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Structural alterations induced by ten disease-causing mutations of human dihydrolipoamide dehydrogenase analyzed by hydrogen/deuterium-exchange mass spectrometry: Implications for the structural basis of E3 deficiency

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ABSTRACT

Pathogenic amino acid substitutions of the common E3 component (hE3) of the human alpha-ketoglutarate dehydrogenase and the pyruvate dehydrogenase complexes lead to severe metabolic diseases (E3 deficiency), which usually manifest themselves in cardiological and/or neurological symptoms and often cause premature death. To date, 14 disease-causing amino acid substitutions of the hE3 component have been reported in the clinical literature. None of the pathogenic protein variants has lent itself to high-resolution structure elucidation by X-ray or NMR. Hence, the structural alterations of the hE3 protein caused by the disease-causing mutations and leading to dysfunction, including the enhanced generation of reactive oxygen species by selected disease-causing variants, could only be speculated. Here we report results of an examination of the effects on the protein structure of ten pathogenic mutations of hE3 using hydrogen/deuterium-exchange mass spectrometry (HDX-MS), a new and state-of-the-art approach of solution structure elucidation. On the basis of the results, putative structural and mechanistic conclusions were drawn regarding the molecular pathogenesis of each disease-causing hE3 mutation addressed in this study.

1. Introduction

Human dihydrolipoamide dehydrogenase (hLADH, hE3) is the common third component enzyme in the mitochondrial 2-oxo acid dehydrogenase complex family comprising the pyruvate dehydrogenase complex (PDHc), the α-ketoglutarate (or 2-oxoglutarate) dehydrogenase complex (KGDHc or OGDHc), and the branched-chain α-keto acid dehydrogenase complex (BCKDHc); hE3 is also part of the glycine cleavage system [1–6]. The hE3 is an obligate homodimer of 474 amino acids in each monomer after cleavage of the mitochondrial signal peptide (35 amino acids); each monomer binds tightly, but non-covalently, a single FAD prosthetic group [7]. The overall stoichiometries (the numbers of E1, E2 and E3 subunits) of the above multienzyme complexes harboring hE3 are generally different and may also vary in different species owing to the specific reaction and the biochemical environment (*e.g.*, substrate provision) [1,3,4,8–12]; optimal stoichiometries of the KGDHc and the PDHc from human and *E. coli* origins were determined recently by us *via* reconstitution experiments using recombinant components [6]. The KGDHc catalyzes a rate-limiting step in the Krebs cycle facilitating the oxidative decarboxylation of α-ketoglutarate and generating succinyl-CoA and NADH, whereas PDHc, with a similar mechanism, converts pyruvate to acetyl-CoA and NADH [13]. The function of the common E3 component in the catalytic mechanism is to reoxidize the reduced dihydrolipoic acid moieties, covalently linked to the respective E2 components,

Abbreviations: KGDHc, alpha-ketoglutarate (also known as 2-oxoglutarate) dehydrogenase complex; PDHc, pyruvate dehydrogenase complex; BCKDHc, branched-chain α-keto acid dehydrogenase complex; E3, dihydrolipoamide dehydrogenase, the E3 component of the 2-oxo acid dehydrogenase complexes shared by all complexes in a particular cell; HDX-MS, hydrogen/deuterium-exchange mass spectrometry; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; NAD^+/NADH, nicotinamide adenine dinucleotide (oxidized/reduced forms); ROS, the reactive oxygen species superoxide anion and hydrogen peroxide; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; wt, wild-type; h, human origin; aa, amino acid; Amp, ampicillin; LB medium, Luria-Bertani medium; IPTG, isopropyl β-D-1-thiogalactopyranoside; NMR, nuclear magnetic resonance.

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while generating NADH. It has been demonstrated that in addition to their physiological catalytic activities, KGDHc and PDHc can generate significant amounts of the reactive oxygen species (ROS) superoxide and, its dismutation product, H_2O_2 under pathologically relevant conditions [6,14–17]. ROS produced by the KGDHc was proved to be a major source of oxidative stress inside the mitochondrion [14,15,18,19] which, together with the impaired activity of the KGDHc under certain pathological conditions, is heavily implicated in the progression of senescence/aging, neurodegenerative diseases (such as Alzheimer's and Parkinson's disease), ischemia-reperfusion, hypoxia- and glutamate-induced cerebral damage, infantile lactic acidosis, Friedreich's ataxia, various atypical E3-deficiencies, among others [20–29]. The ROS-generating activity of the intact KGDHc (and the PDHc) had been attributed to the E3 component [6,14–17]. However, both the isolated hE1 [6, 30] and the hE1-hE2 sub-complexes of hKGDHc [6] could also generate significant amounts of ROS; a feature which is unique to the human complex, and may become a predominant pathomechanism regarding ROS production in acidosis (where a partial detachment of E3 from KGDHc was proposed [16]), or in E2 deficiency [6,30]. The hKGDHc, hPDHc, as well as the isolated hE3 component can consume NADH in the reverse E3 reaction while also generating superoxide and H_2O_2 from O_2; this reaction dominates under elevated NADH/NAD^+ ratios (also a pathologically relevant condition) [6,15–17].

Pathogenic mutations of the *dld* gene (coding for hE3) lead to an inherited, often lethal disease known as E3-deficiency; the clinical course of E3-deficiency is greatly diversified and often involves cardiological and/or neurological symptoms [25,31–42]. The following phenotypes, among many others, for hE3 pathogenic mutations commonly appear in clinical reports: failure to thrive, developmental delay, encephalopathy, hypotonia, seizure, hepatomegaly, liver dysfunction, lactic acidosis, hypoglycemia, microcephaly, and ataxia. The effects of several of the total of 14 clinically reported disease-causing amino acid substitutions of the hE3 component on various biochemical and biophysical parameters, including ROS-generating capacity, of the enzyme have previously been investigated by us and others. Among other findings, it was reported that four amino acid substitutions resulting from the respective pathogenic mutations, P453L, G194C, D444V, and E340K, significantly stimulated ROS generation by the isolated hE3 (and also by hKGDHc reconstituted with G194C-hE3 [6]) in the reverse catalytic direction; in all four cases, the sensitivity of ROS generation by hE3 to a more acidic pH was also augmented relative to the wild-type (wt) enzyme [17]. The E340K, D444V, and G194C substitutions of hE3 all triggered the oxidative deterioration of the lipoic acid (LA) cofactors of both PDHc-E2 and KGDHc-E2 in a yeast model and led to a great reduction in the respiratory function of the yeast cells [43]. Since the decreases in hE3 activity resulting from the pathogenic mutations do not, in general, correlate directly in magnitude to the severity of the respective pathogeneses [25,35], the group at Semmelweis proposed that a missing link in the interpretation of selected pathogeneses is perhaps the enhanced ROS generation stimulated by the respective pathogenic mutations of hE3 [17]. To date, no report has been published regarding the high-resolution structure of any of the pathogenic mutant forms of hE3 determined by a direct structural method such as x-ray crystallography or NMR spectroscopy. Hence, the pathomechanisms of action, including that of the enhanced ROS generation by selected variants, could only be speculated based on the crystal structures of the hE3 [7,44,45], molecular modeling [46,47] and biochemical/biophysical data (*e.g.* [17,43,48,49]).

Hydrogen/deuterium-exchange mass spectrometry (HDX-MS) is a powerful methodology for structural investigation of biomacromolecules in solution, which nevertheless presents considerable methodological and technical challenges (primarily regarding the minimization of the "back exchange" of deuterium (^2H) to protium (^1H) on peptic peptides during analysis). The Rutgers laboratory is equipped with an efficiently performing HDX-MS instrument for this purpose, which has already been successfully used to investigate principally protein-protein interactions in related projects [50–53].

Here we report the results of an HDX-MS investigation performed on ten disease-causing hE3 variants to establish the structural alterations induced by the respective pathogenic substitutions relative to the wild-type protein structure. Based on our findings with the HDX-MS approach, we present plausible mechanisms for enzymatic dysfunction in case of the 10 disease-causing hE3 variants studied.

2. Results

In this study, deuterium incorporation of peptic peptides of hE3 was compared to those of the hE3 disease-causing variants at a peptide resolution to detect conformational perturbations induced by the implicated pathogenic amino acid substitutions; experiments for deuterium incorporation were carried out in solution under physiologically relevant buffer conditions at 25 °C.

Recombinant wild-type and variant proteins for this study were expressed in *E. coli* and purified to homogeneity; the purity of all proteins was >95%, similarly to those reported from the Semmelweis group in previous studies on these hE3 variants [17,54]. There are fourteen pathogenic mutations of hE3 documented in clinical reports to date which are associated with a single amino acid substitution in the mature hE3 monomer (and do not involve exon skipping or a frame shift); pathogenic mutations of the *dld* gene occur frequently in a compound heterozygous state [25,41]. Our protein samples mimic the homozygous forms of the mutations owing to the characteristics of standard heterologous recombinant protein expression protocols. We were able to generate protein samples with adequate concentrations for the HDX-experiment for ten of the above mutations (see below); soluble expression of the remaining four variants (G101del, I12T, M326V, G426E) using all applicable codon synonyms, including the most favorable ones for *E. coli* expression, for each newly incorporated amino acid was also attempted, but proved to be quite inefficient, likely due to structural instability and/or insufficient folding.

It was demonstrated in [7] that each monomer of the catalytically active functional (obligate) hE3 homodimer comprises four domains: one for FAD-binding (residues 1–149), one for NAD^+/NADH-binding (residues 150–282), one referred to as the central domain (residues 283–350), and one referred to as the interface domain (residues 351–474). A single FAD prosthetic group binds non-covalently to each monomer, and each monomer contributes amino acids to the active site of the partner monomer [7]. Of the ten successfully purified hE3 variants, the K37E substitution affects the FAD-binding domain, the G194(C) residue is located in the NAD^+/NADH-binding domain, the E340(K) and the I318(T) residues reside in the central domain, and the remaining substitutions (I445M, R460G, R447G, P453L, D444V, I358T) are all associated with the interface domain.

In this differential HDX-MS investigation, 33 peptic peptides from hE3 were selected for evaluation, constituting an overall sequence coverage of 95% (Fig. S1). Additional peptides originating from the pathogenic hE3 variants were also used for evaluation, as listed in Table S1; these peptides usually contained single amino acid substitutions relative to the above mentioned 33 original peptides. The deuterium incorporation pattern of hE3 is shown in Fig. 1. It is apparent that the regions 81–85 (purple in Fig. 2), 275–289 (light brown in Fig. 2), and 339–351 (red in Fig. 2) display enhanced deuterium incorporation and hence can be stated to be flexible or exposed regions, while dimerization buries and stabilizes the interface domain, in particular fragments 434–441 (dark blue in Fig. 2, with not exclusive coloring) and 459–464 (black in Fig. 2). In fact, the entire interface domain, except the C-terminus, is rather unexposed and rigid in general, as expected. It is also noted that the interior of the central domain is also rather unexposed and rigid and possesses a conserved structure for hE3 and each of its variants studied here (see below and Fig. 3).

The differential deuterium exchange profiles of the ten pathogenic hE3 variants relative to the deuterium exchange profile of the hE3 are shown in Fig. 3; numerical data are displayed in Table S2. As seen, the