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Original article

Crystal structures of the disease-causing D444V mutant and the relevant wild type human dihydrolipoamide dehydrogenase

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ABSTRACT

We report the crystal structures of the human (dihydro)lipoamide dehydrogenase (hLADH, hE3) and its disease-causing homodimer interface mutant D444V-hE3 at 2.27 and 1.84 Å resolution, respectively. The wild type structure is a unique uncomplexed, unliganded hE3 structure with the true canonical sequence. Based on the structural information a novel molecular pathomechanism is proposed for the impaired catalytic activity and enhanced capacity for reactive oxygen species generation of the pathogenic mutant. The mechanistic model involves a previously much ignored solvent accessible channel leading to the active site that might be perturbed also by other disease-causing homodimer interface substitutions of this enzyme.

1. Introduction

The human (h) dihydrolipoamide dehydrogenase (EC 1.8.1.4, LADH, E3) is a flavin-disulfide oxido-reductase that catalyzes the re-oxidation of the covalently bound reduced lipoic acid (LA) cofactor of the E2 subunits of all the mitochondrial α-keto acid dehydrogenase multienzyme complexes including the α-ketoglutarate dehydrogenase complex (hKGDHc), the pyruvate dehydrogenase complex (hPDHc), and the branched-chain α-keto acid dehydrogenase complex (hBCKDHc) [1–4]. hE3 is also part of the glycine cleavage system (GCS) and displays moonlighting functions as a liberated enzyme [5–11]. hE3 is an obligate homodimer formed from ~50 kDa monomers, which binds non-covalently a FAD prosthetic group and contains a redox-active disulfide (Cys45-Cys50, in the mature protein chain of 474 residues) vicinal to the *si* face of the isoalloxazine ring of the FAD in the catalytic center [12]. The LADH reaction is reversible and governed by a ping-pong-type mechanism [1–4]. In the physiological (*forward*) direction, first a reductive half-reaction gives rise to the two-electron reduced enzyme (EH_2) *via* a mixed disulfide intermediate forming between the dihyrolipoate (DHLA) moiety of E2 and Cys45. EH_2 involves three species: a FAD-dithiol (I), a $FADH_2$-disulfide (II), and the predominant FAD-thiolate (Cys50) charge-transfer complex (III); stable flavin radicals are not involved in this mechanism. This part of the reaction is acid-base catalyzed *via* His452' (' for the adjacent monomer), which is positionally oriented towards Cys45 and the entry site of DHLA by Pro453' and Glu457'; His452' promotes proton shuffling between the interchange thiol (Cys45) and DHLA, which is essential for disulfide opening. hE3 undergoes regeneration by NAD^+, which binds to its electron-transfer site on the *re* face of the isoalloxazine ring of the FAD, in the oxidative half-reaction yielding $NADH + H^+$. This second half-reaction involves a transiently formed covalent FAD-C4a-cysteinyl (Cys50) adduct and a [NAD^+-$FADH_2$] intermediate. Under strongly reducing conditions E3 can enter a four-electron reduced state (EH_4, FAD and the redox-active Cys residues are all fully reduced), which is catalytically inactive in the LADH reaction (implicated in the product inhibition of E3 by NADH), but not in the diaphorase and reactive oxygen species (ROS; superoxide and H_2O_2) generating reactions of E3 in the *reverse* catalytic direction (see below) [11]. The EH_4 state is

Abbreviations: h, human; E3, (dihydro)lipoamide dehydrogenase, the common E3 component of the mitochondrial 2-oxo acid dehydrogenase complexes; LADH, (dihydro)lipoamide dehydrogenase; KGDHc, alpha-ketoglutarate (also known as 2-oxoglutarate) dehydrogenase complex; PDHc, pyruvate dehydrogenase complex; BCKDHc, branched-chain α-keto acid dehydrogenase complex; GCS, glycine cleavage system; E3BP, E3-binding protein; SBDb, subunit-binding domain of the E2 subunit in hBCKDHc; ROS, reactive oxygen species (superoxide anion and hydrogen peroxide); LA, lipoic acid; FAD, flavin adenine dinucleotide; NAD^+/NADH, nicotinamide adenine dinucleotide (oxidized/reduced); ASU, asymmetric unit; RMSD, root mean square deviation; wt, wild type; HDX-MS, hydrogen-deuterium-exchange mass spectrometry; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; Bis-Tris, 2-[bis(2-hydroxyethyl)amino] − 2-hydroxymethyl-propane-1,3-diol

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destabilized by NAD^+, yielding LADH-active EH_2, pointing to the physiological role of the NADH/NAD^+ ratio in the regulation of the metabolic efflux and oxidative stress generated by the hE3-harboring multienzyme complexes [8,9,13–22]. ROS generation *via* hE3, principally by hKGDHc, significantly contributes to mitochondrial oxidative stress [15–21].

Pathogenic variants of the hE3 affect several central metabolic pathways simultaneously and result in a clinically severe, often lethal human disease known as E3-deficiency [11]. 14 disease-causing single amino acid substitutions of the mature hE3 have been reported to date leading primarily to neurological, cardiological, and hepatological manifestations. The homozygous state of D444V-hE3 presents with microcephaly, blindness, deafness, mild hypertrophic cardiomyopathy, and metabolic acidosis [23]. The D444V substitution led to compromised LADH activity (15% of the control, beside 10% E3 immunoreactivity, 2% hKGDHc, and 0% hPDHc activities in patient muscle homogenate [23], whereas 35% [22], 80% [10], 100% [24], and 90% [7], under different assay conditions, using recombinant protein). Recombinant, reconstituted D444V-hE3-hPDHc exhibited 12% PDHc activity as compared to the control [24]. The respective mutation significantly stimulated superoxide and H_2O_2 generation by isolated hE3 in the reverse (pathologically more relevant) catalytic direction (by 31% and 152%, respectively); superoxide was the primary ROS generated in this reaction (measured *via* cytochrome c reduction), while H_2O_2 was the spontaneous dismutation product of superoxide (measured *via* Amplex Red fluorescence) [22]. The D444V substitution also augmented the sensitivity of the ROS-generating reverse hE3 reaction to acidic pH shifts *in vitro* [22]. As a result of its enhanced prooxidant status, D444V-hE3 triggered the deterioration of the LA-cofactor of both hPDHc-E2 and hKGDHc-E2 in homozygous patient fibroblasts exposed to oxidative stress contributing to compromised hPDHc and hKGDHc activities [10].

The only experimental structure analysis of the pathogenic hE3 mutants thus far was a hydrogen-deuterium-exchange mass spectrometry (HDX-MS) study performed on ten mutants; seven peptides with altered flexibility/accessibility were identified for D444V-hE3. Three of these peptides are associated with cofactor binding and hence were implicated in the impaired LADH activity and a catalytic advantage for superoxide generation. Another two peptides are involved in subcomplex formation within the multienzyme complexes that points to the greatly compromised hPDHc and hKGDHc activities measured upon the D444V substitution. Significant alteration of the C-terminus could also be observed, while no evidence supported the monomerization of the dimer [25].

A number of E3 crystal structures from various species, including hE3 structures [12,24,26,27], were reported (as reviewed before [28]). Here we determined a unique structure of the hE3, which is of the true canonical sequence and contains no ligands or other proteins. We also report here the first high-resolution structure of a disease-causing hE3 mutant (D444V-hE3). Based on the analysis of the structures and our prior knowledge on the D444V-hE3 variant, a novel molecular pathomechanism is proposed for the compromised catalytic activity and enhanced capacity for ROS generation of this pathogenic mutant. The severely impaired overall multienzyme complex activities measured *in vitro* and *in vivo* upon the D444V substitution can also be explained *via* the revealed structural information.

2. Materials and methods

2.1. Chemicals

All chemicals and accessories were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

2.2. Protein expression and purification

The hE3 and D444V-hE3 proteins were expressed in *E. coli,* with an eight amino acid long Strep-tag fused to their N-termini, and purified to homogeneity, as previously described [25,29,30]; mass spectrometry verified the two protein sequences [22].

2.3. Protein crystallization

Purified hE3 and D444V-hE3 protein samples were buffer-exchanged into 100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0 (prepared from molecular biology grade chemicals and filtered at 0.22 μm) before crystallization.

hE3 crystals were grown at 20 °C using the sitting drop method in 24-well crystallization plates from Hampton Research (Aliso Viejo, CA, USA). The droplets comprised 1 μL protein solution and 1 μL reservoir solution (0.1 M Bis-Tris pH 7.45, 0.2 M $MgCl_2$, 25 w/v% PEG 3350; all from Hampton Research). For cryoprotection 100% Paratone N (Hampton Research) was applied.

D444V-hE3 crystals were grown under identical experimental conditions, but in 96-well plates purchased from Greiner Bio-One (Frickenhausen, Germany) using 0.3 μL protein solution and 0.15 μL reservoir solution (1.6 M NaH_2PO_4/K_2HPO_4 pH 8.1; 2.5 M K_2HPO_4 (Merck, Darmstadt, Germany) was titrated with 2.5 M NaH_2PO_4 (Merck) to pH 8.1 and diluted). For cryoprotection 1.8 M NaH_2PO_4/K_2HPO_4 pH 7.5 (prepared as above), 20 v/v% glycerol (Merck) was used.

All crystals were flash frozen in $N_{2(l)}$ prior to data collection.

2.4. Structure determination

Diffraction data were collected on the BL14.1 beamline operated by the Helmholtz-Zentrum Berlin (HZB) at the BESSY II electron storage ring (Berlin-Adlershof, Germany) [31]. Diffraction data were processed using XDS [32] as implemented in the XDSAPP graphical interface [33,34]. In case of hE3, for adequate completeness, two datasets of the same crystal (in its two orientations) were merged with XSCALE/XDS. Both structures were determined by molecular replacement using the Molrep program [35] inside the CCP4 software suite [36]; the search model in both cases was a previously determined structure of the hE3 [12] (PDB accession code: 1ZMD). Eight monomers were found in the asymmetric unit (ASU) in the 1ZMD structure and one of them (chain A) was used for the purposes of the present work. All atoms of the cofactors and water molecules were removed from the model before molecular replacement. Structure refinements were carried out in repeated cycles using the programs phenix.refine (under the Phenix suite [37]) and REFMAC5 [38] (under CCP4) for the hE3 and D444V-hE3 structures, respectively, with automatically assigned TLS groups. Coot [39] was used for real space refinement. The refined models and structure factor files have been deposited to the Protein Data Bank under the accession codes 5NHG and 5J5Z for the hE3 and D444V-hE3, respectively.

2.5. Structure analysis

Visualization of structures was carried out by PyMol. The presence of the V444 residue in the mutant structure was verified *via* an omit 2mFo-DFc electron density map around the mutation site calculated in Phenix (Fig. S1). For root-mean-square deviation (RMSD) calculations (*e.g.* in Fig. S2) the McLachlan algorithm [40] was applied as implemented in the program ProFit [41]. For structure mapping analysis [42,43], specific interatomic distances of functional relevance were compared in the hE3 and the D444V-hE3 structures. Characterization of the dimer interface was performed using the PDBePISA server [44].

Analysis of the channels in the hE3 and D444V-hE3 structures was carried out using the CAVER 3.0 PyMol plugin [45]. The LA-binding