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Alpha-ketoglutarate dehydrogenase: a target and generator of oxidative stress

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Alpha-ketoglutarate dehydrogenase (α-KGDH) is a highly regulated enzyme, which could determine the metabolic flux through the Krebs cycle. It catalyses the conversion of α-ketoglutarate to succinyl-CoA and produces NADH directly providing electrons for the respiratory chain. α-KGDH is sensitive to reactive oxygen species (ROS) and inhibition of this enzyme could be critical in the metabolic deficiency induced by oxidative stress. Aconitase in the Krebs cycle is more vulnerable than α-KGDH to ROS but as long as α-KGDH is functional NADH generation in the Krebs cycle is maintained. NADH supply to the respiratory chain is limited only when α-KGDH is also inhibited by ROS. In addition being a key target, α-KGDH is able to generate ROS during its catalytic function, which is regulated by the NADH/NAD^+ ratio. The pathological relevance of these two features of α-KGDH is discussed in this review, particularly in relation to neurodegeneration, as an impaired function of this enzyme has been found to be characteristic for several neurodegenerative diseases.

Keywords: alpha-ketoglutarate dehydrogenase; reactive oxygen species; mitochondria; oxidative stress; neurodegeneration

Alpha-ketoglutarate dehydrogenase (α-KGDH) is a Krebs cycle enzyme, which catalyses the non-equilibrium reaction converting α-ketoglutarate, coenzyme A and NAD^+ to succinyl-CoA, NADH and CO_2, requiring thiamine pyrophosphate as a cofactor. α-KGDH is not simply one of the enzymes of the Krebs cycle. There are a number of features that makes this enzyme distinct from other enzymes important in the bioenergetic processes. First of all, it is highly regulated and is the primary site of control of the metabolic flux through the Krebs cycle (Hansford 1980). The mammalian enzyme is inhibited by its end products, succinyl-CoA and NADH (Garland 1964; Smith *et al.* 1974). Regulation of α-KGDH is complex involving ATP/ADP ratio, NADH/NAD^+ ratio, calcium and the substrate availability in mitochondria; therefore, it is also related to the activity of NAD^+-isocitrate dehydrogenase (ICDH; Hansford 1980). The apparent K_m (Michaelis constant) of the enzyme for α-ketoglutarate in the mitochondria is 0.67 mM (Hansford 1980).

α-KGDH is a complex enzyme consisting of multiple copies of three subunits (see figure 2), a thiamine pyrophosphate-dependent dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2) and dihydrolipoamide dehydrogenase (E3), the latter being a flavoprotein (Koike *et al.* 1974; Koike & Koike 1976).

Calcium plays a key role in activating three mitochondrial dehydrogenases, pyruvate dehydrogenase (PDH), isocitrate dehydrogenase and α-KGDH in the 0.1–10 μM range (Denton *et al.* 1978; Denton & McCormack 1980) and by this, in adjusting the rate of metabolism to an increased demand. Activation of these dehydrogenases could increase the supply of NADH to the respiratory chain and, indeed, an increased reduction of NAD^+ by α-ketoglutarate was monitored in rat heart mitochondria (Hansford & Castro 1981). The first evidence that an increase in the intracellular calcium concentration is able to activate mitochondrial dehydrogenases resulting in an increase in the NADH level in intact cells was provided by Duchen (1992) and Pralong *et al.* (1992), demonstrating that NADH level is increased in cells in a calcium-dependent manner in response to depolarization. Calcium lowers the apparent K_m of α-KGDH for α-ketoglutarate (McCormack & Denton 1979), this mechanism being different from that underlying the activation of PDH, where calcium promotes the dephosphorylation of the enzyme (Denton *et al.* 1972; Pettit *et al.* 1972). Activation of dehydrogenases occurs at relatively low calcium concentrations (less than 20 μM). Calcium, in high concentrations (greater than or equal to 100 μM), however, inhibits α-KGDH (Lai & Cooper 1986), ICDH (Bulos *et al.* 1984) and PDH (Sheu *et al.* 1985). This effect could significantly contribute to the deleterious effects of high calcium concentration in neurons under pathological conditions.

α-KGDH could be a crucial target of reactive oxygen species (ROS) in cells and, being an important regulatory site in the mitochondrial metabolism, could play a key role in the bioenergetic deficit evolving in oxidative stress. On the other hand, it has been revealed recently that the enzyme itself is able to generate ROS, therefore could contribute to the induction of oxidative stress. This review will focus on these two features of α-KGDH and discuss the relevance of α-KGDH in

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One contribution of 18 to a Theme Issue 'Reactive oxygen species in health and disease'.

oxidative stress, concentrating mainly on the brain enzyme and its relation to neurodegeneration.

Early studies performed with brain mitochondria demonstrated that the activity of α-KGDH was the lowest among the Krebs cycle enzymes (Lai *et al.* 1977), indicating that, similarly to peripheral tissues (Hansford 1980), it could also be a rate-limiting enzyme in the brain regulating the flux through the cycle. The regional distribution of α-KGDH in the brain is not uniform but follows the pattern of other citric acid cycle and glycolytic enzymes (Leong *et al.* 1981; Leong & Clark 1984); the activity is highest in cerebral cortex and lowest in the phylogenetically older parts of the brain (Lai & Cooper 1986). The properties of the brain α-KGDH appear to differ, in many respects, from those of the enzyme originating from non-neuronal tissues. For instance, brain enzyme is inhibited by ammonia, whereas the enzyme in isolated rat heart or purified from bovine heart was much less sensitive to inhibition (Lai & Cooper 1986). Inhibitors of the bovine heart enzyme, β-methylene-D,L-aspartate or D,L-vinylglycine, were without effect on the brain mitochondrial α-KGDH but L-aspartate, which did not influence the activity of the purified bovine heart α-KGDH, significantly inhibited the enzyme in rat brain mitochondria (Lai & Cooper 1986).

1. α-KGDH IS A CRUCIAL TARGET OF REACTIVE OXYGEN SPECIES IN THE MITOCHONDRIA

Oxidative stress involves an increased production and/or a decreased elimination of ROS in cells and has been shown to contribute to the cell damage caused by ischaemia/reperfusion, trauma, ageing and several neurodegenerative diseases (Halliwell & Gutteridge 1984, 1985; Cao *et al.* 1988; Schmidley 1990; Halliwell 1992; Hall *et al.* 1993; Olanow 1993; Siesjo *et al.* 1995). The effect of ROS (superoxide, hydrogen peroxide and hydroxyl radical) could be damaging on virtually all cellular components but membrane lipids are particularly sensitive to free radicals due to the presence of polyunsaturated fatty acids, which preferentially undergo lipid peroxidation (Halliwell & Gutteridge 1984, 1985). The fragmentation products of lipid peroxidation are highly toxic aldehydes, of which 4-hydroxy-2-nonenal (HNE) is the major and probably the most reactive product (Esterbauer *et al.* 1991). Exposure of cardiac mitochondria to HNE resulted in a reduced α-KGDH activity and an inhibition of the NADH-linked state three respiration (Humphries *et al.* 1998). Besides α-KGDH only PDH was inhibited by HNE; other mitochondrial dehydrogenases and complexes of the respiratory chain were unaltered by HNE (Humphries *et al.* 1998). Investigation of the effect of HNE on isolated α-KGDH and PDH revealed that the target of HNE is lipoic acid bound covalently to the E2 subunit of both α-KGDH and PDH (Humphries & Szweda 1998).

α-KGDH is also inactivated by other reactive products. Exposure of soleus muscle to the superoxide donor xanthine and xanthine oxidase inhibited the activity of α-KGDH in muscle extracts but nitric oxide alone had no effect (Andersson *et al.* 1998). However, superoxide and nitric oxide together, which generate peroxynitrate, were more effective than superoxide alone, in inactivating the enzyme (Andersson *et al.* 1998). Consistent with this, isolated α-KGDH as well as the enzyme present in the microglia were inactivated by peroxynitrate and nitric oxide and proved to be at least 10-times more sensitive to these agents than another mitochondrial enzyme, glutamate dehydrogenase (Park *et al.* 1999). Peroxynitrate nitrated tyrosine residues of the α-KGDH subunits and reduced the immunoreactivity to antibodies against the E1 and E2 but not the E3 subunits (Park *et al.* 1999).

The toxic amyloid-β-peptide, which accumulates in the plaques in Alzheimer's disease, increases oxidative stress in cultured neurons (Harris *et al.* 1995; Mark *et al.* 1997) and directly inhibits PDH and α-KGDH in brain mitochondria (Casley *et al.* 2002), pointing to the possible involvement of these enzymes in the defective energy metabolism characteristic to the affected brain regions in Alzheimer's disease.

Our group, using isolated nerve terminals (synaptosomes), demonstrated first that α-KGDH is sensitive to H_2O_2 (Chinopoulos *et al.* 1999; Tretter & Adam-Vizi 2000), and this was confirmed for fibroblasts (Gibson *et al.* 2000) and cardiomyocytes (Nulton-Persson & Szweda 2001). α-KGDH in nerve terminals exposed to 0.1 or 0.5 mM H_2O_2 for 10 min was inhibited by 40 and 50%, respectively (Chinopoulos *et al.* 1999). In a detailed study addressing the sensitivity of the Krebs cycle enzymes to oxidative stress, we found that three enzymes were inhibited by H_2O_2: aconitase, α-KGDH and succinate dehydrogenase, but the vulnerability of these enzymes to H_2O_2 and the metabolic consequences of their impaired function were markedly different (Tretter & Adam-Vizi 2000). The least significant was the effect of H_2O_2 on succinate dehydrogenase, which is not a rate-limiting enzyme in the Krebs cycle and was only inhibited by *ca* 30% in the presence of the highest concentration of H_2O_2 (0.5 mM). In contrast, aconitase showed a high sensitivity to H_2O_2, being completely inactivated at already very small H_2O_2 concentrations (less than 50 μM). In fact, aconitase was the only enzyme which could be inhibited by 100% upon acute exposure to H_2O_2. Yet, the impaired bioenergetic consequences in the Krebs cycle under the early phase of an H_2O_2-induced oxidative stress are not due to inhibition of aconitase but rather that of α-KGDH. The key question here is whether the overall function of the Krebs cycle, as shown by the generation of NADH, is impaired by H_2O_2 and if so, which enzyme(s) limits the generation of NADH under oxidative stress.

For the clarification of the impact and the metabolic consequences of the inhibition of α-KGDH under oxidative stress it is important first to consider the role of aconitase in oxidative stress. Gardner & Fridovich (1992) and Gardner *et al.* (1995) reported first that mammalian aconitase was inhibited by superoxide and later it has been shown that nitric oxide (Andersson *et al.* 1998), peroxynitrate (Andersson *et al.* 1998) and, as shown by our group, H_2O_2 (Chinopoulos *et al.* 1999; Tretter & Adam-Vizi 2000) also inhibited this enzyme, most likely due to interaction with the iron–sulphur clusters. Generally, iron–sulphur centres of proteins are primary sites for ROS to attack mitochondrial enzymes

and this may be the mechanism by which succinate dehydrogenase is also inhibited by ROS. The extreme sensitivity of aconitase to ROS allows the measurement of this enzyme to be an indicator of superoxide (Patel *et al*. 1996) and H_2O_2 production (Tretter & Adam-Vizi 2000; Sipos *et al*. 2003*a*,*b*) in cells.

Aconitase was inhibited in cortical cell culture treated with excitotoxic concentrations of NMDA or kainate and this was prevented by the cell-permeable superoxide dismutase mimetic manganese(III) 5,10, 15,20-tetra(4-pyridyl)-21H,23H-porphine chloride terakis (methochloride) (MnTBAP) indicating that the active species was superoxide (Patel *et al*. 1996). Conversion of citrate to isocitrate catalysed by aconitase is thought to be a near-equilibrium reaction *in vivo*, therefore not believed to be a regulatory site in the Krebs cycle. However, inhibition of aconitase could lower the flux through the cycle due to accumulation of citrate and a consequent inhibition of citrate synthase, or could block the flux when inhibited by 100%. Patel *et al*. (1996) indeed found an inactivation of aconitase that paralleled the amount of cell death after exposure of cortical cells to excitotoxic insults. However, when we addressed specifically the question whether inhibition of aconitase limits NADH production in the Krebs cycle, it was found that in the early period of oxidative stress the function of the cycle is dependent on the inhibition of α-KGDH and not that of aconitase (Tretter & Adam-Vizi 2000). Parallel measurements of aconitase and NAD(P)H generation revealed that aconitase could be inhibited by 100% in the presence of H_2O_2 (up to 50 μM concentration) without any change in the maximum available NAD(P)H level in nerve terminals. NAD(P)H level decreased only at higher H_2O_2 concentrations (greater than or equal to 50 μM), when aconitase was already completely inactivated, therefore no correlation was seen between the inhibition of this enzyme and the impaired NAD(P)H generation in the Krebs cycle. At higher H_2O_2 concentrations, α-KGDH was also inhibited and this showed a correlation with a fall in the NAD(P)H level in nerve terminals (Tretter & Adam-Vizi 2000). A clear conclusion offered by these results is that though α-KGDH is less sensitive to H_2O_2 than aconitase, because it is inhibited at higher oxidant concentrations and is never inactivated by 100%, yet this is the key enzyme that limits the generation of NAD(P)H in the Krebs cycle under acute exposure to oxidative stress. α-KGDH, as discussed above, is one of the key regulatory enzymes, which, together with citrate synthase and isocitrate dehydrogenase, is thought to determine the overall rate of the Krebs cycle (Cooney *et al*. 1981; McCormack *et al*. 1990; Moreno-Sanchez *et al*. 1990). Nonetheless, it has to be resolved how the Krebs cycle can function and generate NAD(P)H when one of the enzymes, aconitase, even though not rate-limiting, is completely blocked. The explanation for this apparent contradiction is that when the input to Krebs cycle from pyruvate is inhibited due to the block of aconitase, a segment of the cycle between α-ketoglutarate and oxaloacetate can still function if glutamate is available and feeds the cycle via α-ketoglutarate (figure 1). This segment of the Krebs cycle has been suggested to function in the absence of glucose, when most likely glutamate is converted to α-ketoglutarate (Yudkoff *et al*. 1994; Erecinska *et al*. 1996). In this respect oxidative stress, in a phase when aconitase is already completely blocked but α-KGDH is still functional, is similar to glucose-free condition. We found that the amount of glutamate present in nerve terminals decreased when aconitase was fully blocked by H_2O_2 (Tretter & Adam-Vizi 2000). Fuelling the Krebs cycle from glutamate via α-ketoglutarate is possible by transamination due to the presence of aspartate aminotransferase, rather than glutamate dehydrogenase, which has limited activity in nerve terminals (Cheeseman & Clark 1988; Yudkoff *et al*. 1994). This rescue route could explain that in the early phase of an oxidative stress, NAD(P)H generation in the Krebs cycle is maintained in spite of a blockage of aconitase (figure 1). When at a higher degree of oxidative stress α-KGDH is also inhibited, NAD(P)H generation becomes limited (Chinopoulos *et al*. 1999; Tretter & Adam-Vizi 2000). The oxidative stress-induced inhibition of α-KGDH is critical because it decreases the provision of NAD(P)H for complex I, thus decreasing the rate of the respiratory chain. Under this condition, the ATP production decreases in nerve terminals (Tretter *et al*. 1997) and the membrane potential of mitochondria could be maintained only with the contribution of the F_0F_1-ATPase working in reverse and hydrolysing ATP. This was apparent from $\Delta\Psi$m measurements, which revealed that as long as the F_0F_1-ATPase was active $\Delta\Psi$m was maintained under H_2O_2-induced oxidative stress, but when the enzyme was inhibited by oligomycin, $\Delta\Psi$m decreased indicating that the respiratory chain was insufficient to maintain $\Delta\Psi$m (Chinopoulos *et al*. 1999). Nerve terminals, with an impaired α-KGDH activity, produce less ATP by *ca* 30% in the early phase of oxidative stress (Tretter *et al*. 1997), but this is still adequate to maintain the resting function of the ATP-driven ion pumps in the plasma membrane. When oxidative stress is superimposed on some other burden such as a $[Na^+]_i$ load occurring during excessive stimulation of glutamate receptors or in ischaemia/reperfusion, the inability of mitochondria to meet an additional ATP demand, mainly due to inhibition of α-KGDH, becomes apparent and the $[Na^+]$ and $[Ca^{2+}]$ gradients across the plasma membrane (Chinopoulos *et al*. 2000) as well as the mitochondrial membrane potential rapidly collapse (Scanlon & Reynolds 1998).

α-KGDH was found to be inhibited in cardiac mitochondria isolated after myocardial ischaemia and reperfusion (Lucas & Szweda 1999). The reperfusion-induced inactivation of α-KGDH involved the oxidative damage of lipoic acid essential for the catalytic activity of the enzyme and was specific; the activities of other dehydrogenases were not influenced by ischemia/reperfusion (Sadek *et al*. 2002). It has been suggested that free radicals are crucial in mediating reperfusion-induced injuries (Ambrosio *et al*. 1991, 1993; Blasig *et al*. 1995; Bolli *et al*. 1988) but the nature of radicals acting under this condition has not been defined yet. The effect of H_2O_2 on α-KGDH might be significant during reperfusion for it is formed in a relatively high concentration (0.1 mM), as detected by microdialysis

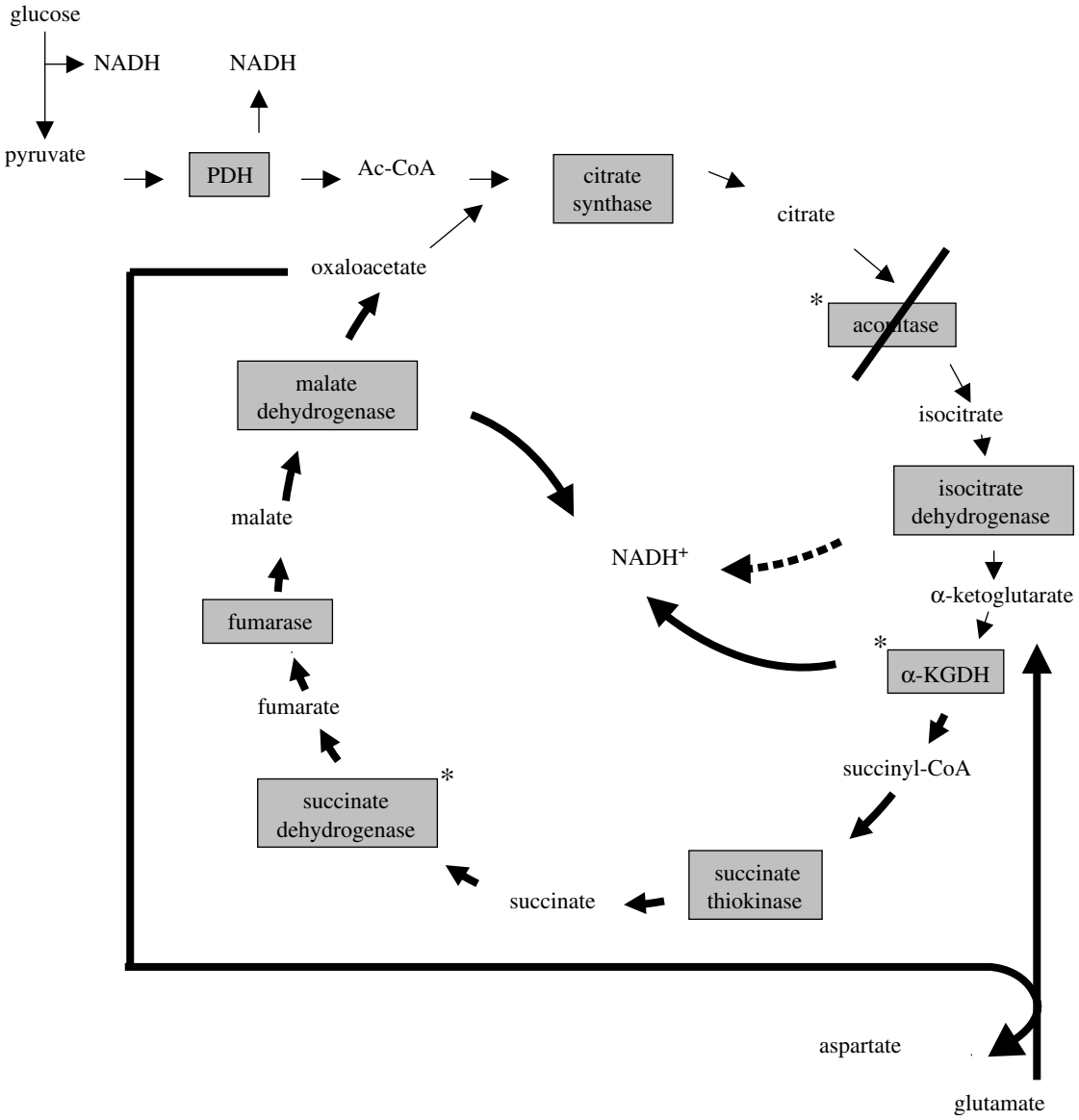

Figure 1. Metabolic flux in the Krebs cycle during oxidative stress when aconitase is completely inactivated. * Enzymes inhibited by H_2O_2. When aconitase, the most sensitive enzyme, is inhibited by 100% but α-KGDH is still functional in the presence of H_2O_2, a segment of the Krebs cycle (bold arrows) is maintained by glutamate, which is converted to α-ketoglutarate via transamination. This segment is inhibited only when α-KGDH is also inhibited by ROS. PDH, pyruvate dehydrogenase.

in the striatum during reperfusion following an ischaemic period (Hyslop *et al.* 1995).

Another factor that might play a role in the inhibition of α-KGDH during ischaemia/reperfusion could be Zn^{2+}, which is present in elevated concentrations in cells under this condition (Tonder *et al.* 1990; Koh *et al.* 1996). It has been reported that Zn^{2+}, in submicromolar concentrations, induces reversible inhibition of the purified α-KGDH and inhibits respiration in liver mitochondria, the α-ketoglutarate-dependent respiration showing the highest Zn^{2+}-sensitivity (Brown *et al.* 2000). Zn^{2+} also inhibited glutamate dehydrogenase and PDH, but not succinate dehydrogenase or malate dehydrogenase (Brown *et al.* 2000). Zn^{2+} has been implicated in the pathogenesis of excitotoxic injury (Weiss *et al.* 2000; Dineley *et al.* 2003; Frederickson *et al.* 2004), another condition in which oxidative stress is a key event (Coyle & Puttfarcken 1993; Lafon-Cazal *et al.* 1993; Reynolds & Hastings 1995; Patel *et al.* 1996; Wang & Thayer 1996; White & Reynolds 1996). It has been demonstrated that Zn^{2+} interacts with lipoamide dehydrogenase and inhibits both the forward and the reverse reaction (see below) catalysed by the isolated enzyme (Gazaryan *et al.* 2002), pointing to the site of effect of Zn^{2+} on α-KGDH.

The susceptibility of α-KGDH to a variety of conditions somehow related to oxidative stress strongly suggests that the deficiency of this enzyme in oxidative stress could be crucial in the metabolic defects caused by ROS.

α-KGDH appears to be more sensitive to disturbed homeostatic factors than other enzymes. In post-mortem mouse brain samples, the activity of α-KGDH is quickly lost whereas the activity of another TPP-dependent enzyme, PDH, remains unaltered for at least 24 h (Gibson *et al.* 1988; Mizuno *et al.* 1990). Mastrogiacomo *et al.* (1993) found a correlation between the lactate level in the brain samples and the decrease in the activity of α-KGDH, which could indicate that α-KGDH is more sensitive to antemortem hypoxia than other enzymes involved in metabolism

(Terwel *et al.* 1998). It is of note here that the pH optimum of the brain mitochondrial α-KGDH is very narrow, between 7.2 and 7.4, and at pH values below 7.2 or above 7.8 the activity of the enzyme markedly decreased (Lai & Cooper 1986). α-KGDH purified from pig heart showed a broader pH optimum, between pH 6.6 and 7.4 when the maximum activity of the enzyme was measured, but the K_m of the enzyme was significantly increased in this pH range (McCormack & Denton 1979).

Sulphydryl groups which are critical to the catalytic activity of α-KGDH on the E2 subunit of the enzyme may be responsible for the increased vulnerability of the enzyme to ROS or nitric oxide, whereas tyrosine residues make this enzyme a target for peroxynitrate-induced nitration.

2. α-KGDH AS A GENERATOR OF OXIDATIVE STRESS

It is firmly believed that mitochondrial electron transport chain is a rich source of ROS (Boveris *et al.* 1972; Loschen *et al.* 1973). Large number of studies using different substrates supporting the respiration and different inhibitors of the respiratory complexes established that ROS can be produced at complex I (NADH ubiquinone oxidoreductase; Cadenas *et al.* 1977; Takeshige & Minakami 1979; Korshunov *et al.* 1997; Herrero & Barja 2000; Votyakova & Reynolds 2001; Liu *et al.* 2002; Starkov & Fiskum 2003; for review see Turrens 2003) and the Q-cycle of the respiratory chain (Boveris & Chance 1973; Loschen *et al.* 1974; Boveris *et al.* 1976; Turrens & Boveris 1980; Starkov & Fiskum 2001). With a critical view one has to admit that it is impossible to determine at present how and where in the respiratory chain ROS are generated in *in situ* mitochondria under resting condition, when during the cellular metabolism the respiratory chain receives electrons both from $FADH_2$ and NADH. *In situ* mitochondria in isolated nerve terminals react with an increased ROS generation to an already small degree of complex I inhibition, which suggest that this could be physiologically more important than ROS production in response to complex III inhibition, which requires more than 70% inhibition of this complex (Sipos *et al.* 2003*a*).

Recently, the generally accepted idea that complexes of the respiratory chain are the 'major' site of ROS production in the mitochondria has been challenged. Independent studies have demonstrated that α-KGDH could be an important source of ROS (Starkov *et al.* 2004; Tretter & Adam-Vizi 2004).

Earlier it has been reported that flavoproteins, generally, can activate oxygen, resulting in the production of superoxide and/or hydrogen peroxide (Ballou *et al.* 1969; Chan & Bielski 1980; Kakinuma *et al.* 1987; Massey 1994; Zhang *et al.* 1998). Lipoamide dehydrogenase, the E3 subunit of α-KGDH, is a flavoenzyme, being responsible for the transfer of reducing equivalents from lipoate, which is bound to the E2 subunit, to NAD^+, the final electron acceptor in the α-KGDH catalysed reaction (figure 2). Lipoamide dehydrogenase is not unique for α-KGDH, it is also a component of PDH and branched-chain ketoacid dehydrogenase. With dehydrogenases, generally, the flavoprotein-mediated ROS generation was found to be very slow with a primary product being poorly defined (Massey 1994). The E3 subunit of α-KGDH can be studied either as part of α-KGDH by using dihydrolipoate (plus NAD^+) instead of α-ketoglutarate as substrate, or as an isolated enzyme with the same substrate. Under this condition dihydrolipoate ($L(SH)_2$) is oxidized to lipoate (LS_2) [$L(SH)_2 + NAD^+ \rightarrow LS_2 + NADH$]. This reaction is reversible, in the presence of NADH, free lipoate is reduced to dihydrolipoate [$LS_2 + NADH \rightarrow L(SH)_2 + NAD^+$]. It was reported that isolated lipoamide dehydrogenase generates hydrogen peroxide in the reverse reaction exhibited under a non-physiological condition, when in the presence of oxygen only NADH but no lipoate, the substrate, is present (Huennekens *et al.* 1955; Massey *et al.* 1969; Bando & Aki 1991; Gazaryan *et al.* 2002). In the lipoamide dehydrogenase reaction, superoxide was also produced and the ratio of superoxide and hydrogen peroxide generated in the reaction was found to be 1 : 9 (Bando & Aki 1991). Interestingly, it has been reported that Zn^{2+}, which could inhibit α-KGDH (Brown *et al.* 2000), is able to stimulate the oxidase activity of the isolated lipoamide dehydrogenase (Gazaryan *et al.* 2002). In light of the report that Zn^{2+} is also able to induce ROS generation in the mitochondria (Sensi *et al.* 1999), it is possible that Zn^{2+}-induced ROS generation by lipoamide dehydrogenase could be of pathological importance.

Recently, our group (Tretter & Adam-Vizi 2004) and Starkov *et al.* (2004) have demonstrated that α-KGDH, during the physiological catalytic function of the enzyme, generates hydrogen peroxide and to a smaller extent superoxide. H_2O_2 generation by α-KGDH was inhibited by 80% in the presence of NAD^+ and accelerated by addition of NADH (Tretter & Adam-Vizi 2004). The most important regulator of the α-KGDH-mediated ROS generation appeared to be the NADH/NAD^+ ratio. When this was increased the physiological catalytic function and the ROS generation by α-KGDH were clearly dissociated, the physiological reaction by the enzyme being inhibited and the ROS generation becoming dominant (Tretter & Adam-Vizi 2004). With the isolated enzyme, ROS generation by the enzyme working in the physiological direction could be distinguished from that attributed to a reverse mode of the enzyme. When the physiological substrates (α-ketoglutarate and CoA) are present, but NAD^+, the physiological electron acceptor, is not available, electrons derived from the substrate oxidation reduce oxygen and produce ROS in the forward reaction. For the stimulation of ROS generation by NADH, no added substrates were required, only the presence of the enzyme and NADH was sufficient (Tretter & Adam-Vizi 2004). This clearly suggested that ROS is generated on the E3 subunit and for this electrons can be provided either by the oxidation of α-ketoglutarate involving the E1 and E2 subunits (forward, physiological direction) or by NADH in the non-physiological reverse reaction (figure 2).

α-KGDH-mediated ROS production demonstrated with the isolated enzyme could be significant in mitochondria as shown in studies performed with

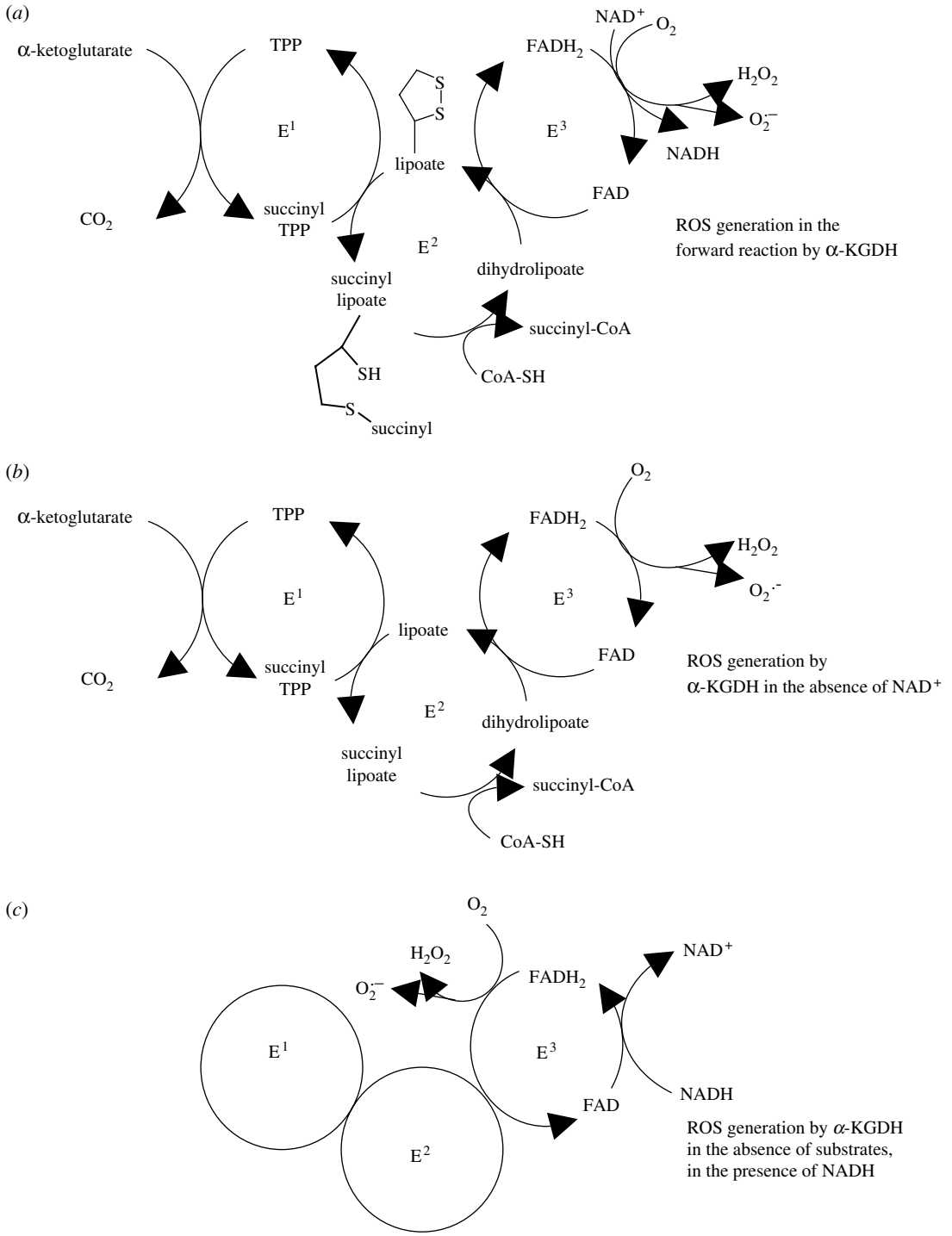

Figure 2. Generation of reactive oxygen species by α-KGDH. Subunit composition of α-KGDH and generation of ROS in the physiological forward reaction of the enzyme, when substrates and cofactors and oxygen are present (*a*). ROS generation in the forward reaction by α-KGDH is enhanced in the absence of NAD^+ (*b*). ROS generation by the E3 subunit induced by NADH in the absence of substrates (*c*). E1, α-ketoglutarate dehydrogenase; E2, dihydrolipoamide succinyltransferase; E3, dihydrolipoamide dehydrogenase; TPP, thiamine pyrophosphate.

isolated rat brain mitochondria (Starkov *et al.* 2004), or intact synaptosomes where ROS generation in *in situ* mitochondria can be investigated (Tretter & Adam-Vizi 2004). In the experiments by Starkov *et al.* (2004) ROS production in isolated mitochondria respiring on different substrates correlated with the reduction level of mitochondrial NAD(P)H, with the exception of α-ketoglutarate, which supported the highest rate of ROS generation. In the presence of the complex I inhibitor, rotenone, the rate of H_2O_2 generation was three times higher with α-ketoglutarate than with succinate (Starkov *et al.* 2004), which might be consistent with the effect of an increased NADH/NAD^+ ratio in the mitochondria under this condition.

In situ synaptic mitochondria present in isolated nerve terminals (synaptosomes) produce H_2O_2 in the presence of glucose under resting conditions, which can be detected by measuring H_2O_2 release from synaptosomes into the medium or following the activity of endogenous aconitase (Sipos *et al.* 2003*a*,*b*).

α-Ketoglutarate, which is taken up by synaptosomes, could maintain the mitochondrial respiration and ATP production by directly fuelling α-KGDH (Shank & Campbell 1984; Willoughby *et al.* 1989). When α-ketoglutarate maintained the mitochondrial metabolism the rate of H_2O_2 release from synaptosomes was 2.5 times higher than that measured in the presence of glucose, and consistent with this, the activity of endogenous aconitase as an indicator of ROS generation was significantly decreased (Tretter & Adam-Vizi 2004). This observation strongly supports the suggestion that α-KGDH present in mitochondria *in situ* could generate ROS in significant amount to interact with aconitase, which is arranged in the close proximity of α-KGDH in the Krebs cycle.

In the study performed by Starkov *et al.* (2004), ROS formation by α-KGDH was also attributed to the E3 subunit as brain mitochondria prepared from heterozygous knock-out mice deficient in the E3 subunit produced significantly less H_2O_2 than mitochondria prepared from their littermate wild-type mice (Starkov *et al.* 2004). The rate of H_2O_2 generation in the presence of α-ketoglutarate in response to rotenone was also significantly reduced in mitochondria isolated from E3-deficient mice. This result together with our observation that an increase in the NADH/NAD^+ ratio favours ROS generation by α-KGDH raises the possibility that generation of ROS by α-KGDH could contribute to the accumulation of ROS in mitochondria in response to inhibition of complex I. It is not possible to estimate, as yet, the extent of contribution of α-KGDH to the ROS generation in mitochondria, but it could be assumed that when complex I is inhibited, like in the case of Parkinson's disease (see below), the oxidative stress that is characteristic for the pathology of the disease (Beal 1996, 2001; Mizuno *et al.* 1998; Schapira 1999) is partly due to ROS generation by this enzyme. A further factor that makes this picture more intricate is that complex I itself is vulnerable to ROS (Zhang *et al.* 1990; Chinopoulos & Adam-Vizi 2001) and would be inhibited under oxidative stress creating a vicious cycle, eventually building up a large degree of oxidative stress.

Different radical species as side products in the 2-oxo acid dehydrogenase reaction was detected by spin trapping involving superoxide in the presence of oxygen and thiyl radical of the complex-bound dihydrolipoate in the absence of oxygen, and the latter was suggested to mediate the inactivation of the enzyme on the E1 subunit (Bunik & Sievers 2002). The physiological importance of this self-regulated inactivation occurring when the concentration of NAD^+ is low in the mitochondria could be the sparing of 2-oxo acid and prevention of production of radical species, as proposed by Bunik (2003). Given the sensitivity of α-KGDH to ROS discussed earlier, it is feasible to suggest that the enzyme is also inactivated when radicals are produced by the enzyme itself in the immediate vicinity of the ROS-sensitive components. However assuming that this happens *in vivo*, it may indeed spare α-ketoglutarate but radicals could still be generated even when E1 or E2 subunits are inhibited. Conditions involving a decrease in NAD^+ concentration in the mitochondria usually parallel an increase in NADH level, and ROS generation by α-KGDH induced by NADH, as shown in our study (Tretter & Adam-Vizi 2004), would require only the E3 subunit by which ROS generation could be maintained.

A beneficial aspect of ROS generation by α-KGDH in the physiological direction, when α-ketoglutarate is oxidized, could be also envisaged under specific pathological conditions. In particular, when reoxidation of NADH in the respiratory chain is impaired due to complex I deficiency it could be important to channel electrons to the respiratory chain through complex II and maintain ATP production. ROS generation by α-KGDH could enable the conversion of α-ketoglutarate to succinyl-CoA to a certain extent even when the physiological electron acceptor, NAD^+, is not sufficiently available. At present this is only a hypothesis and it needs further clarification as to what extent ROS generation by α-KGDH could serve the maintenance of the flow through the Krebs cycle, thus being of use for bioenergetics, particularly when complex I is inhibited. It is also unclear at present to what extent the α-KGDH-mediated ROS generation could contribute to the accumulation of ROS in mitochondria under conditions when ROS generation by the enzyme becomes dominant over the physiological NADH generation.

3. α-KGDH IN NEURODEGENERATION

Diminished activity of α-KGDH has been linked to the mitochondrial deficiency that is crucial in the neurodegenerative process in several neurodegenerative diseases.

A characteristic change in the bioenergetic parameters in the brain of patients suffering from Parkinson's disease is a decrease in the activity of complex I of the respiratory chain (Parker *et al.* 1989; Schapira *et al.* 1989, 1990). It has been hypothesized that this could be crucial in the initiation of degeneration in the nigro-striatal dopaminergic neurons. Interestingly, the only other enzyme that has been found to be altered in the post-mortem substantia nigra samples from these patients was α-KGDH (Mizuno *et al.* 1990, 1994). The role of this enzyme in the pathology of the disease is underlined by the inhibitory effect of MPP^+ and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine; Joffe *et al.* 1998) and other structurally related isoquinoline derivatives on this enzyme (McNaught *et al.* 1995). α-KGDH activity is reduced also in the brain of patients suffering from Wernicke–Korsakoff syndrome (Butterworth *et al.* 1993).

A consistent finding was that α-KGDH activity was decreased in the brain of Alzheimer's disease patients, involving regions affected by the disease as well as regions that remained normal (Gibson *et al.* 1988, 2000; Butterworth & Besnard 1990; Mastrogiacomo *et al.* 1996). The change in the α-KGDH activity was as consistent and characteristic as the abnormal amyloid metabolism (Gibson *et al.* 1988, 1998). α-KGDH in fibroblasts from patients with presenilin-1 mutation was either reduced (Sheu *et al.* 1994) or had increased vulnerability to oxidative stress (Gibson *et al.* 1999). Recently, it has been reported that α-KGDH-enriched neurons are lost in the temporal cortex in Alzheimer's

disease (Ko *et al*. 2001). The reduced enzyme activity may not be a causative event in the pathophysiology of Alzheimer's disease but the mitochondrial dysfunction due to the inhibition of α-KGDH could be a critical factor in the degeneration process (Gibson *et al*. 2000). The role of α-KGDH in the pathogenesis of neurodegenerative diseases is underlined by the observation that in rare cases of genetic defects of α-KGDH (Gibson *et al*. 2000) progressive neural degeneration was observed in patients, involving a deficient E3 subunit of α-KGDH (Kohlschutter *et al*. 1982).

The key role of a decreased α-KGDH activity in the events leading to cell death in the central nervous system is very clearly indicated in animal models of thiamine deficiency, in which the activity of α-KGDH is reduced in brain regions where the neurons die, whereas in regions that survive the activity of the enzyme is spared (Heroux & Butterworth 1995).

The exact role and the pathological significance of α-KGDH in the neurodegenerative processes are not clear at present, but given the crucial role in limiting the metabolism during oxidative stress as well as the ROS generation by this enzyme, it would be important to address in future studies the means by which α-KGDH can be prevented from producing excess amount of ROS and/or can be protected against oxidative damage.

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