Human dihydrolipoamide dehydrogenase (E3) deficiency: novel insights into the structural basis and molecular pathomechanism

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Abstract

This review summarizes our present view on the molecular pathogenesis of human (h) E3-deficiency caused by a variety of genetic alterations with a special emphasis on the moonlighting biochemical phenomena related to the affected (dihydro)lipoamide dehydrogenase (LADH, E3, gene: *dld*), in particular the generation of reactive oxygen species (ROS). E3-deficiency is a rare autosomal recessive genetic disorder frequently presenting with a neonatal onset and premature death; the highest carrier rate of a single pathogenic *dld* mutation (1:94-1:110) was found among Ashkenazi Jews. Patients usually die during acute episodes that generally involve severe metabolic decompensation and lactic acidosis leading to neurological, cardiological, and/or hepatological manifestations. The disease owes its severity to the fact that LADH is the common E3 subunit of the alpha-ketoglutarate (KGDHc), pyruvate (PDHc), and branched-chain α-keto acid dehydrogenase complexes and is also part of the glycine cleavage system, hence the malfunctioning of LADH simultaneously incapacitates several central metabolic pathways. Nevertheless, the clinical pictures are usually not unequivocally portrayed through the loss of LADH activities and imply auxiliary mechanisms that exacerbate the symptoms and outcomes of this disorder. Enhanced ROS generation by disease-causing hE3 variants as well as by the E1-E2 subcomplex of the hKGDHc likely contributes to selected pathogeneses of E3-deficiency, which could be targeted by specific drugs or antioxidants; lipoic acid was demonstrated to be a potent inhibitor of ROS generation by hE3 *in vitro*. Flavin supplementation might prove to be beneficial for those mutations triggering FAD loss in the hE3 component. Selected pathogenic hE3 variants lose their affinity for the E2 component of the hPDHc, a mechanism which warrants scrutiny also for other E3-haboring complexes.

Keywords:

E3-deficiency; dihydrolipoamide dehydrogenase; alpha-ketoglutarate dehydrogenase complex; pyruvate dehydrogenase complex; pathogenic mutation

Abbreviations

KGDHc, alpha-ketoglutarate (also known as 2-oxoglutarate) dehydrogenase complex; KG, alpha-ketoglutarate; PDHc, pyruvate dehydrogenase complex; BCKDHc, branched-chain α-keto acid dehydrogenase complex; GCS, glycine cleavage system; OADH(c), 2-oxo acid dehydrogenase (complexes); E3, dihydrolipoamide dehydrogenase, the common E3 component of the 2-oxo acid dehydrogenase complexes; LADH, (dihydro)lipoamide dehydrogenase; LA, lipoic acid; DHLA, dihydrolipoic acid; DCPIP, 2,6-dichlorophenolindophenol; MSUD, maple syrup urine disease; HDX-MS, hydrogen/deuterium-exchange mass spectrometry; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; NAD^+/NADH, nicotinamide adenine dinucleotide (oxidized/reduced forms); ROS, reactive oxygen species (superoxide anion and hydrogen peroxide); wt, wild-type; h, human origin; NMR, nuclear magnetic resonance; HRP, horseradish peroxidase; LC, liquid chromatography; MD, molecular dynamics; ThDP, thiamin diphosphate.

Highlights

- Dihydrolipoamide dehydrogenase (E3) deficiency is a recessive genetic disorder.
- Metabolic decompensation leads primarily to neurological symptoms and often death.
- ROS generation triggered by *dld* mutations likely exacerbates the symptoms.
- Selected E3 variants lose their affinity for FAD or other multienzyme components.
- Specific drugs, antioxidants, and flavins could be proposed for therapy.

1. Introduction

Dihydrolipoamide dehydrogenase (LADH, E3; gene: *dld*) deficiency is a rare autosomal recessive genetic disorder (Quinonez and Thoene, 2014). The highest carrier rate for a pathogenic mutation of the *dld* gene is found in the Ashkenazi Jewish population (1:94-1:110, in case of the G194C-E3 pathogenic variant) with a disease frequency of 1:35,000-1:48,000 (Scott et al., 2010; Shaag et al., 1999). In this review we use an amino acid numbering scheme that is based on the 474 residues of the mature enzyme lacking the mitochondrial leader sequence of 35 residues (Brautigam et al., 2005). This disorder affects primarily tissues with high oxygen expenditure and hence involves mainly neurological and cardiological manifestations, but a hepatological involvement is also frequently diagnosed in the disease (Quinonez and Thoene, 2014). The phenotypic spectrum includes failure to thrive, developmental delay, encephalopathy, hypotonia, seizure, hepatomegaly, liver dysfunction, lactate acidosis, hypoglycemia, Leigh syndrome, hypertrophic cardiomyopathy, hyperammonemia, vision impairment/optic atrophy, microcephaly, and ataxia, among others (Cameron et al., 2006; Carrozzo et al., 2014; Cerna et al., 2001; Grafakou et al., 2003; Hong et al., 1996; Hong et al., 1997; Hong et al., 2003; Liu et al., 1993; Odievre et al., 2005; Quinonez et al., 2013; Quinonez and Thoene, 2014; Quintana et al., 2010; Shaag et al., 1999; Shany et al., 1999). The symptoms may arise early in life (early-onset; even in the neonatal period), but in selected probands the disease manifests itself only in adulthood. The early-onset form of the disease appears generally with hypotonia and lactate acidosis; the patients often die at a very young age during their first or recurrent metabolic decompensation, whereas those who survive this initial period usually remain developmentally retarded with neurological deficits (intellectual impairment, ataxia, seizures, spasticity) (Quinonez and Thoene, 2014). Hepatological consequences may present in isolation and even decades later (Barak et al., 1998; Brassier et al., 2013); this is often associated with encephalopathy and/or

coagulopathy and can be lethal even when the liver failure arises in adulthood (Elpeleg et al., 1997; Shaag et al., 1999).

In this review we summarize the current knowledge on the pathomechanism of the compromised LADH activity and also on the auxiliary molecular mechanisms that could exacerbate the symptoms and outcomes of E3 deficiency. Out of the moonlighting biochemical phenomena reported for LADH we focus on the generation of reactive oxygen species.

2. Characteristics of E3-deficiency

Diagnosis of the disease is made on the basis of relevant symptoms and the biochemical evidence for the defect of either or all of the E3-harboring complexes (the alpha-ketoglutarate (also known as 2-oxoglutarate) dehydrogenase complex (KGDHc (or OGDHc)), the pyruvate dehydrogenase complex (PDHc), and the branched-chain α-keto acid dehydrogenase complex (BCKDHc) (Ambrus et al., 2015b; Hansford, 1980; Koike et al., 1974; Massey, 1960a; Perham, 1991; Pettit and Reed, 1967; Reed, 1974; Reed and Oliver, 1968; Sheu and Blass, 1999)); interestingly, the glycine cleavage system (GCS) (Kikuchi and Hiraga, 1982), which also contains the LADH protein, appears to remain unaffected in patients suffering from E3-deficiency (Quinonez and Thoene, 2014). Due to the presence of LADH in BCKDHc, E3-deficiency is also referred to as maple syrup urine disease (MSUD; also called branched-chain ketoaciduria) type 3. Impaired activities of BCKDHc, PDHc, and KGDHc may result in lactate acidosis, elevated levels of α–ketoglutarate (KG) and branched-chain ketoacids in urine, branched-chain amino acids (Leu, Ile, Val) and/or allo-Ile in plasma (these changes might be intermittent); in the early-onset neurological and hepatic presentations elevated levels of transaminases are also frequently found (Quinonez and Thoene, 2014). Residual activity of the E3 component is measured for diagnostic purposes in

fibroblasts, lymphocytes, or tissue samples of liver or muscle (Cameron et al., 2006; Quinonez et al., 2013; Robinson et al., 1977). Data obtained in patients are not always compatible with *in vitro* results measured with purified (mutant) proteins due mainly to frequent compound heterozygosity (Ambrus et al., 2011). The only definite diagnosis for the disease is the verification of the homozygosity or compound heterozygosity for pathogenic mutations of the *dld* gene; heterozygous individuals present no symptoms and do not develop E3-deficiency. Pathogenic variants include missense or nonsense mutations, splice site variants, and ones with (small) deletions/insertions (see below and in (Cameron et al., 2006; Quinonez et al., 2013)). So far, there are 14 clinically relevant pathogenic hE3 protein variants with single amino acid substitution or deletion in the mature protein: P453L-, G194C-, M326V-, D444V-, R460G-, R447G-, I445M-, I318T-, K37E-, I12T-, E340K-, I358T-, G426E-, and G101del-E3 (Brassier et al., 2013; Cameron et al., 2006; Carrozzo et al., 2014; Cerna et al., 2001; Grafakou et al., 2003; Hong et al., 1996; Hong et al., 1997; Hong et al., 2003; Liu et al., 1993; Odievre et al., 2005; Quinonez et al., 2013; Quinonez and Thoene, 2014; Quintana et al., 2010; Sansaricq et al., 2006; Shaag et al., 1999; Shany et al., 1999) (see Figure 1). X-ray crystallographic analysis of hE3 revealed that the disease-causing single residue substitutions are located in either the cofactor-binding sites (e.g. I12T, K37E, G194C, M326V, I358T), or the disulfide-exchange site (e.g. P453L, G101del), or on the homodimer interface (e.g. E340K, D444V, R460G) of the protein (Brautigam et al., 2005).

3. Factors involved in the pathomechanisms of E3-deficiency

The clinical severity of E3-deficiency proved to be unpredictable on the sole basis of the genotypic characteristics or the degree of the loss of enzymatic activity of the E3 component (Ambrus et al., 2015b; Ambrus et al., 2011; Ambrus et al., 2016; Cameron et al., 2006; Quinonez et al., 2013; Shany et al., 1999; Vaubel et al., 2011). Recent results suggest

that the missing clues could be i) the elevated reactive oxygen species (ROS) generation by selected pathogenic mutants of hE3 (Ambrus et al., 2015b; Ambrus et al., 2011; Vaubel et al., 2011), especially in acidosis (see below and (Ambrus et al., 2011)), ii) the dissociation of selected pathogenic hE3 variants from the harboring multienzyme complexes (KGDHc, PDHc, BCKDHc) (Ambrus et al., 2016; Brautigam et al., 2006; Ciszak et al., 2006; Park and Patel, 2010; Patel et al., 2009), and iii) perhaps the ROS generation by the E1-E2 subcomplex of the hKGDHc upon dissociation of a pathogenic hE3 variant from the hKGDHc (see below and (Ambrus et al., 2015b)), especially in acidosis (Ambrus et al., 2009c). In the corresponding multienzyme complexes the common E3 subunit catalyzes the re-oxidation of the dihydrolipoate moieties covalently linked to the respective E2 components and the reduction of NAD^+ to NADH (LADH activity, forward reaction). The decreased enzymatic LADH activities of the pathogenic hE3 variants can theoretically be originated from unfavorable conformational changes, loss of FAD (the prosthetic group of E3 (Brautigam et al., 2005; Kalse and Veeger, 1968)), abolished critical amino acid interactions, or monomerization of the functional (obligate) homodimer of the E3 subunit (Ambrus and Adam-Vizi, 2013; Ambrus et al., 2015a; Ambrus et al., 2011; Ambrus et al., 2009c; Ambrus et al., 2016; Babady et al., 2007; Brautigam et al., 2005; Klyachko et al., 2005; Shany et al., 1999).

It has to be considered that most *in vitro* studies use proteins obtained by heterologous expression that mimics exclusively the homozygous forms of the mutations. This limitation, however, could also be an advantage: the obtained information can be associated clearly with a single type of mutation.

Hydrogen/deuterium-exchange mass spectrometry (HDX-MS) is a powerful technique for structural investigation of biomacromolecules in solution (Ambrus et al., 2016; Arjunan et al., 2014; Chandrasekhar et al., 2013; Wang et al., 2014; Wang et al., 2015). This method is

particularly useful when high-resolution structural techniques (like X-ray crystallography and NMR spectroscopy) fail on a given system, which is the case for all disease-causing hE3 variants (Ambrus and Adam-Vizi, 2013; Ambrus et al., 2015a). In the course of HDX-MS in a mutant analysis, deuterium incorporations of peptides of the wild-type and variant proteins are compared at a peptide resolution to detect conformational perturbations induced by amino acid substitutions. HDX-MS analysis for structural alterations in the hE3 protein induced by pathogenic mutations has been carried out for ten disease-causing *dld* mutations (leading to K37E-, R460G-, E340K-, I445M-, R447G-, P453L-, G194C-, D444V-, I358T-, and I318T-hE3) (Ambrus et al., 2016). This appears to be hitherto the only investigation in the literature that experimentally addressed the structural changes induced by the disease-causing mutations of the human *dld* gene in the hE3 protein.

ROS generation by E3, its harboring complexes, and pathogenic hE3 variants

ROS generation by the KGDHc is a major source of the mitochondrial oxidative stress (Adam-Vizi and Tretter, 2013; Andreyev et al., 2005; Andreyev et al., 2015; Mailloux et al., 2016; Quinlan et al., 2014; Rimessi et al., 2016; Shi et al., 2008; Starkov, 2013; Starkov et al., 2004; Tretter and Adam-Vizi, 2004). This, together with the compromised activity of the KGDHc, a key enzyme in the Krebs cycle (Gibson et al., 2000; Hansford, 1980; Lai et al., 1977; Massey, 1960a; Reed, 1974; Sheu and Blass, 1999), is highly implicated in hypoxia- and glutamate-induced cerebral damage, Wernicke-Korsakoff syndrome, neurodegenerative diseases, Friedreich's ataxia, ischemia-reperfusion, progressive supranuclear palsy, senescence/aging, infantile lactate acidosis, cancer, and E3-deficiency, among others (Albers et al., 2000; Anderson et al., 2016; Bunik et al., 2007; Burr et al., 2016; Butterworth and Besnard, 1990; Butterworth et al., 1993; Cameron et al., 2006; Chen et al., 2016; Droge and Schipper, 2007; Gibson et al., 2003; Gibson et al., 2000; Gibson et al., 1988; Gibson et al.,

2010; Graf et al., 2009; Klivenyi et al., 2004; Lucas and Szweda, 1999; Mastrogiacomo et al., 1996a; Mastrogiacomo et al., 1996b; Mizuno et al., 1990; Park et al., 2001; Peiris-Pages et al., 2015; Starkov, 2008; Starkov and Adam-Vizi, 2010; Tretter and Adam-Vizi, 2004, 2005; Zundorf et al., 2009). ROS generation by the PDHc appears to be significant only *in vitro* (Ambrus et al., 2015b; Fisher-Wellman et al., 2013; Mailloux et al., 2016; Quinlan et al., 2014; Starkov et al., 2004).

Interestingly, both PDHc and KGDHc are also sensitive to ROS and various other oxidants (Andersson et al., 1998; Chinopoulos et al., 1999; Contreras and Vasquez, 2010; Gibson et al., 2000; Humphries and Szweda, 1998; Kumar et al., 2003; Nulton-Persson and Szweda, 2001; Park et al., 1999; Pocernich and Butterfield, 2003; Rokutan et al., 1987; Tretter and Adam-Vizi, 2000; Vereczki et al., 2006; Xu et al., 2001). Self–regulation of 2-oxo acid dehydrogenase complexes, and the KGDHc in particular, during catalysis was demonstrated *via* intrinsic radical species formation mediated by the complex-bound lipoic acid (LA) and acting at the E1 subunit (Bunik, 2003; Bunik and Sievers, 2002). H_2O_2-mediated reversible glutathionylation of the LA cofactor at E2 that leads to the reversible inactivation of the KGDHc was shown to be an antioxidant response in oxidative stress protecting the KGDHc (Applegate et al., 2008; Esterbauer et al., 1991; Humphries and Szweda, 1998; McLain et al., 2011).

ROS generation by the KGDHc can occur in the forward catalytic reaction in case the physiological electron acceptor NAD^+ is scarce or absent (*in vitro*), or in the reverse direction, when the NADH/NAD^+ ratio is high (Ambrus et al., 2015b; Ambrus et al., 2009c; Starkov et al., 2004; Tretter and Adam-Vizi, 2004). ROS generation by the intact KGDHc (and PDHc) is ascribed to the flavoenzyme E3 component (Ambrus et al., 2015b; Starkov et al., 2004; Tretter and Adam-Vizi, 2004); flavins and flavoenzymes have been suggested as being able to reduce molecular oxygen generating superoxide (*via* single electron reduction) (Ballou et al.,

1969; Massey et al., 1969) and H_2O_2, generally as a secondary product (Bando and Aki, 1991; Behar et al., 1970; Bielski et al., 1985; Bunik et al., 2007; Fridovich, 1975; Ghisla and Massey, 1989; Massey, 1994). A supporting evidence for the above concept is that isolated mitochondria originating from heterozygous E3 knock-out mice generate ROS at a significantly lower rate than the control (Starkov et al., 2004). Similarly to KGDHc, the isolated E3 component (Ambrus et al., 2009a, b) can also generate ROS (oxidase reaction) in either direction of the catalytic mechanism (Ambrus et al., 2015b; Ambrus et al., 2011; Ambrus et al., 2009c; Bando and Aki, 1991; Gazaryan et al., 2002; Huennekens et al., 1955; Kareyeva et al., 2012; Massey et al., 1969; Tahara et al., 2007), though most measurements address the reverse reaction avoiding the methodological difficulties that would arise from the pro-oxidant property of dihydrolipoates in the ROS detection systems (Ambrus et al., 2009c; Kooyman, 1967; Mottley and Mason, 2001; Tretter and Ambrus, 2014). The ROS-generating oxidase reaction of E3 is potentiated by Zn^{2+}, which appears to be in higher concentrations in ischemia/reperfusion and Alzheimer's disease (Brown et al., 2000; Gazaryan et al., 2002).

The LADH reaction follows a ping-pong mechanism that involves a two-electron-reduced enzyme (Ambrus et al., 2011; Gazaryan et al., 2002; Ide et al., 1967; Kiselevsky et al., 1990; Lai and Cooper, 1986; Massey et al., 1960; Moxley et al., 2014; Reed, 1973; Wang et al., 2007; Williams Jr, 1992); under strongly reducing conditions LADH enters a four-electron-reduced state that is highly implicated in its ROS-generating oxidase reaction (see Figure 2~~1~~) (Bando and Aki, 1991; Gazaryan et al., 2002; Visser and Veeger, 1968). The stimulatory effect of Zn^{2+} on ROS generation by E3 in the reverse reaction is due to the binding of Zn^{2+} to the reduced catalytic thiols that prevents delocalization of the reducing equivalents between the thiols and FAD (see Figure 2~~1~~); ROS is generated exclusively *via* FAD (upon its reduction by NADH), not the disulfides, in the reverse E3 reaction (Gazaryan et al., 2002). Depending on the direction of the reaction and the derivative of LA applied to

measure the normal LADH reaction, the pH optimum of the reaction may vary between 5.5 (DL-LA) and 8.3 (DL-dihydrolipoamide) (Ide et al., 1967; Klyachko et al., 2005; Massey, 1960b). Interestingly, mild acidosis appears to be favorable for the forward (physiological) KGDHc reaction (Ambrus et al., 2015b; Gupta and Dekker, 1980; Hirashima et al., 1967; McCormack and Denton, 1979; Nemeria et al., 2014), except for brain KGDHc which exhibits a very narrow optimal pH-range of 7.2-7.4 (Lai and Cooper, 1986). In the reverse reaction in lieu of LA E3 accepts alternative substrates like various organic dyes (e.g. 2,6-dichlorophenolindophenol (DCPIP)) or inorganic complexes (e.g. $K_3[Fe(CN)_6]$); these reactions are collectively referred to as the diaphorase activity of the LADH (Corran et al., 1939; Gazaryan et al., 2002; Kalse and Veeger, 1968; Klyachko et al., 2005; Massey, 1960b; Straub, 1939). The pH optimum of the diaphorase reaction of E3 is in the acidic range (Klyachko et al., 2005; Massey, 1960b)). Relevant to this is the observation that acidosis stimulates ROS generation in the reverse reaction of the isolated E3 component and also in the reverse, but not in the forward reaction of KGDHc (Ambrus et al., 2009c). Sensitivity to acidosis of ROS-generation by selected isolated pathogenic hE3 variants possessing enhanced ROS-generating capacities in the reverse E3 reaction (D444V-, E340K-, P453L-, and G194C-hE3) was reported to be even more pronounced (Ambrus et al., 2011). Enhanced ROS-generation *via* selected pathogenic hE3 variants can manifest also when the hE3 variant is bound to the harboring complexes (e.g. G194C-hE3 to the hKGDHc (Ambrus et al., 2015b)). The D444V-, E340K-, and G194C-hE3 disease-causing hE3 variants, and other pathogenic hE3 variants (R447G-, and R460G-hE3), were found to oxidatively damage the LA-cofactors of the KGDHc and PDHc in a yeast model, and in case of D444V-hE3, in human homozygous fibroblasts (Vaubel et al., 2011). To note, the LA cofactors have previously been shown to be highly susceptible to oxidative damage (see above and in (Cabiscol et al., 2000; McLain et al., 2011; Sadek et al., 2002; Tretter and Adam-Vizi, 2005)).

In mutants displaying enhanced ROS generation LADH activity was usually compromised both in the forward and reverse catalytic directions (Patel et al., 1995); it is remarkable that in the P453L-hE3 variant the physiological enzyme activity was almost completely lost with a ROS-producing activity becoming dominant, whereas the G194C substitution led to no alteration in LADH activity, but to a gain in the ROS-producing capacity (Ambrus et al., 2011). The clinical picture in case of the P453L substitution is very severe (Liu et al., 1993; Sakaguchi et al., 1986) and excessive ROS generation was proposed to contribute to this (Ambrus et al., 2011). The G194C substitution in hE3 leads generally to adult-onset manifestations, which is in concord with the unaltered LADH activity and the considerably elevated ROS production leading likely to an accumulation of the oxidative damages caused by ROS over the years in the affected patients (Ambrus et al., 2011).

The localization of the pathogenic substitutions in hE3 is apparently unrelated to the stimulation of ROS generation; the substitutions that stimulated ROS generation could be at the dimerization surface (E340K, D444V), the disulfide-exchange site (P453L), or the cofactor-binding site (G194C), however, other substitutions at the same locations failed to augment the ROS-generating capacity of hE3 (Ambrus et al., 2011). Circular dichroism (CD) spectroscopy showed no significant overall structural changes for the hE3 mutants exhibiting elevated ROS generation (Ambrus et al., 2011) (see below). In the same study, the FAD contents of E340K-hE3, D444V-hE3, and normal hE3 were practically indistinguishable, whereas P453L-hE3 and G194C-hE3 displayed approximately 30% loss of FAD (see below). These data show that an altered FAD content would not be the underlying mechanism of ROS generation by the pathogenic mutants of hE3 (Ambrus et al., 2011).

The E3 component also exists as a free enzyme *in vivo* (Ambrus et al., 2009c; Constantinescu et al., 1995; Jiang et al., 2016; Reed and Hackert, 1990; Reed and Oliver, 1968; Yan et al., 2013), and it is the most abundant flavoprotein in muscle and brain

mitochondria (Kunz and Gellerich, 1993). It is to note that E3 binds ~30 times weaker to the KGDHc than to the PDHc (Erfle and Sauer, 1969; Poulsen and Wedding, 1970; Reed and Oliver, 1968) and interaction with the KGDHc may be further weakened in acidosis (Ambrus et al., 2009c). Selected pathogenic hE3 mutants display significantly decreased affinity for the hPDHc (and perhaps also for the other OADH complexes), likely accounting for the greatly impaired overall hPDHc activities (Ambrus et al., 2016; Brautigam et al., 2006; Park and Patel, 2010; Patel et al., 2009), and also strengthening the notion that hE3 and its selected pathogenic variants may indeed be present in their liberated forms *in vivo* in abundant concentrations.

When a suboptimal quantity of hE3 is present or bound to the hKGDHc, as in selected cases of E3-deficiency or acidosis, the E1-E2 subcomplex of the enzyme can also potentially generate substantial amounts of ROS, a phenomenon displayed *in vitro* by the hKGDHc, but not hPDHc (Ambrus et al., 2015b). Given the pro-oxidant properties of DHLA (Bunik, 2003; Bunik and Sievers, 2002; Kooyman, 1967; Mottley and Mason, 2001), and our recent results on E1-E2 (Ambrus et al., 2015b) together with preliminary experimental evidence collected thereafter (unpublished results) we propose that the E2-bound DHLA in the absence of the E3 component may readily react with molecular oxygen producing superoxide (see Figure 2~~1~~ for further details). Altogether, under pathological conditions when hE3 or its mutants partially or completely dissociate from the hKGDHc, ROS production may arise simultaneously from E3 (preferably in its reverse reaction) as well as from the E1-E2 subcomplex (in its forward reaction), provided that the supply of alpha-ketoglutarate is adequate (Ambrus et al., 2015b; Qi et al., 2011). Even under these conditions a remaining intact KGDHc (and PDHc) population may preserve some functionality (Quinonez et al., 2013), unless their LA-cofactors are incompetent due to oxidative damage (Vaubel et al., 2011). Following an earlier study on the ROS generation by the isolated E1 components of OADH complexes from different

species (Frank et al., 2008), we found that the rate of ROS generation by E1 of hKGDHc was exceedingly more significant than those by E1 components of other OADH complexes including the hPDHc (Ambrus et al., 2015b; Nemeria et al., 2014). Given that the E1-E2 complex is far more stable than the (E1-E2)-E3 complex (Erfle and Sauer, 1969; Poulsen and Wedding, 1970; Reed and Oliver, 1968), ROS generation by the free E1 component of hKGDHc might gain pathological significance only when E1 is quantitatively in excess *in vivo* (e.g. in E2 deficiencies) (Ambrus et al., 2015b; Cruts et al., 1995; Diaz-Munoz et al., 2015; Dumont et al., 2009; Gibson et al., 1998; Nakano et al., 1994; Nemeria et al., 2014; Shi et al., 2005; Starkov, 2013).

Lipoic acid, which is the common prosthetic group of the E2 components of all the mitochondrial OADH complexes and also a dietary antioxidant supplement, inhibits ROS generation by the KGDHc in the forward as well as in the reverse reaction, but in the latter the effect was significant only at pH < 6.8. It is to note that such low pH could be observed in the ischemic brain (Csiba et al., 1983). Similar pattern characterized the inhibitory effect of LA on the reverse ROS-generating E3 reaction obtained with the isolated E3 component, which accounted for the potential (at least partial) detachment of the E3 component from the KGDHc at pH<6.8 (Ambrus et al., 2009c); the binding of E3 to the KGDHc was reported to be easily disruptable (Bunik et al., 2000; Massey, 1960a; Reed and Oliver, 1968). In fact, free LA can serve as a relatively effective electron-acceptor substrate for the isolated E3 in its reverse LADH reaction, but only in the acidic pH range (see above). Indeed, addition of free LA augmented the NADH consumption by isolated E3 (and KGDHc) while blocking the NADH-mediated ROS generation at pH<6.8 (Ambrus et al., 2009c). The above data suggest that LA is a better substrate than O_2 for isolated E3 at pH<6.8. Hence it can be considered as an efficient antioxidant partly due to its capacity to prevent the formation of ROS by E3. Another advantage of the application of LA for inhibiting ROS generation by E3 could be that

the conversion of LA produces DHLA, another antioxidant and an effective redox regenerator of several endogenous antioxidants (Packer et al., 1995). The benefit of generating free DHLA *in vivo* can however be argued (Scott et al., 1994) because DHLA could also be a pro-oxidant (Mottley and Mason, 2001) (and see above the formation of ROS potentially *via* the DHLA moiety of the E1-E2 subcomplex) for which we have also found indirect evidence (Ambrus et al., 2009c). It appears that DHLA becomes pro-oxidant *via* the formation of the very reactive and unstable primary thiyl radical preferably in the presence of free radical sources (including horseradish peroxidase (HRP) (Ambrus et al., 2009c; Mottley and Mason, 2001), flavins/flavoproteins (Bunik, 2003), or O_2 (under specific circumstances) (Kooyman, 1967)). Nevertheless, LA can cross the blood–brain barrier and was proposed to exert neuroprotective effects (Tirosh et al., 1999); it decreased ROS generation and improved mitochondrial functions in rats (Hagen et al., 1999).

As for the mechanism of enhanced ROS generation by selected pathogenic hE3 variants, certain conclusions could be drawn from HDX-MS measurements. The rate of ROS generation by hE3 can likely be stimulated by conformational changes in the LA-binding cavity on the *si* face of the isoalloxazine ring of FAD whence the approach of lipoic moieties or O_2 is most likely in the course of the normal catalytic activity or superoxide generation, respectively (Ambrus et al., 2016). In the absence of such changes, which is the case for the D444V substitution, ROS generation by E3 is likely stimulated by other mechanisms, also involving the isoalloxazine ring of FAD; however this cannot be elucidated by HDX-MS. HDX-MS detected significant changes in exposure/dynamics of peptides in the LA-binding cavity, which could potentially lead to enhanced ROS-generation, in case of the G194C and P453L pathogenic hE3 substitutions, but not of the D444V substitution. For the enhanced ROS generation of E340K-hE3 a peptide (275-289), located outside the LA-binding cavity, was proposed to be responsible; the perturbation of this peptide may potentially affect the

conformation or reactivity of the isoalloxazine ring of FAD (Ambrus et al., 2016). Out of those pathogenic hE3 variants whose ROS-generating capacities have not been reported only I445M-hE3 displayed considerable conformational changes in the LA-binding pocket in HDX-MS studies (R447G- and I318T-hE3 did not). It would be important to perform site-directed mutagenesis studies in the future to determine the residues of hE3 that participate in the ROS-generation and to map at higher resolution the modulations of the local structure in the LA-binding cavity upon the pathogenic substitutions leading to elevated ROS generation. These pieces of information can potentially lead to targeting ROS generation of each of the above hE3 mutants by specific inhibitors. Six of the 14 pathogenic hE3 variants have not been addressed yet for their ROS-generating capacities, which is also to be done in the future.

Monomerization of E3 and FAD loss under pathological conditions

In addition to elevated ROS generation, monomerization of E3 and FAD loss were also considered as underlying mechanisms for the impaired enzyme function under pathological conditions. The E3 subunit forms a non-covalent, functional (obligate) homodimer that requires amino acids from both monomers for its physiological LADH activity (Brautigam et al., 2005; Gazaryan et al., 2002; Kim and Patel, 1992; Liu et al., 1995; Qi et al., 2011), but not for its diaphorase activity (Klyachko et al., 2005; Tsai et al., 1981; Visser and Veeger, 1968). Pathogenic homodimer interface mutations of hE3 were suggested to cause monomerization (Brautigam et al., 2005; Shany et al., 1999), and monomerization of E3 *via* mild acidification or a disease-causing dimerization surface mutation (as in D444V-hE3) was proposed to stimulate the diaphorase (Klyachko et al., 2005) or a moonlighting protease activity (Babady et al., 2007) of E3. Calibrated size-exclusion chromatography was applied to address monomerization of E3 under mild acidosis or pathogenic dimerization surface mutations; soft-ionizing nano-LC MS and DOSY NMR spectroscopy (Ambrus et al.,

2006; Ambrus and Yang, 2007) verified the gel filtration data (Ambrus et al., 2011; Ambrus et al., 2009c). After careful analysis, a conclusion was drawn that neither mild acidosis nor any of the pathogenic dimerization surface substitutions investigated (R460G, D444V, and E340K) triggered monomerization of the E3 dimer (Ambrus et al., 2011; Ambrus et al., 2009c). Molecular dynamics (MD) simulations of 13 pathogenic hE3 variants led to the same conclusion (Ambrus and Adam-Vizi, 2013; Ambrus et al., 2015a). HDX-MS also demonstrated no monomerization for any of the ten pathogenic hE3 variants investigated (six of the variants examined have their substitutions at the interface domain) (Ambrus et al., 2016). This suggests that monomerization is not the underlying mechanism of the enhanced diaphorase/ROS-generating activity of E3 in acidosis or that of the stimulated proteolytic activity due to pathogenic amino acid substitutions at the dimer interface. It was postulated that the above phenomena might rather be the consequences of specific local or propagated structural changes in the intact functional homodimer of hE3 (Ambrus et al., 2011; Ambrus et al., 2009c; Brautigam et al., 2005; Massey, 1960b; Visser and Veeger, 1968), which could also result in FAD loss (Ambrus and Adam-Vizi, 2013; Ambrus et al., 2015a; Ambrus et al., 2011; Ambrus et al., 2016) (see below). This view is also consistent with interface domain mutations of hE3 influencing the three activities of hE3 (LADH-, diaphorase-, and the proteolytic activities) independently (Vaubel et al., 2011).

A single FAD prosthetic group is bound very tightly, but non-covalently to each of the E3 monomers (Ambrus et al., 2011; Ide et al., 1967; Kalse and Veeger, 1968; Wang et al., 2008; Wang et al., 2007) in a way that at least 36 residues in each monomer contribute to the binding (Brautigam et al., 2005). Several (single) pathogenic substitutions in hE3, some even taking place outside the cofactor-binding domains, resulted in a significantly lower affinity for FAD and this can contribute to the impaired enzymatic activities (Ambrus et al., 2011; Ambrus et al., 2016; Brautigam et al., 2005; Liu et al., 1995). The following data are available

for compromised FAD contents in pathogenic hE3 variants (in mol FAD/mol E3 monomer) using expressed hE3 proteins: P453L (0.66), G194C (0.72), E340K (0.99), D444V (0.95) (Ambrus et al., 2011), K37E (0.76) (Liu et al., 1995) or (0.67) (Ambrus et al., 2011). Many of the structural changes in pathogenic hE3 variants detected by HDX-MS elucidated perturbation of the exposure to solvent or the dynamics of selected residues involved in FAD-binding in hE3. A variable percentage of the FAD-binding residues affected by the pathogenic substitutions in hE3 were determined by HDX-MS in our recent study for the D444V, G194C, P453L, K37E, E340K, R460G, R447G, I318T, I445M, and I358T variants (see below) (Ambrus et al., 2016). For the remaining four pathogenic substitutions not examined by HDX-MS (G101del (Hong et al., 1997; Kim, 2012; Quinonez et al., 2013), I12T (Cameron et al., 2006; Yuan et al., 2009), M326V (Cerna et al., 2001; Yuan et al., 2008), and G426E (Carrozzo et al., 2014)) FAD contents have not been reported in the literature.

It would be intriguing to investigate *in vitro* how hE3 mutants with experimentally confirmed or hypothesized FAD loss would respond to elevated FAD concentrations; this could raise the possibility of a beneficial dietary supplementation with any vitamers/precursors of Vitamin B2 for affected patients, particularly in light of earlier reports on the beneficial effects of Vitamin B2 supplementation in selected clinical cases (Carrozzo et al., 2014; Hong et al., 2003; Quinonez and Thoene, 2014).

Structural alterations in pathogenic hE3 variants

The crystal structure of hE3 displays 35% helices (18 helices) and 26% β-sheets (31 strands); the structure can be considered to be very compact. There are four domains in hE3: the FAD-binding domain (1-149), the NAD^+/NADH-binding domain (150-282), the central domain (283-350), and the interface domain (351-474) (Brautigam et al., 2005). Circular dichroism (CD) spectroscopy has been successfully applied in the conformational analysis of

proteins and other biomolecules (Fasman, 1996) including the E3 protein (Ambrus et al., 2011; Brady and Beychok, 1969; Brady and Beychok, 1971; Liu et al., 1995; Templeton et al., 1980; Visser et al., 1995). As for the pathogenic hE3 variants no significant changes in the CD spectra relative to hE3 were found for the K37E-, G194C-, M326V-, I358T-, P453L-, E340K-, D444V-, and R460G-hE3 variants (Ambrus et al., 2011; Liu et al., 1995) implying that the respective mutations induce no major overall conformational changes in the hE3 structure (Ambrus et al., 2011). Although multiple crystal structures have already been published for hE3 (Brautigam et al., 2005; Brautigam et al., 2006; Brautigam et al., 2011; Ciszak et al., 2006), no crystal structure for mutated hE3 is available at present. We analyzed the structural effects of 13 disease-causing mutations on the low-pH conformation of hE3 by *in silico* molecular dynamics (MD) simulations (Ambrus and Adam-Vizi, 2013; Ambrus et al., 2015a). The best correlations between MD simulation and HDX-MS data revealing ~~fine~~ structural alterations in the hE3 structure triggered by ten pathogenic *dld* mutations (Ambrus et al., 2016) were found for the K37E-, G194C-, P453L-, R460G-, and I445M-hE3 mutants; for correlation analysis the considerably affected residues and peptides were used. These information further support the conclusion that specific local or relayed structural changes ought to be sought explaining the effects of pathogenic substitutions in hE3.

4. Concluding remarks

The ability to generate ROS in excess by selected pathogenic variants (D444V, P453L, G194C, and E340K) of the hE3 component as well as by the E1-E2 subcomplex of the hKGDHc could justify an antioxidant therapy in the management of human E3-deficiency. Supplementation with lipoic acid could also be beneficial due to inhibition of ROS generation by hE3, and perhaps by selected pathogenic hE3 variants, in acidosis. In particular, it could generate dihydrolipoic acid, an arguably efficient other antioxidant, which could be another

advantage of this rather unspecific inhibitory strategy. Specific structure-based drug design against selected malfunctioning and predominantly ROS-generating hE3 variants is not possible for the time being as structures of the hE3 variants are unavailable. A selective drug candidate should potentially work only temporally and/or locally in acidosis (to protect the residual normal LADH function between acute episodes). Based also on recent *in vitro* data, flavins might be another rational supplementation in the therapy for those mutations triggering FAD loss in the hE3 component.

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Figure Legends

Fig. 1. Structure of the obligate homodimer of the hE3 protein with pathogenic substitution sites (side chains in red) indicated in one of the monomers. FAD (cyan) and active site Cys residues (orange) are highlighted by colors, but also only in one of the monomers, for clarity.

Fig. 2~~1~~. Current (simplified) view of the mechanisms of superoxide generation by the E1 (Nemeria et al., 2014), E1-E2 (Ambrus et al., 2015b; Bunik, 2003; Kooyman, 1967; Mottley and Mason, 2001), and the E3 (Bando and Aki, 1991; Gazaryan et al., 2002; Ghisla and Massey, 1989; Massey, 1994; Massey et al., 1969) components/sub-complexes of the hKGDHc and the fully assembled/intact hKGDHc. H_2O_2 could always be detected besides superoxide in these systems (unpublished data of the authors for E1-E2) due to the spontaneous disproportionation of superoxide in aqueous solutions. In the free E1 component the relatively stable enzyme-bound enamine is the likely substrate for oxidation by O_2, leading to the superoxide anion radical formation. The E1-E2 sub-complex generates superoxide likely *via* the E2-bound reduced lipoic acid moiety. The detailed mechanism which initiates the ROS-generating reaction of very significant rate at the E1-E2 sub-complex of the hKGDHc, but not of the hPDHc ought to be the subject of further investigations. ROS generation by the free or complexed E3 component is facilitated by the FAD prosthetic group in either direction of the catalytic mechanism of the E3 reaction. KG: alpha-ketoglutarate, ThDP: thiamin diphosphate, Lip: lipoic acid moiety of E2, FAD: flavin adenine dinucleotide, NAD^+/NADH, nicotinamide adenine dinucleotide (oxidized/reduced forms).

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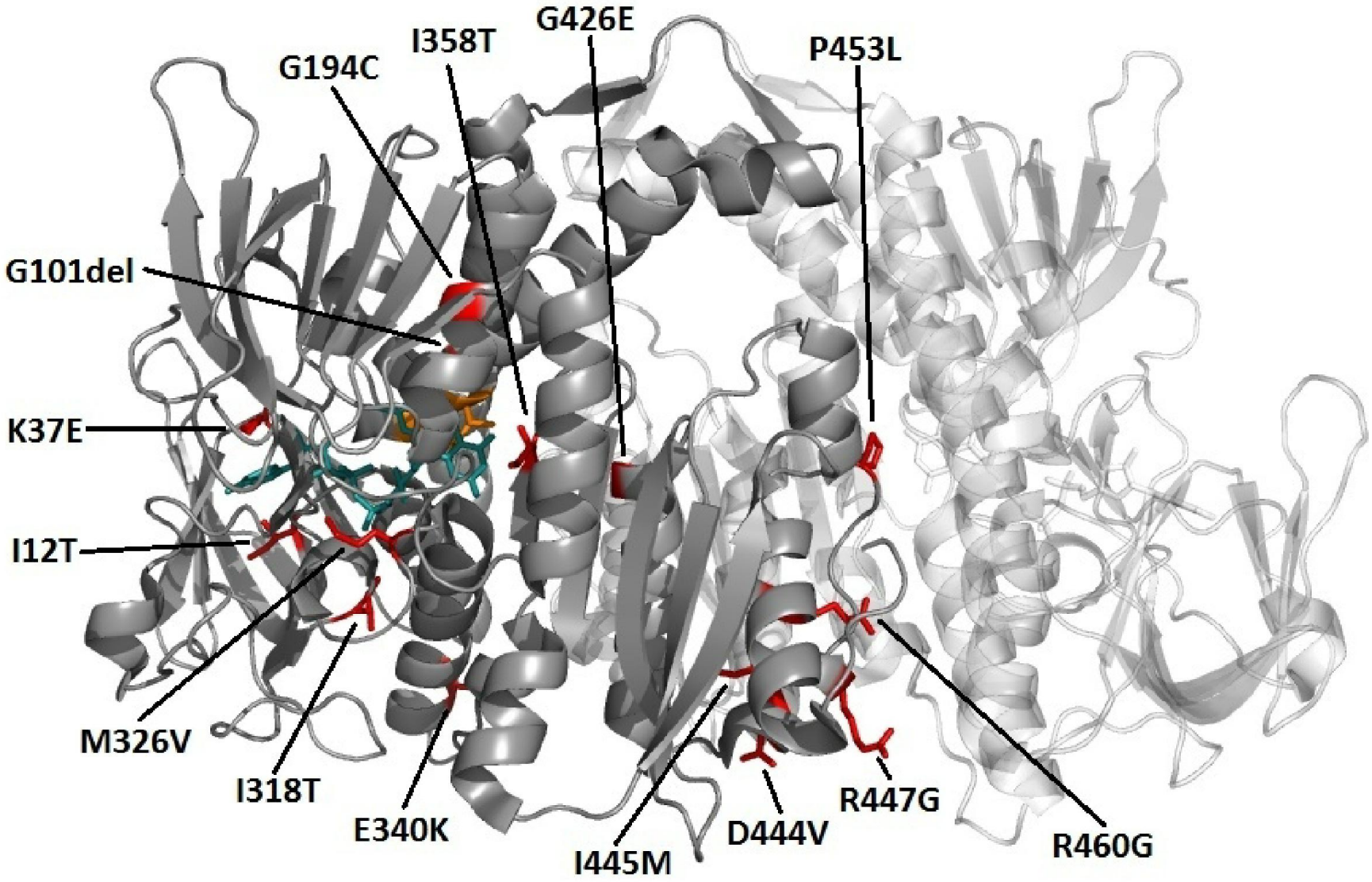
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