The Human Krebs Cycle 2-Oxoglutarate Dehydrogenase Complex Creates an Additional Source of Superoxide/Hydrogen Peroxide from 2-Oxoadipate as Alternative Substrate.

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Abbreviations used: hE1o, human 2-oxoglutarate dehydrogenase; hE2o, human dihydrolipoyl succinyltransferase; hE3, human dihydrolipoyl dehydrogenase; hOGDHc, human 2-oxoglutarate dehydrogenase complex; PDC, pyruvate dehydrogenase complex; ecE1o, *E. coli* 2-oxoglutarate dehydrogenase; ecE2o, *E. coli* dihydrolipoyl succinyltransferase; DHTKD1, gene coding for dehydrogenase E1 and transketolase domain-containing protein 1; HRP, horseradish peroxidase; SOD, superoxide dismutase; ThDP, thiamin diphosphate; DCPIP, 2,6-dichlorophenol-indophenol; OG, 2-oxoglutarate; OA, 2-oxoadipate; hLDo, human lipoyl domain of hE2o; KMV, α -keto- β -methyl-n-valeric acid; FT-MS, Fourier transform mass spectrometry; CD, circular dichroism; EPR, Electron Paramagnetic Resonance.

Abstract

Recently, we reported that the human 2-oxoglutarate dehydrogenase (hE1o) component of the 2-oxoglutarate dehydrogenase complex (OGDHc) could produce the reactive oxygen species superoxide and hydrogen peroxide (detected by chemical means) from its substrate 2-oxoglutarate (OG), most likely concurrently with one-electron oxidation by dioxygen of the thiamin diphosphate (ThDP)-derived enamine intermediate to a C2 α -centered radical (detected by Electron Paramagnetic Resonance) [Nemeria *et al.*, 2014; Ambrus *et al.* 2015]. We here report that hE1o can also utilize the next higher homologue of OG, 2-oxoadipate (OA) as a substrate according to multiple criteria in our toolbox: (i) Both E1o-specific and overall complex activities (NADH production) were detected using OA as a substrate; (ii) Two post-decarboxylation intermediates were formed by hE1o from OA, the ThDP-enamine and the C2 α -hydroxyalkyl-ThDP, with nearly identical rates for OG and OA; (iii) Both OG and OA could reductively acylate lipoyl domain created from dihydrolipoyl succinyltransferase (E2o); (iv) Both OG and OA gave α -ketol carboligaton products with glyoxylate, but with opposite chirality; a finding that could be of utility in chiral synthesis; (v) Dioxygen could oxidize the ThDP-derived enamine from both OG and OA, leading to ThDP-enamine radical and generation of superoxide and H₂O₂. While the observed oxidation-reduction with dioxygen is only a side reaction of the predominant physiological product glutaryl-CoA, the efficiency of superoxide/H₂O₂ production was 7-times larger from OA than from OG, making the reaction of OGDHc with OA one of the important superoxide/ H₂O₂ producers among 2-oxo acid dehydrogenase complexes in mitochondria.

Keywords: 2-oxoglutarate dehydrogenase, 2-oxoglutarate dehydrogenase complex, 2-oxoadipate, ThDP-enamine radical, EPR, superoxide and H₂O₂ generation, α -ketol carboligaton products.

1. Introduction

The human 2-oxoglutarate dehydrogenase multienzyme complex (hOGDHC) is a key enzyme in the tricarboxylic acid (TCA) cycle and consists of multiple copies of three enzymes (Scheme 1): a thiamin diphosphate (ThDP)-dependent 2-oxoglutarate dehydrogenase (hE1 α ; EC1.2.4.2), dihydrolipoyl succinyl transferase (hE2 α ; EC 2.3.1.61), and dihydrolipoyl dehydrogenase (hE3; EC1.8.1.4) (Scheme 1) [1-4]. A diminished activity of hOGDHC has been linked to numerous neurodegenerative diseases, such as Alzheimer's disease [5, 6], Parkinson disease [7], Huntington's disease [8, 9], and progressive supranuclear palsy [10]. Recently, it was reported that OGDHC is involved in cellular regulation of mitochondrial proteins via succinylation of lysine residues [11] and the gene encoding OGDHC was identified as being responsible for stabilization of the hypoxia-inducible transcription factors (HIF's) under aerobic conditions [12]. Much attention has been focused on the generation of the reactive oxygen species (superoxide /H₂O₂), by hOGDHC [13-21]. It is known that hOGDHC has the highest capacity of H₂O₂ generation within the NADH/NAD⁺ isopotential group in mammalian mitochondria, which also includes the pyruvate dehydrogenase complex (PDC), branched-chain α -ketoacid dehydrogenase complex (BCKDHC), and more recently, the putative 2-oxoadipate dehydrogenase complex (OADHC) (see ref.19 for review). All 2-oxo acid dehydrogenase complexes share the same flavin-containing E3 component in a particular cell, the component, which is suggested to be the major source of superoxide/H₂O₂ generation by mammalian mitochondria [13, 14, 22, 23]. The E3-bound reduced flavin is believed to undergo two single-electron oxidation steps by oxygen,

leading to production of superoxide and H_2O_2 [16, 24, 25]. There are two possible ways to flavin reduction within OGDHc: (i) from OG oxidation in the forward physiological reaction, or (ii) from NADH oxidation in the reverse reaction. Independently of E3, which is not specific for any of the 2-oxo acid dehydrogenase complexes, the hE1o by itself and the hE1o-hE2o sub-complex were also shown to be able to produce superoxide/ H_2O_2 from decarboxylation of OG in the physiological direction, with no such activity detected for hE2o by itself [17, 18]. The major source of superoxide/ H_2O_2 on hE1o was shown to be the ThDP-derived enamine radical, detected by electron paramagnetic resonance spectroscopy (EPR) in the active centers of both hE1o [17] and *E. coli* E1o [26] upon reaction of E1o's with OG [17]. The specific activity of superoxide and H_2O_2 production by hE1o on its own was less than 1% of the E1o-specific activity [17], and was consistent with the fraction of the radical intermediate species occupying the hE1o active centers (0.2% occupancy for hE1o on its own and 0.59% occupancy for hE1o assembled into hOGDHc). The data indicated that hE1o does produce both the ThDP-enamine radical and superoxide/ H_2O_2 that could contribute to oxidative stress involved in Alzheimer's disease pathogenesis, especially under conditions of hE2o deficiency [27, 28].

It was of importance to further investigate whether or not the substrate specificity of the hE1o and hOGDHc would tolerate the homologue 2-oxoadipate (OA), differing only by an additional CH_2 group from OG. Recent studies have reported that rat skeletal muscle mitochondria could produce superoxide/ H_2O_2 when using 2-oxoadipate as a substrate [29]. This function was assigned to a putative 2-oxoadipate dehydrogenase complex (OADHc), specifically to its FAD-containing E3 component, rather than to OGDHc, due to its potential inhibition at high concentrations of OA [29]. Taking into account that there are already eleven distinct sites known to be associated with superoxide/ H_2O_2 generation by mammalian mitochondria [19], it is

a great challenge to evaluate each site's individual contribution from OA. Our first question is whether hOGDHc decarboxylates OA with production of NADH and glutaryl-CoA. Earlier, the 2-oxoadipate dehydrogenase activities by OGDH complexes from bacterial [31] and mammalian sources [32, 33] were reported but no such studies were reported for the human OGDHc. This is important in view of the controversy regarding which enzyme system, hOGDHc or the putative OADHc is involved in the conversion of OA to glutaryl-CoA in mammalian mitochondria [30, 34-36]. Our study is also important to our understanding of the role of hOGDHc in glutaric aciduria type I, the neurodegenerative disorder associated with accumulation of neurotoxic glutaric and 3-hydroxyglutaric acid, that is caused by the deficiency of glutaryl-CoA dehydrogenase, which plays a role in the degradation pathways of L-lysine and L-tryptophan [30,35].

We here report that according to numerous independent experiments, OA behaves very similarly to OG as an alternate substrate (see Scheme 1 for OA), but not inhibitor of the hOGDHc. Perhaps the most significant finding is that H_2O_2 generation efficiency of hE1o and an assembled hOGDHc from OA was about 7-times higher compared to that with the physiological substrate OG under similar conditions. Once more, as with OG, the superoxide/ H_2O_2 is being derived from one-electron oxidation of the ThDP-bound enamine intermediate derived from OA on hE1o by O_2 . These results also make a significant contribution to our understanding of an important current topic of interest, the efficiency of superoxide/ H_2O_2 production from OA by the hOGDHc. Our studies were made possible by our successful expression of the hE1o and hE2o components [17].

2. Materials and methods

2.1. Reagents.

ThDP, NAD⁺, CoA, DTT and IPTG were from Affimetrix; hydrogen peroxide, peroxidase from horseradish, superoxide dismutase, thiamin·HCl, DCPIP, benzamidine·HCl, 2-oxoglutaric acid and 2-oxoadipic acid were from Sigma-Aldrich; AmplexTM UltraRed was from Invitrogen via Thermo Fisher Scientific.

2.2. Protein expression and purification.

Expression and purification of hE1o, hE2o and hE3 were as reported earlier [17].

2.3. Overall OGDHc activity of NADH production.

The reaction medium contained the following in 1.0 ml: 0.10 M Tris·HCl (pH 7.5), 0.50 mM ThDP, 2.0 mM MgCl₂, 2.0 mM DTT, 2.5 mM NAD⁺, 0.1-0.2 mM CoA and hOGDHc assembled from independently expressed and purified hE1o, hE2o and hE3 components at 37 °C. The reaction was initiated by addition of 2 mM OG or 5 mM OA and CoA. For hOGDHc assembly, the hE1o (0.125 mg) was incubated with hE2o (0.250 mg) and hE3 (1.25 mg) with mass ratio of hE1o:hE2o:hE3 of 1:2:10 in 50 mM KH₂PO₄ (pH 7.5) containing 0.20 M NaCl, 0.5 mM ThDP, and 2.0 mM MgCl₂ at 25 °C. After 30 min of incubation, an aliquot of the reaction mixture containing 5 µg of hE1o and the corresponding amounts of hE2o and hE3 was withdrawn for the overall OGDHc activity measurement. Steady-state velocities were taken from the linear part of the progress curves recorded at 340 nm. One unit of activity is defined as the amount of NADH produced (µmol·min⁻¹·mg hE1o⁻¹).

2.4. E1o-specific assay.

The E1o-specific activity was measured with the external oxidizing agent 2,6-dichlorophenol-indophenol (DCPIP) by monitoring its reduction at 600 nm. The reaction medium in 1.0 ml

contained: 50 mM KH₂PO₄ (pH 7.0), 0.25 mM ThDP, 2.0 mM MgCl₂, 2 mM OG or 5 mM OA and DCPIP (0.08 mM) at 37 °C. The reaction was initiated by the addition of 0.015 mg of hE1o. Steady-state velocities were taken from the linear portion of the progress curves recorded at 600 nm. One unit of activity is defined as the amount of reduced DCPIP produced (μmol· min⁻¹· mg hE1o⁻¹).

2.5. Dependence of the overall hOGDHc and hE1o-specific activities on concentration of OA.

The hOGDHc and hE1o activities were measured as presented above using OA in 0.125– 10.0 mM range for hOGDHc and in 0.025–7.0 mM range for hE1o. The data points were fit to a modified Hill equation (equation 1)

$$\{y = y_0 + ((\text{rate}_{\max} * x^n) / (S_{0.5}^n + x^n))\}. \quad (1)$$

2.6. Measurement of the rate of H₂O₂ production.

The rate of H₂O₂ production by hE1o and by assembled hOGDHc was measured using a fluorescent Amplex UltraRed reagent, in an assay highly recommended in the literature [29,37-39]. The reaction medium in 2.5 ml contained: 50 mM KH₂PO₄ (pH 7.0), 0.5 mM ThDP, 1.0 mM MgCl₂, 50 μM Amplex UltraRed, 5 units/ml of horseradish peroxidase (HRP), 25 units/ml of superoxide dismutase (SOD), 0.4 mM ADP (when needed) and 0.0992 mg of hE1o (concentration of active centers = 0.357 μM) at 37 °C. Time-dependent H₂O₂ production was measured by using a Varian Cary Eclipse spectrofluorimeter in the kinetic mode with an excitation wavelength of 560 nm and emission wavelength of 590 nm. The reaction was initiated by OG (2 mM) or OA (5 mM) after 2-3 min recording of background due to the spontaneous oxidation of Amplex UltraRed. The amount of H₂O₂ produced was calculated from a calibration curve that was generated with known concentrations of H₂O₂ using a reaction assay similar to that used in the experiments. The slope due to background oxidation of Amplex UltraRed was

subtracted from all experimental curves. The activity is defined as the amount of H_2O_2 produced (amount of Amplex UltraRed oxidized), taking into account that Amplex UltraRed reacts with HRP- H_2O_2 at a 1:1 stoichiometry ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg hE1o}^{-1}$) [38]. For hOGDHc assembly, hE1o, hE2o, and hE3 were mixed in 50 mM KH_2PO_4 (pH 7.0) containing 0.50 mM ThDP and 1 mM MgCl_2 with a 0.222 mM concentration of subunits for each protein (ratio of hE1o:hE2o:hE3 subunits = 1:1:1) for 30 min at 25 °C. Aliquots containing 0.0992 mg of hE1o and the corresponding amounts of hE2o and hE3 were withdrawn for measuring of the H_2O_2 production in the reaction assay as presented above. A calibration curve was generated with known amounts of H_2O_2 at the end of each experiment.

2.7. Reductive acylation of hLDo by hE1o and OG or OA in vitro as detected by Fourier transform mass spectrometry.

Reductive acylation of the E2o lipoyl domain (hLDo) was as reported by us earlier [40,41]. In a steady-state experiment, the hLDo (100 μM) was incubated with hE1o (0.025 μM hE1o with OG and 0.10 μM hE1o with OA) in 35 mM NH_4HCO_3 (pH 7.5) containing 0.5 mM MgCl_2 and 0.10 mM ThDP. The reaction was initiated by addition of 2.0 mM OG or 2.5 mM OA. An aliquot of 10 μL of the reaction mixture was withdrawn at different times and was quenched into 1 mL of 50% methanol, 0.1% formic acid. Samples were analyzed on relative amounts of the succinylated (or glutarylated) and unsuccinylated (or unglutarylated) forms of LDo by Fourier transform mass spectrometry (FT-MS). The ratio of intensity of the succinylated hLDo versus that of the total intensity (sum of succinylated and unsuccinylated hLDo) was plotted against time. The rates of the reaction were calculated from the initial slope.

2.8. Synthesis of carboligation products by hE1o.

The reactions were performed in 1.2 ml of 50 mM KH_2PO_4 (pH 7.5) containing 0.20 M NH_4Cl , 2 mM MgCl_2 , 0.50 mM ThDP, 30 mM OG or 30 mM OA, 20 mM glyoxylate and 5% DMSO. Reactions were initiated by addition of hE1o (the final concentration of 0.7 mg/ml) and were performed at 25 °C and 300 rpm using Thermomixer (Eppendorf). After 24h of incubation, 200 μL of the reaction mixtures were withdrawn and circular dichroism (CD) spectra were recorded on a Chirascan spectrometer (Applied Photophysics, Leatherhead, UK) in the near-UV CD region (270-450 nm). The CD spectra show a negative band with $\lambda_{\text{max}}=278$ nm for (*R*)-2-hydroxy-3-oxoadipate and a positive CD band with $\lambda_{\text{max}}=278$ nm for (*S*)-2-hydroxy-3-oxoheptanedioic acid. For NMR analysis, the pH of the entire reaction mixture after 48 h of incubation was adjusted to pH 3.0 with 50% formic acid (25 μL) and products were extracted with 600 μL of CDCl_3 as reported in the literature [42].

2.9. Near-UV CD spectra of hE1o.

CD spectra of hE1o (1.5 mg/ml, 13.3 μM active centers) in 50 mM KH_2PO_4 (pH 7.5) containing 0.15 M NaCl, 0.20 mM ThDP, 2.0 mM MgCl_2 and 3 mM OA were recorded in the near-UV region (290-450 nm) using a 1 cm path cell at 37 °C.

2.10. Stopped-flow CD spectroscopy.

Stopped-flow CD spectroscopy was as reported by us recently [17]. Kinetic traces were recorded on a Pi*-180 stopped-flow CD spectrometer (Applied Photophysics) using 10 mm path length.

The hE1o (4.0 mg/ml, 35.9 μM active centers) in 50 mM KH_2PO_4 (pH 7.5) containing 0.15 M NaCl, 0.20 mM ThDP and 2.0 mM MgCl_2 in one syringe was mixed in the reaction cell with OG (2 mM) or with OA (10 mM) in the same buffer system from the second syringe and the reaction was monitored at 365 nm or at 313 nm for 30-35 s at 6 °C. The data points were fit using a double exponential (equation 2):

$$CD_{365} = a * (\exp(-k_1 * x) + c * (\exp(-k_2 * x)) + y_o, \quad (2)$$

or a single exponential with slope (equation 3): (3)

$$CD_{313} = a * (\exp(-k_1 * x)) + c * x + y_o.$$

2.11. Electron Paramagnetic Resonance Spectroscopy.

For hE1o sample preparation, the following protocol was used: hE1o (30.00 - 40 mg/ml, 0.267 - 0.355 mM hE1o active centers) in 0.30 ml of 50 mM HEPES (pH7.5) containing 0.15 M NaCl, 2.0 mM MgCl₂, 0.5 mM ThDP and 1.0 mM benzamidine·HCl was mixed with 10 mM OG or 10 mM OA at room temperature. The mixture was immediately transferred into an EPR tube and was flash-frozen in liquid nitrogen after approximately 30-40 s of incubation of all components.

For preparation of hOGDHc for EPR experiments, hOGDHc was assembled by mixing of the hE1o (0.20 mM subunits), hE2o (0.18 mM subunits), and hE3 (0.18 mM subunits) in 50 mM HEPES (pH 7.5), containing 0.50 mM ThDP, 1.0 mM MgCl₂, 0.15 mM NaCl and 1.0 mM benzamidine·HCl. Reaction was started by addition of OA to 10 mM final concentration at room temperature. The mixture was immediately transferred into an EPR tube and was flash-frozen in liquid nitrogen after approximately 40 s of incubation of all components.

X-band (9 GHz) EPR measurements were made using a Varian E-112 spectrometer equipped with a TE₁₀₂ cavity and interfaced to a PC using custom written software. The sample temperature was held at 77K using an immersion finger dewar. Spectrometer parameters used to acquire the spectra are as follows: modulation amplitude: 1x10 G; microwave power, 0.20 milliwatt; receiver gain, 3.20 x 10⁴; microwave frequency, 9.1 GHz, scan time 2.0 min; time constant, 1 s; number of scans 4. The field was calibrated using a standard sample of manganese doped in MgO [43].

3. Results

3.1. The 2-oxoadipate homologue of OG could serve as a substrate for hOGDHc.

First, we wish to address the question whether or not hOGDHc decarboxylates OA, an analogue of OG differing by an additional CH₂ group from OG, with production of NADH and glutaryl-CoA. Earlier, 2-oxoadipate dehydrogenase activities were reported by OGDH complexes from bacterial [31] and some mammalian sources [32,33]. However, no such studies could be carried out by human OGDHc, due to lack of availability of the human E1o and E2o components until our success in creating them in active functional form [17]. The steady-state kinetic parameters determined for OA transformation by hOGDHc assembled from hE1o, hE2o and hE3 components and by hE1o individually are summarized and compared with results on OG in Table 1 (see also SI Fig. S1 for progress curves of NADH formation from OA). The rate constants of NADH production in the overall OGDHc assay using different preparations of hE1o assembled with hE2o and hE3 into hOGDHc were in the 12-25% range of that with OG. According to E1-specific assay, the activity was in the 74-100% range compared to OG. Taking into account the $K_{m,OG}$ of 0.15 mM [17] and the $K_{m,OA}$ of 0.52 mM (Fig. 1), both determined in the overall OGDHc reaction, ranges of values of $k_{cat}/K_{m,OG}$ ($46 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ – $142 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) and $k_{cat}/K_{m,OA}$ ($1.7 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ - $10 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) were calculated, and revealed that the catalytic efficiency of hOGDHc with OA was about 14-30-fold smaller than with OG (Table 1). It is also important to note that no inhibition of OGDHc or hE1o-specific activity was observed at concentrations of OA up to 8 mM (Fig. 1). The pH dependence of the overall hOGDHc activity with OA displayed maximum activity at pH 7.5 with apparent pK_a values of 6.89 and 8.34 (Fig. 2), which correlate well with the pH optimum of 6.5-7.5 for OG, and an apparent pK_a of 8.48

reported by us earlier [17]. On the basis of these experiments, our subsequent experiments with OA were conducted at pH 7.5.

3.2. Evidence for reactivity of 2-oxoadipate in communication between hE1o and hE2o via the reductive acyltransferase reaction.

The enamine intermediate formed on hE1o after decarboxylation of OG or OA reductively acylates the lipoamide groups on hE2o with the formation of S8-succinyldihydrolipoyl-E2 or S8-glutaryldihydrolipoyl –E2 (see Scheme 1 for OA). Reductive succinylation or glutarylation of hE2o by hE1o and OG or OA, respectively, could be analyzed by FT-MS in a model reaction, in

Table 1. Steady-state kinetic parameters for hOGDHc and hE1o with OA and OG.

hE1o ^a	Overall OGDHc activity, OG ^b (units·mg E1o ⁻¹)	k_{cat} (s ⁻¹)	$k_{cat}/K_{m,OG}$ (M ⁻¹ s ⁻¹) ^c	Overall OGDHc activity, OA ^b (units· mg E1o ⁻¹)	k_{cat} (s ⁻¹)	$k_{cat}/K_{m,OA}$ (M ⁻¹ s ⁻¹) ^c
1	4.86 ± 0.59	18.4	123 x10 ³	0.95 ± 0.09	3.64	7.0 x10 ³
2	2.34 ±0.19	8.9	59 x10 ³	0.28 ± 0.02	1.06	2.0 x10 ³
3	1.82 ±0.33	6.9	46 x10 ³	0.23 ± 0.02	0.87	1.7 x10 ³
5	5.63 ±0.59	21.3	142 x10 ³	1.38 ±0.07	5.23	10 x10 ³
hE1o ^a	E1o-specific activity, OG ^b (units· mg E1o ⁻¹)	k_{cat} (s ⁻¹)	$k_{cat}/K_{m,OG}$ (M ⁻¹ s ⁻¹) ^c	E1o-specific activity, OA ^b (units· mg E1o ⁻¹)	k_{cat} (s ⁻¹)	$k_{cat}/K_{m,OA}$ (M ⁻¹ s ⁻¹) ^c
1	1.10 ± 0.05	4.2	247 x10 ³	0.92 ± 0.06	3.48	12.9 x10 ³
2	0.43 ± 0.02	1.6	94 x10 ³	0.32 ± 0.02	1.21	4.5 x10 ³
3	0.36 ± 0.02	1.36	80 x10 ³	0.41 ± 0.01	1.55	5.7 x10 ³
4	0.33 ± 0.01	1.2	71 x10 ³	0.26 ± 0.01	0.98	3.6 x10 ³

^a Five different preparations of hE1o were analyzed. Similar numbers indicate similar preparation of hE1o. ^bOne unit of activity is defined as the amount of NADH produced by the

assembled hOGDHc or the amount of DCPIP_{reduced} by hE1o ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg hE1o}^{-1}$). The $K_{m,OG}$ of 0.15 mM and $K_{m,OA}$ of 0.52 mM were determined in the overall assay; the $K_{m,OG}$ of 0.017 mM and $K_{m,OA}$ of 0.27 mM were determined in DCPIP assay.

Fig. 1. Dependence of the overall hOGDHc and hE1o-specific reactions on [2-oxoadipate]. Data points were fit to a modified Hill equation $1\{y = y_0 + ((\text{rate}_{\text{max}} * x^n) / (S_{0.5}^n + x^n))\}$; the lines are the regression fit trace. For experimental conditions, see Materials and methods.

Fig. 2. pH-Dependence of the OGDHc activity with OA. The reaction was carried out in 0.1 M Tris·HCl in the pH range of 6.5-9.3. The values of activity were plotted to a curve defined by two ionizing groups according to $\log(\text{activity}) = \log(\text{activity}_{\text{max}}) - \log(1 + 10^{\text{pK}_1 - x}) + 10^{x - \text{pK}_2}$, where x is the value of pH.

in which the hE2o was replaced by an independently expressed lipoyl domain (hLDo) as reported by us [40,41]. Herein is provided direct evidence that reaction intermediates are transferred between the active centers of hE1o and hE2o, i.e., its hLDo. First, on incubation of hLDo with hE1o and OG, two forms of hLDo were detected: reduced form of hLDo with mass of 11,248.1 Da (the theoretical mass of LDo = 11,246.2 Da) and reductively succinylated form of hLDo with mass of 11,349.1 Da. The fraction of reductively succinylated hLDo versus total hLDo (succinylated plus unsuccinylated hLDo) was plotted versus time and led to $k_{\text{succinylation}}$ of 77.6 s^{-1} (Fig. 3), which could be compared with the highest value of k_{cat} of 21.3 s^{-1} in Table 1 calculated for NADH production by hOGDHc from OG. The results indicate that reductive succinyl transfer between hE1o and hE2o is not a rate-limiting step in the hOGDHc reaction with its natural substrate, OG. Next, in a similar experiment with OA, a rate constant for reductive glutarylation of hLDo of 1.8 s^{-1} was calculated, approximately 43-fold lower than that for OG under similar conditions and approximately 3-fold lower compared to the highest value of k_{cat} of 5.23 s^{-1} detected for NADH production from OA (Table 1). With no significant difference between their K_m values ($K_{m,\text{OG}} = 0.15 \text{ mM}$, $K_{m,\text{OA}} = 0.52 \text{ mM}$, both measured in the overall OGDHc assay), the data indicate that in hOGDHc, the hLDo is responsible for specificity of acyl recognition and that glutaryl transfer to hE2o could be a rate-limiting step. For comparison, rate constants of 20 s^{-1} and 22 s^{-1} were obtained for reductive glutarylation of the *E. coli* LDo by ecE1o and OA, approximately 5-fold lower than that for OG, with no reduction in glutaryl transfer rate between ecE1o and ecE2o (a k_{cat} of 2.8 s^{-1} was calculated for NADH production from OA) (to be presented elsewhere).

Fig. 3. Time dependence of fraction of acylated hLDo measured during steady-state reaction. The hLDo (100 μM) in 35 mM NH_4HCO_3 (pH 7.5) containing 0.5 mM MgCl_2 and 0.10 mM ThDP was acylated by hE1o (0.025 μM for OG and 0.10 μM for OA) in the presence of 2.0 mM OG or 2.5 mM OA. Samples were analyzed for relative amounts of the acylated and unacylated forms of hLDo by FT-MS.

Scheme 1. Mechanism of OGDHc with 2-oxoadipate showing enamine side reactions.

3. Formation of the carboligation products by hE1o from OG or OA and glyoxylate.

Alternatively, in the absence of hE2o or hLDo for reductive acylation, the enamine intermediate formed after decarboxylation of OG or of OA on hE1o, could add to an aldehyde acceptor to form what are known as carboligation products (Scheme 1) [44-46]. Since carboligation results in a chiral product by virtue of the newly formed α -ketol functional group, they could be detected by circular dichroism (CD) spectroscopy. A negative CD band with λ_{max} at 278 nm was observed in the near-UV CD spectra of hE1o with OG as a substrate donor and glyoxylate as a substrate acceptor suggesting the formation of (*R*)-2-hydroxy-3-oxohexanedioic acid [(*R*)-2-hydroxy-3-oxoadipate] (Fig. 4). An activity of $0.054 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg hE1o}^{-1}$ ($k = 0.2 \text{ s}^{-1}$) for (*R*)-2-hydroxy-3-oxoadipate formation could be determined from the linear slope of the progress curves recorded in kinetic CD experiment (not shown). With OA as a substrate and glyoxylate as acceptor, the formation of (*S*)-2-hydroxy-3-oxoheptanedioic acid (2-hydroxy-3-oxopimelic acid) could be detected as a positive CD band with λ_{max} at 278 nm (Fig. 4) [47]. A ^1H NMR analysis of the carboligation reaction with OG assigned the product as 2-hydroxy-3-oxohexanedioic acid (SI Fig.S2), while with OA as 2-hydroxy-3-oxoheptanedioic acid (SI Fig. S3). The studies presented above provided additional evidence for the formation of the ThDP-enamine intermediate on hE1o from OA.

3.4. Circular dichroism evidence of the presence of ThDP-bound post-decarboxylation intermediates from 2-oxoadipate.

Earlier, we reported that on CD titration of the hE1o by OG, two positive CD bands were in evidence: one broad band around 347 nm that could be assigned to the post-decarboxylation ThDP-enamine intermediate, and a second one at 302 nm that could also be assigned to the

Fig. 4. CD detection of the carboligation products formed by hE1o from 2-oxoglutarate or 2-oxoadipate and glyoxylate. The hE1o (0.72 mg/ml) in 1.2 ml of 50 mM KH_2PO_4 (pH 7.5) containing 0.20 M NH_4Cl , 2 mM MgCl_2 , 0.5 mM ThDP and 5% DMSO was incubated with 30 mM OG or 30 mM OA and 20 mM glyoxylate at 25 °C before recording CD spectra.

Fig. 5. Circular dichroism spectra of hE1o with and without 2-oxoadipate. CD spectra of hE1o (1.5 mg/ml, 13.3 μM active centers) in 50 mM KH_2PO_4 (pH 7.5) containing 0.15 M NaCl, 0.1 mM ThDP, and 0.5 mM MgCl_2 were recorded in the absence and presence of 3 mM OA at 37 °C.

second post-decarboxylation ThDP-bound intermediate (C2- α -hydroxy- δ -carboxypropyl-ThDP) [17]. These assignments were based on analogy with observations made with ecE1o, where similar CD bands at 351 nm (ThDP-enamine intermediate) and at 303 nm (C2- α -hydroxy- γ -carboxypropyl-ThDP) were detected and assigned by NMR [17]. It was also reported that the ThDP-enamine intermediate is formed on ecE1o about three-four times faster than the second post-decarboxylation intermediate (C2- α -hydroxy- γ -carboxypropyl-ThDP), confirming that the positive CD band at 351 nm is a signature for the ThDP-enamine intermediate [17].

Next, similar CD experiments were carried out with hE1o and OA. On titration of hE1o by OA, two positive CD bands centered at 301 nm and at 351 nm were detected, suggesting the formation of two post-decarboxylation ThDP intermediates, similar to those reported with OG earlier (Fig. 5) [17]. However, the ThDP intermediates from OA are less stabilized on hE1o,

making their detection by steady-state CD difficult not only at 37 °C, but even at 6 °C. The rates of formation of the ThDP intermediates from OG and OA were therefore determined in pre-steady state experiments by using stopped-flow CD experiments at 365 nm and at 313 nm (Fig. 6). The choice of wavelengths was necessitated by the need to use optimal intensity of the lamp nearest to the wavelength of interest in Fig. 5. In the experiment at 365 nm, where the rate of ThDP-enamine formation was measured, a rate constant of $0.85 \pm 0.33 \text{ s}^{-1}$ was calculated for hE1o with OG (Fig. 6). In comparison, an average rate constant of $0.52 \pm 0.08 \text{ s}^{-1}$ was calculated for formation of enamine from hE1o and OA, indicating less than two-fold difference between the two substrates for formation of the ThDP-enamine (Table 2). Related to conversion of the enamine-ThDP intermediate to the second post-decarboxylation intermediate observed at 313 nm (presumably, protonation at C2 α of the C2-(α -hydroxy) - δ -carboxy-butyldiene ThDP in Scheme 1), rate constants of $0.53 \pm 0.02 \text{ s}^{-1}$ (OG) and $0.40 \pm 0.01 \text{ s}^{-1}$ (OA) were calculated indicating that these rate constants are similar for the two substrates (Table 2). As this reaction is off-pathway, this near identity of the rate constants forming them for OG and OA is expected and confirms the mechanism suggested earlier for OG as presented for OA in Scheme 1 [17].

Table 2. Pre-steady-state kinetic constants for the formation of the post-decarboxylation ThDP-bound covalent intermediates by hE1o from OG and OA.

Rate constants at 365 nm (s^{-1}) ^a		Rate constants at 313 nm (s^{-1}) ^a	
2-oxoglutarate ^b	2-oxoadipate ^b	2-oxoglutarate ^b	2-oxoadipate ^b
$k_1 = 0.85 \pm 0.33$	$k_1 = 0.52 \pm 0.08$	$k_1 = 0.53 \pm 0.02$	$k_1 = 0.40 \pm 0.01$
$k_2 = 0.26 \pm 0.13$		$k_2 = 0.043$	

^aAn average rate constant presented from three experiments. ^bData were fit using a double exponential equation (OG) or a single exponential equation (OA).

Fig. 6. Pre-steady-state kinetics of ThDP-enamine formation on hE1o. (Top) Time-dependence of the ThDP-enamine formation from OG.

3.5. EPR detection of the ThDP-enamine radical on hE1o from 2-oxoadipate.

Earlier we reported that hE1o could generate the ThDP-enamine radical from OG that could be detected by EPR [17]. To extend these studies to OA, X-band EPR measurements were made for the reactions where hE1o was aerobically incubated with 10 mM OG at room temperature. After different times of incubation indicated in Fig. 7 (left), the samples were flash-frozen in liquid nitrogen and were analyzed by EPR. As demonstrated in Fig. 7, the thiamin-enamine radical species were detected at all analyzed times, although they displayed lower amplitude at longer times of

incubation. The time-dependence of the amplitude in Fig. 7 (right), with the exception of the amplitude at 40 s, represents an exponential decay plot that allowed estimation of $\tau_{1/2}$ of ~ 1 min for the half-life of the ThDP-enamine radical on hE1o. These results ensure us that in most of the EPR experiments where the mixing time was in the 30-40 s range, more than half of the ThDP-enamine radical is still bound on hE1o. Perhaps most important is that the X-band continuous wave EPR spectra of hE1o (0.268 mM concentration of active centers) with 0.5 mM ThDP and 10 mM OA displayed a line shape consistent with ThDP-enamine radical species, similar to that detected in hE1o-ThDP-OG system and reported by us earlier (Fig. 8, spectrum B). However, the spectra of hE1o with OA exhibited the characteristics of slight saturation, making the

determination of absolute radical concentrations difficult. The ThDP-enamine radical species was also detected in samples of hOGDHc with OA, where hE1o was assembled with hE2o and hE3 into hOGDHc (Fig. 8, spectrum A). A comparison using peak to peak height rather than

Fig. 7. X-band EPR spectra of the ThDP-enamine radical species generated on hE1o from OG. (Left) Stack-plot of EPR spectra generated at different times of incubation: 28s (A); 35s (B); 40s (C); 76 s (D); 180s (E). The hE1o (31.0 mg/ml, 0.275 mM concentration of active centers) in 50 mM HEPES (pH 7.5) containing 0.15 M NaCl, 2.0 mM MgCl₂, 0.5 mM ThDP and 1.0 mM benzamidine·HCl was mixed with 10 mM OG. The mixture was immediately transferred into an EPR tube and was flash-frozen in liquid nitrogen after the indicated times of incubation. (Right) Time-dependence of the EPR amplitudes.

integrated intensity, indicates that formation of ThDP-enamine radical from OA was unaffected on hOGDHc assembly. The EPR spectra of the ThDP-enamine radical generated using OG and OA under similar conditions are shown in Fig. 9. The radical generated from OA is ~3

times lower in concentration and has a slightly broader line width than that using OG. Earlier we reported the concentration of ThDP-enamine radical intermediate of 0.9 μ M for the hE1o/OG/ThDP system (0.449 mM concentration of hE1o active centers, 0.2% occupancy) and ~1.3 μ M for the hOGDHc assembled from hE1o, hE2o and E3 (0.222 mM concentration of hE1o active centers, 0.59% occupancy) indicating that less than 1% of active centers of hE1o were occupied by the radical species. The data clearly indicate formation of ThDP-enamine radical species on hE1o from OA by itself or when it was assembled into hOGDHc.

Fig. 8 X-band EPR spectra of the ThDP-enamine radical species generated from OA by hE1o on its own and by hE1o assembled into hOGDHc. **A.** EPR spectra of hOGDHc with OA. The concentration of subunits: hE1o (0.225 mM); hE2o (0.200 mM) and E3 (0.178 mM) in 50 mM HEPES (pH 7.5) containing 0.15 M NaCl, 0.50 mM ThDP, 2.0 mM MgCl_2 and 1.0 mM benzamidine·HCl. **B.** EPR spectra of hE1o with OA. The hE1o (30.18 mg/ml, 0.268 mM concentration of subunits) was mixed with 10 mM OA at room temperature in the same buffer system. In both experiments, the mixture was immediately transferred into an EPR tube and was flash-frozen in liquid nitrogen after 40 s of incubation with OA.

Fig. 9 Comparison of the X-band EPR spectra of the ThDP-enamine radical species generated on hE1o from OG and from OA. **A.** EPR spectra of the ThDP-enamine radical species generated form OG. The hE1o (25.33 mg/ml, 0.225 mM concentration of active centers) in 50 mM HEPES (pH 7.5) containing 0.15 M NaCl, 0.50 mM ThDP, 2.0 mM MgCl_2 and 1.0 mM benzamidine·HCl was mixed with 10 mM OG at room temperature. **B.** EPR spectra of the ThDP-enamine radical species generated from OA. The hE1o (30.18 mg/ml, 0.268 mM concentration of active centers) was mixed with 10 mM OA in the same buffer system. Both samples were flash-frozen in liquid nitrogen after 40s of incubation.

3.6. Evidence for H_2O_2 production by hE1o and hOGDHc from OA.

It was reported by us earlier that hE1o could generate superoxide/ H_2O_2 from OG with the activities comprising 0.25% (H_2O_2) and 0.12% (superoxide) compared to hE1o-specific activity of $1.78 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg hE1o}^{-1}$ [17]. It was also reported that hE1o by itself and hE1o assembled into hE1o-hE2o sub-complex produce superoxide from OG with similar rates of ~ 2.0

nmol · min⁻¹ · mg hE1o⁻¹, indicating that hE2o does not contribute to superoxide production [18].

In this paper the rates of H₂O₂ production were measured by using the fluorescence Amplex UltraRed- horseradish peroxidase method reported in the literature [38,39] in the presence of exogenous superoxide dismutase, which converts two molecules of superoxide into one molecule of H₂O₂. The primary product is proposed to be superoxide generated by ThDP-bound enamine radical via a one electron reduction of O₂ to superoxide, the H₂O₂ present in the assay could be considered to be the product of dismutation of superoxide by exogenous superoxide dismutase.

The following activities of H₂O₂ production by hE1o were calculated (see Table 3): (i) with hE1o and OA, the activity of 1.958 nmol · min⁻¹ · mg hE1o⁻¹ for H₂O₂ production was about 7-fold higher than that for hE1o with OG (0.279 nmol · min⁻¹ mg hE1o⁻¹) under similar conditions; (ii) in the presence of 0.4 mM ADP in the reaction assay, both activities in (i) were increased by two-fold resulting to 4.093 nmol · min⁻¹ · mg hE1o⁻¹ with OA (comprises 0.5 - 1.2% of the E1o-specific activity with OA) and to 0.546 nmol · min⁻¹ · mg hE1o⁻¹ with OG (comprises 0.06-0.15% of the E1o-specific activity with OG). Again, H₂O₂ generation by hE1o from OA was ~ 7.5-fold higher than that from OG. The first order rate constants for H₂O₂ generation by hE1o could be estimated as: 7.4x10⁻³ s⁻¹ (OA) and 15.5 x10⁻³ s⁻¹ (OA + 0.4 mM ADP). These constants are the same order of magnitude as the rate constant of 7.8x10⁻³ s⁻¹ calculated for superoxide generation by hE1o from OG in the cyt c assay [18]. Taking into account that the second order rate constant for the reaction of superoxide with superoxide dismutase is ~ 2x10⁹ M⁻¹ s⁻¹ [48], we can conclude that superoxide generation by hE1o from OA is a rate-limiting step.

On assembly of hE1o with hE2o and E3 into hOGDHc, the rates of H₂O₂ production from both substrates were independent of ADP (Table 3; the progress curves of H₂O₂ production see in SI Fig. S4). The rates of 0.345 nmol · min⁻¹ · mg hE1o⁻¹ (OG) and of 2.668 nmol · min⁻¹ · mg

hE1o⁻¹ (OA) were obtained for assembled hOGDHc and represent only a 1.2-1.4-fold increase in the rates compared to hE1o by itself. These rates were not changed significantly on addition of 2.5 mM NAD⁺ into the reaction assay (2.18 ± 0.087 nmol·min⁻¹·mg hE1o⁻¹ for hE1o with OA). When both NAD⁺ (2.5 mM) and CoA (0.06 mM) were present in the reaction assay, the rate of H₂O₂ production from OA was about 3.14 ± 0.10 nmol·min⁻¹·mg hE1o⁻¹ compared to 2.66 nmol·min⁻¹·mg hE1o⁻¹ in their absence. However, with CoA present, the progress curves displayed a significant background slope even before substrate was added to start reaction, which needed to be taken into account. The data above verify generation of superoxide/H₂O₂ by hE1o from the alternative substrate OA.

Table 3. hE1o activities in the reaction producing H₂O₂.

Enzyme ^a	2-oxoglutarate ^b (2 mM)	2-oxoglutarate ^b +0.4 mM ADP	2-oxoadipate ^b (5 mM)	2-oxoadipate ^b +0.4 mM ADP
hE1o	0.279 ± 0.055	0.546 ± 0.057	1.958 ± 0.099	4.093 ± 0.444
hOGDHc	0.345 ± 0.043	0.347 ± 0.020	2.668 ± 0.188	2.086 ± 0.126

^a The hE1o by itself or hE1o assembled with hE2o and E3 into hOGDHc were used for activity measurement. ^b The rates of H₂O₂ production were measured by using a fluorescent Amplex UltraRed –horseradish peroxidase assay reported in the literature [refs. 29,37-39]. Activity is defined as the H₂O₂ produced (nmol·min⁻¹·mg hE1o⁻¹).

The following controls were performed for the Amplex UltraRed fluorescence assay in support of our conclusions: (a) First, it was demonstrated that superoxide/H₂O₂ production from OA was sensitive to inhibition of hE1o by α -keto- β -methyl-n-valeric acid (KMV) in agreement with data reported earlier [49]. Our values of $K_{i,KMV}$ of 0.73 mM (NADH assay) and of 1.12 mM (H₂O₂ assay) were similar to the $K_{m,OA}$ of 0.52 mM (NADH assay) (Fig. 10). Saturation by inhibitor could not be reached because of reversible binding of KMV in the active centers of hE1o and the

presence of OA (5 mM). (b) When catalase was present in the Amplex UltraRed assay, significant reduction of the fluorescence signal resulted, confirming the presence of H_2O_2 in the reaction assay (Fig. 11). (c) In the Amplex UltraRed assay, no H_2O_2 production was demonstrated in the absence of OA in the reaction assay, as evident from a small background slope detected in progress curves (Fig. 11).

Fig. 10. Inhibition of OGDHc activities by KMV. Steady-state rates were measured at different concentrations of KMV (0.5-20 mM) with 5 mM 2-oxoadipate present. The *black curves* are the regression fit of data to Hill plot.

4. Discussion

Our findings demonstrate that hOGDHc behaves as a 2-oxoadipate dehydrogenase, in addition to its usual 2-oxoglutarate dehydrogenase activity in the TCA. According to our findings, hE1o

Fig.11. Progress curves of H_2O_2 production by OGDHc in the absence and presence of catalase in the reaction assay. See Materials and methods for details. The reaction was initiated by OA (5 mM) after ~ 2 min of equilibration. The 50 μl of catalase from stock solution (21 mg/ml, 1 mg/ml = 43,107 units/ml) was added to the reaction when needed.

by itself and when assembled into hOGDHC can serve as a novel source of superoxide/H₂O₂ generation in mitochondria from OA, as its role in ROS generation from OG is well established [13-18]. The superoxide generating activity of hE1o from OG is unique and was not evident for human pyruvate dehydrogenase component (hE1p) by itself or in hE1p-hE2p sub-complex [18]. The H₂O₂ generating activity from OA of 2.668 nmol · min⁻¹ · mg hE1o⁻¹ was estimated for assembled hOGDHC (concentrations of hE1o, hE2o and hE3 subunits were similar) and was more than 7-fold higher than that with OG (0.345 nmol · min⁻¹ · mg hE1o⁻¹) with no further activation by ADP. It needs to be noted that all H₂O₂ activities in this paper were measured in the forward physiological direction with no NADH present in the reaction assay (forward electron flow direction). Our studies suggest that superoxide /H₂O₂ generation by hOGDHC from OG and/or from OA in the forward physiological direction can play a role even in normal mitochondria with known low μM concentration of NADH (concentration of NAD⁺ in the mitochondrial matrix is approximately 400 μM and a predicted ratio of NAD⁺: NADH = 8:1) [21]. While it is difficult to compare H₂O₂ activities in our study with that reported for rat skeletal muscle mitochondria [29], both using OA as substrate, we recently reported superoxide generating activities of 2.06 nmol · min⁻¹ · mg hE1o⁻¹ (hE1o by itself), and 1.76 nmol · min⁻¹ · mg hE1o⁻¹ (for the hE1o-hE2o sub-complex), all using OG as substrate [17]. A striking finding is that the X-band continuous wave EPR spectra of hE1o with OA (hE1o-ThDP-OA system) displayed a line shape consistent with the ThDP-enamine radical species, similar to that detected in hE1o-ThDP-OG system and reported by us earlier [17]. The ThDP-enamine radical species were also detected from OA in samples of hE1o assembled with hE2o and hE3 into hOGDHC. Our data indicate that hOGDHC could contribute significantly to superoxide /H₂O₂ generation from OA as an alternative substrate to OG, and could lead to a better understanding of the role

which hOGDHc plays in numerous neurological disorders. Recently, in search for the proteins forming functional complexes with mitochondrial glutaryl-CoA dehydrogenase, the E2o component of the OGDHc was identified as a potential partner among others, suggesting its involvement in formation of glutaryl-CoA [35]. Based on our findings, the OGDHc could play a role in glutaric aciduria type I, the neurodegenerative disorder associated with accumulation of neurotoxic glutaric acid and 3-hydroxyglutaric acid caused by the deficiency of glutaryl-CoA dehydrogenase [30,35]. The OGDHc could play an additional role in the degradation pathways of L-lysine, L-hydroxylysine, and L-tryptophan, a currently controversial topic [29,30,34,36], which our data begin to resolve.

Scheme 2. Proposed role of the hOGDHc in the mitochondrial (saccharopine) and peroxisomal (pipecolate) L-lysine degradative pathways. AASS, 2-aminoadipate-6-semialdehyde synthase; PIPOX, pipecolate oxidase; AASDH, 2-aminoadipate-6-semialdehyde dehydrogenase; AADAT, 2-aminoadipate transaminase; GCDH, glutaryl-coenzyme A dehydrogenase (modified from ref 50).

3. In the model reaction mimicking communication between hE1o and hE2o, rate constants for acyl transfer (k_4+k_5) of 78 s^{-1} (OG) and of 1.8 s^{-1} (OA) were detected (Scheme 3), indicating that reductive glutarylation could be a rate-limiting step in OGDHc reaction with OA, and that communication between hE1o and hE2o was affected by changing substrate.
4. The hE1o produced a carboligation side product with glyoxylate (Scheme 3 *right middle*) as acceptor with both OG and OA, but (and of potential value for chemo-enzymatic synthesis), the reaction led to α -ketol products of opposite chirality from OA and OG.
5. Most importantly, the ThDP-enamine radical species were detected from both OG and OA on hE1o, and on assembled hOGDHc (assembled from hE1o, hE2o and E3), however, the radical concentration was about three-times lower with OA than with OG. The data on OG also enabled us to estimate the half-life of the ThDP-enamine radical on hE1o of approximately 1 min, similar in magnitude to the (approximately) 4 min reported for pyruvate:Ferredoxin oxidoreductase, a very different ThDP enzyme with iron sulfur clusters as oxidizing agent [51].
6. Also significantly, the rate of H_2O_2 production by hOGDHc was approximately >seven-fold higher from OA than from OG indicating that hOGDHc could contribute significantly to superoxide/ H_2O_2 generation from OA in the forward physiological reaction in the mitochondrion.
7. Finally, it needs to be emphasized that superoxide / H_2O_2 in our studies is being produced by hE1o with oxidation of the enamine by dioxygen, and the latter's concomitant reduction to superoxide, and thence spontaneous dismutation to peroxide (Scheme 3, lower right).

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