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Structural insight into the calcium ion modulated interdomain electron transfer in cellobiose dehydrogenase

Alan Kadek [a,b], Daniel Kavan [a,b], Alfons K.G. Felice [c], Roland Ludwig [c], Petr Halada [a], Petr Man [a,b,*]

[a] Institute of Microbiology, The Czech Academy of Sciences, Videnska 1083, Prague, Czech Republic
[b] Department of Biochemistry, Faculty of Science, Charles University in Prague, Hlavova 8, Prague, Czech Republic
[c] Department of Food Sciences and Technology, Food Biotechnology Laboratory, BOKU – University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria

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ABSTRACT

Cellobiose dehydrogenase (CDH) from wood degrading fungi represents a subclass of oxidoreductases with unique properties. Consisting of two domains exhibiting interdomain electron transfer, this is the only known flavocytochrome involved in wood degradation. High resolution structures of the separated domains were solved, but the overall architecture of the intact protein and the exact interface of the two domains is unknown. Recently, it was shown that divalent cations modulate the activity of CDH and its pH optimum and a possible mechanism involving bridging of negative charges by calcium ions was proposed. Here we provide a structural explanation of this phenomenon confirming the interaction between negatively charged surface patches and calcium ions at the domain interface.

1. Introduction

Cellobiose dehydrogenase (EC 1.1.99.18, CDH) is an intriguing oxidoreductase produced and secreted by several wood degrading and phytopathogenic fungi. CDH is composed of two domains which are covalently linked by a flexible linker. At the C-terminus a flavin adenin dinucleotide (FAD) bearing dehydrogenase domain (DH) performs the oxidation of carbohydrates, e.g. cellobiose or cello-oligosaccharides. The electrons obtained during this reaction are stored on the FAD and can be either transferred to soluble electron acceptors or by interdomain electron transfer (IET) to the second domain of the enzyme – a heme *b* containing cytochrome domain (CYT). CYT can further transport electrons to cytochrome *c* (cyt *c*, an artificial substrate) or reduce lytic polysaccharide monooxygenase (LPMO, the proposed natural substrate) which in turn depolymerizes cellulose [1].

Despite the high interest in CDH, the exact structure and organization of the whole protein remains elusive. High-resolution structure of individual domains of CDH from *Phanerochaete chrysosporium* were already solved a decade ago [2,3] and based on them a possible assembly of the full length protein and the mechanisms underlying IET were drawn [4]. In this model the two domains face each other in a way which allows contact between the DH domain and the CYT domain. The crystal structure of the isolated CYT domain showed that the heme *b* propionates are facing outwards and thus are available for a close contact with FAD in the DH domain. In such orientation, the interacting surfaces are complementary and the buried surface area between the domains is rather large [4]. However, it is also known, that the IET in CDHs is pH dependent and that the electron transfer is blocked at a pH above 6 for most CDHs. Based on these facts the generally accepted view of the domain interaction is such, that at higher pH, the surfaces of both domains are negatively charged due to the deprotonation of amino acid side chains, which in turn causes

Abbreviations: CDH, cellobiose dehydrogenase; DH, dehydrogenase domain; CYT, cytochrome domain; HDX-MS, hydrogen/deuterium exchange coupled to mass spectrometry; IET, interdomain electron transfer; TCEP, tris(2-carboxyethyl)phosphine; EDTA, ethylenediaminetetraacetic acid

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* Corresponding author at: Institute of Microbiology, Videnska 1083, Prague 4, 142 20, Czech Republic.
E-mail address: pman@biomed.cas.cz (P. Man).

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electrostatic repulsion and results in the separation of the DH and CYT domain. As pH decreases, amino acid side chains become protonated and this renders the surfaces neutral. In this situation the domains can get into a close contact and IET may occur. Recently, it was found that divalent cations influence this pH dependent behavior by enhancing the enzymatic activity of CDH [5]. Interestingly, the effect of calcium differed between individual cellobiose dehydrogenases from various sources which pointed on a relation to the individual protein sequence/structure rather than to the binding of calcium by a specific site in CDH structure. A recent study explored this phenomenon in detail and screened CDHs from twelve different fungi [6]. It was shown, that any divalent alkali metal cation (regardless its atomic radius or electronegativity) at concentrations above 3 mM increases the IET of most cellobiose dehydrogenases. The divalent cation-dependent IET enhancing effect can be seen at pH 5.5 and 7.5, but is more pronounced at pH 7.5, with the most striking increase in activity observed for cellobiose dehydrogenase from *Myriococcum thermophilum* (*Mt*CDH). In addition to these observations, monovalent cations and anions were shown to have no impact, which rules out the effect of ionic strength alone. The lack of divalent cation selectivity together with the requirement for their rather high concentration (mM) and with the differences between the levels of high pH IET activation among individual CDHs pointed again on the possible elimination of negative charges by calcium cations *via* the cation bridging effect. This hypothesis was further explored by molecular modeling and domain docking and suggested an explanation by highlighting much higher number of possible divalent cation interacting residues in *Mt*CDH in contrast to other CDHs from *Phanerochaete sordida* or *Corynascus thermophilus* [6].

Hydrogen/deuterium exchange coupled to mass spectrometry (HDX-MS) is nowadays a well-established technique for fast and straightforward monitoring of protein dynamics and protein interactions [7]. It has virtually no limitation in terms of size or flexibility of the studied proteins and thus even quite complex and dynamic systems can be investigated [8–10]. Based on the measured time-resolved kinetics of backbone amide hydrogen exchange for two or more states of the protein we can identify the regions of a protein that are influenced by e.g. ligand binding. Here we used HDX-MS to provide structurally localized answer to the question: how calcium ions bind to the *Mt*CDH?

2. Materials and Methods

2.1. Materials

All chemicals were from Sigma–Aldrich unless otherwise stated. Endoglycosidase Endo Hf (1.000.000 U/mL) was purchased from New England Biolabs. The immobilization of porcine pepsin A followed the procedure described previously [11].

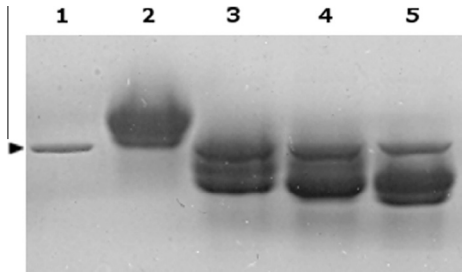

Fig. 1. Deglycosylation of *Mt*CDH by Endo Hf prior to the HDX-MS. 1 – Endo Hf; 2 – native *Mt*CDH; 3 – 5 *Mt*CDH – deglycosylation of *Mt*CDH by Endo Hf after 1 h, 4 h and overnight treatment. Position of Endo Hf is indicated by an arrowhead.

2.2. Protein preparation

Recombinant full length cellobiose dehydrogenase from *M. thermophilum* (Uniprot accession number A9XK88) was expressed in *Pichia pastoris* and purified as described previously [12]. Prior to the analyses, *Mt*CDH was deglycosylated overnight by Endo Hf under non-denaturing conditions (15 U Endo Hf/1 μg *Mt*CDH, at 37 °C in 50 mM sodium acetate buffer pH 5.5).

2.3. Hydrogen/deuterium exchange

Deglycosylated *Mt*CDH was pre-incubated for 30 min in an H_2O-based 50 mM 4-morpholinepropanesulfonic acid (MOPS) buffer pH 7.4, alone or in the presence of the studied ions. The buffers contained either 30 mM $CaCl_2$, 90 mM KCl or 9 mM EDTA-Na_2 (disodium ethylenediaminetetraacetate) to reach identical ionic strength under all the added ion conditions tested. The deuterium labeling was initiated by a 10-fold dilution of the protein into a deuterated buffer (50 mM MOPS, pD 7.4) alone or including the ions. The final *Mt*CDH concentration during the labeling was 5 μM. The exchange was left to proceed at 21 °C and aliquots (50 μL) were removed after 0.33, 1, 3, 10, 30, 60, 180 and 300 min. In these aliquots the exchange was quenched by the addition of 50 μL of a buffer containing 6 M guanidine, 0.9 M tris-(2-carboxyethyl)phosphine (TCEP) and 1 M glycine pH 2.4. The quenched mixture was incubated for 10 min on ice before being rapidly frozen in liquid nitrogen.

2.4. Digestion and liquid chromatography

Each sample was quickly thawed and injected onto an immobilized pepsin column (bed volume 66 μL). Digestion was driven by a flow of 0.4% formic acid in water at a flow rate of 100 μL/min (LC-20AD pump, Shimadzu). The resulting peptides were trapped and desalted online on a peptide microtrap (Michrom Bioresources). After a desalting step (4 min), the peptides were eluted onto a Jupiter C18 analytical column (0.5 × 5 mm, 5 μm, 300 Å, Phenomenex) and separated by a linear gradient of 10–35% B in 12 min, followed by a quick jump to 99% B, where A was 0.2% formic acid/2% acetonitrile in water and B was 95% acetonitrile/0.2% formic acid in water. The solvent was delivered at a constant flow rate of 15 μL/min (Agilent Technologies 1200). For peptide mapping of non-deuterated samples the same conditions were used. All the valves, capillaries as well as protease, desalting and analytical columns were kept at 0 °C to minimize the deuterium back-exchange.

2.5. Mass spectrometry and data analysis

The outlet of the LC system was interfaced to an electrospray ionization source of a Fourier transform ion cyclotron resonance mass spectrometer (9.4 T Apex-Qe, Bruker Daltonics). For peptide mapping (LC–MS/MS) the instrument was operated in data-dependent mode, where each MS scan was followed by up to six MS/MS collision-induced fragmentations of the most intense ions. Data were searched using MASCOT against a single protein database containing the sequence of *Mt*CDH. Identified peptides were plotted using the DrawMap script (MSTools) [13].

To determine the amount of deuterium incorporated into the peptides after the HDX, the instrument was operated in an LC–MS mode and the acquired data were processed using an in-house developed program DeutEx. The deuterium content of each peptide was reported as a percentage of maximal achievable deuteration based on the number of exchangeable amide hydrogens in each peptide.