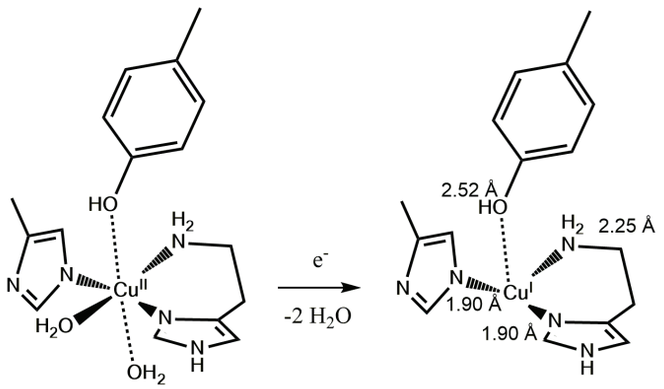

Fig. 5. Proposed structural change upon reduction of Cu(II)-NCU08746 to Cu(I)-NCU08746.

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