BBA - General Subjects 1862 (2018) 1031–1039

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

Structural investigation of cellobiose dehydrogenase IIA: Insights from small angle scattering into intra- and intermolecular electron transfer mechanisms

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ARTICLE INFO

Keywords:
Intramolecular electron transfer (IaET)
Intermolecular electron transfer (IeET)
Redox complex
Oxidative cellulose degradation
Small-angle scattering
Modeling

ABSTRACT

Background: Cellobiose dehydrogenases have gained interest due to their potential applications in sectors from biofuel production to biomedical devices. The CDHIIA variant is comprised of a cytochrome domain (CYT), a dehydrogenase domain (DH), and a carbohydrate-binding module (CBM) that are connected by two flexible linkers. Upon cellobiose oxidation at the DH, intramolecular electron transfer (IaET) occurs from the DH to the CYT. *In vivo*, CDHIIA CYT subsequently performs intermolecular electron transfer (IeET) to a lytic polysaccharide monooxygenase (LPMO). The relevant solution-state CDH domain conformations for IaET and IeET have not been fully characterized.

Methods: Small-angle X-ray and neutron scattering measurements of oxidized CDHIIA from *Myriococcum thermophilum* and *Neurospora crassa* were performed to investigate the structural landscape explored in solution by *Mt*CDHIIA and *Nc*CDHIIA in response to cations, pH, and the presence of an electron acceptor, LPMO9D from *N. crassa*.

Results: The scattering data complemented by modeling show that, under oxidizing conditions, *Mt*CDHIIA undergoes global conformational rearrangement in the presence of Ca^{2+}. Oxidized *Nc*CDHIIA exhibits conformational changes upon pH variation and, in the presence of *Nc*LPMO9D, primarily adopts a compact conformation.

Conclusions: These results demonstrate different conformational responses of oxidized *Mt*CDHIIA and *Nc*CDHIIA to changes in environment. The results also reveal a shift in the oxidized *Nc*CDHIIA conformational landscape toward interdomain compaction upon co-incubation with *Nc*LPMO9D.

General significance: The present study is the first report on the structural landscapes explored in solution by oxidized cellobiose dehydrogenases under various cation concentrations, pH conditions and in the presence of an electron-accepting LPMO.

1. Introduction

The cellulose and other polysaccharides present in biomass are appealing raw materials for producing renewable materials and biofuels. Currently, biorefineries utilize cellulase mixtures to breakdown cellulose into monomeric glucose for downstream production into biofuels. However, one of the biggest hurdles with cellulose degradation is overcoming its crystallinity, which makes it recalcitrant to degradation and enzymatic attack (Cellobiose dehydrogenases are not currently included in commercially available mixtures).

Fungal cellulases frequently utilized for cellulose breakdown employ both hydrolytic and oxidative processes [1]. The hydrolytic system includes three types of enzymes: cellobiohydrolases (CBHs) processively hydrolyze cellulose strands into cellobiose; endoglucanases (EGs) cleave amorphous cellulose; and β-glucosidases hydrolyze the glycosidic bond of cellobiose to form two glucose molecules. Characterized oxidative cellulases include lytic polysaccharide monooxygenases (LPMOs) and cellobiose dehydrogenases (CDHs). With the recent discovery of these oxidative enzymes, research has shifted toward better understanding the oxidative mechanisms fungal cellulases utilize to breakdown cellulose.

CDHs are extracellular flavocytochrome enzymes classified into the glucose-methanol-choline (GMC) oxidoreductase family. CDHs are co-secreted with a consortium of other biomass-degrading enzymes by most wood-degrading fungi in the presence of cellulose [2, 45]. CDHs comprise as much as 2% (by mole) or more of the secreted cellulases in

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https://doi.org/10.1016/j.bbagen.2018.01.016
Received 24 August 2017; Received in revised form 18 December 2017; Accepted 23 January 2018
Available online 31 January 2018
0304-4165/

fungal secretomes indicating a natural importance of these enzymes in fungal cellulose degradation [3,4]. When CDH genes were deleted in *N. crassa,* a significant decrease in cellulase activity was observed. Upon addition of CDH from *M. thermophilum* to the *N. crassa* Δ*cdh-1* strain, cellulase activity was recovered [4]. These results emphasize a potential role of CDHs in cellulase cocktails and the "mix-and-match" optimization possibilities for CDHs and LPMOs from various organisms.

CDH activity was first described in the mid-1970s by Westermark and Eriksson [5–8]. Structural characterization from small angle X-ray scattering and X-ray crystallography was later performed on full length and truncated domains, respectively [9–11, 46]. CDHs are comprised of an N-terminal cytochrome domain (CYT) with a coordinated heme b and a dehydrogenase domain (DH) containing a flavin adenine dinucleotide (FAD) cofactor. The CDHIIA variant also has a C-terminal family 1 carbohydrate-binding module (CBM). Long, flexible linkers connect each of these domains [12–14]. The DH carries out the two-electron oxidation of cellobiose, or other small sugars, converting cellobiose into cellobiono-1,5-lactone and reducing the FAD cofactor to $FADH_2$. Electrons from $FADH_2$ reoxidaiton are passed *via* direct single-electron transfer to the heme group within the CYT. According to Moser–Dutton theory, the DH and CYT must be positioned such that the $FADH_2$ and heme are within 14 Å of each other (edge-to-edge) to perform the intramolecular electron transfer (IaET) without involvement of other redox cofactors [15]. Each transferred electron subsequently undergoes either single-electron intermolecular electron transfer (IeET) to a final electron acceptor, such as LPMO, or direct electron transfer (DET) to an electrode in an electrochemical cell.

Potential applications of CDHs expand beyond biofuel production to sensor technology and biomedical devices [16–19]. Each of these applications requires CDH to maintain activity at varied pH and under less-than-favorable enzymatic conditions, thus necessitating a robust, tunable enzyme that operates across a broad spectrum of environments. Previous work grouped CDHs from ascomycetes into acidic, intermediate, and alkaline classes based on their optimum pH for activity [3]. Alkaline CDHs have pH optima within the neutral to alkaline range. Acidic CDHs have a pH optimum around pH 5.0 and are only active in a narrow pH range [3]. *Mt*CDHIIA is characterized as an acidic CDH [20]. The intermediate CDHs, including *Nc*CDHIIA, have optimum pH between 5.0 and 6.0 but are capable of maintaining activity over a broader pH range than acidic CDHs. For example, *Nc*CDHIIA maintains 50–70% of its maximum activity at pH 7.5 [3]. However, biochemical assays have shown that IaET can be increased or regained in CDHs at pH conditions non-optimal for activity [21–23]. Initial work investigated immobilized class I CDH from *Phanerochaete chrysosporium*, and class II CDHs from both *Humicola insolens* and *M. thermophilum* [23]. A two-fold increase in catalytic current for *Mt*CDHIIA was measured in the presence of the monovalent cation salt KCl whereas a five-fold increase was observed when the divalent cation salt $CaCl_2$ was added to the buffer. Kracher et al. [22] further investigated the Ca^{2+} enhancement of IaET in *Mt*CDHIIA by assaying CDH activity in the presence of Mg^{2+}, Sr^{2+}, Ba^{2+}, and Cd^{2+}. The IaET rate did not change as a function of atomic radius or electronegativity. Furthermore, out of twelve CDHs investigated, ten had increased IaET rates when away from optimal pH conditions in the presence of $CaCl_2$, with *Sclerotium rolfisii* and *N. crassa* IIA CDHs being the exceptions. Based on these activity studies and complementary docking models, the authors hypothesized that the divalent cations form complexes with CDH at the interface of CYT and DH, promoting interactions between these domains and more efficient IaET [22,23].

The first structural model of a full length CDH was reported by Lehner et al. [46] who used SAXS and modeling to investigate the size and shape of *Phanerochaete chrysosporium* CDH in solution. *Pc*CDH was modeled in an elongated conformation with the largest dimension being about 180 Å. The SAXS data informed advanced *Ab initio* reconstructions of the shape of *Pc*CDH which confirmed the elongated shape of *Pc*CDH in solution [47]. Recently, the structures of full-length *Mt*CDHIIA and *Nc*CDHIIA have been solved by X-ray crystallography [13]. In the crystals, *Mt*CDHIIA was captured in a closed conformation, while *Nc*CDHIIA adopted an extended conformation. Tan et al. noted the high flexibility of *Nc*CDHIIA and the difficulty of obtaining electron density for the CYT. This observation suggests that the enzyme can adopt a continuum of conformations. Complementary small-angle X-ray scattering (SAXS) studies were performed to understand the conformational landscape of these two enzymes. Ensemble optimization modeling (EOM) of the SAXS data supported the existence of CDHs in an ensemble of conformations [13]. While this structural investigation suggested that CDH would adopt a closed conformation for IaET and an extended conformation for IeET, it did not however explain the pH and cation responses of CDHs from different organisms.

Electrostatic mapping at the interface of DH and CYT using the crystal structures reveals that pH variation has different effects on the surface charges of *N. crassa* and *M. thermophilum* CDHs (Supplementary Information Fig. S1). The *Nc*CDHIIA DH interface transitions from positive at pH 5.5 to neutral at pH 7.5. The *MtCDHIIA* CYT has a negative interface at pH 5.5 which becomes more negative at pH 7.5. We hypothesize that the interface electrostatics directly affect domain interaction and, therefore, play a role in the variations of IaET rates in response to changes in pH and cations measured for CDHs from different organisms [3].

The enzymatic electron acceptor for CDH is LPMO, a cellulase responsible for oxidizing and cleaving cellulose chains. Tan et al. [13] demonstrated that LPMO accepts electrons only from the CYT of CDH. Docking studies have proposed binding sites for potential interactions between LPMO and the CYT domain; however, scarce experimental evidence exists for the complex [13,24]. NMR experiments performed by Courtade et al. [25] have shown that the presence of CDH perturbed chemical shifts of LPMO residues around the copper binding site indicating that the CYT domain interacted directly with the LPMO active site rather than a secondary site previously proposed by Li et al. [24].

Small-angle scattering can elucidate conformational rearrangements that occur in solution due to changes in the environment. We have previously characterized oxidized *Nc*CDHIIA using small-angle neutron scattering (SANS) [26]. In the present work, we characterize the effect of the chemical environment (pH and cations) on the structures of oxidized CDHs from *M. thermophilium* and *N. crassa*. The structural response of oxidized *Nc*CDHIIA in the presence of *Nc*LPMO9D is also investigated. Using the recently solved structures of *Mt*CDHIIA and *Nc*CDHIIA single- or multistate models (where appropriate) are proposed for each condition. This combination of SANS, SAXS, and modeling provides a new experimental glimpse of the CDH–LPMO interaction under oxidizing conditions.

2. Results

In this study, we characterize the conformational landscape of fully oxidized CDHIIA in solution. X-ray photoreduction of redox groups has been previously reported to affect the flexibility and conformational state of multi-domain proteins [27,28]. Since cold neutrons (≤ 25 meV) do not induce photoreduction in biological samples, SANS measurements were initially performed on *Nc*CDHIIA to ensure that only the fully oxidized state of *Nc*CDHIIA was measured. The dimensions obtained from SANS measurements were then compared to dimensions derived from laboratory-source SAXS measurements (Supplementary Information Fig. S2). SANS and laboratory-source SAXS measurements were consistent thus supporting the use of both radiation sources to complete the study (Supplementary Information Table S1).

2.1. Effect of pH variation on NcCDHIIA and MtCDHIIA

The SANS and SAXS data from *Nc*CDHIIA and *Mt*CDHIIA, respectively, plotted on a log(*I*) *vs* log(*Q*) scale are shown in Supplementary Information Figs. S3 and S4. Both CDHs were measured at protein