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Kinetic characterization of human hydroxyacid–oxoacid transhydrogenase: Relevance to D-2-hydroxyglutaric and γ-hydroxybutyric acidurias

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Summary: We investigated the presence of hydroxyacid–oxoacid transhydrogenase (HOT), which catalyses the cofactor-independent conversion of γ-hydroxybutyrate (GHB) to succinic semialdehyde coupled to reduction of 2-ketoglutarate (2-KG) to D-2-hydroxyglutarate (D-2-HG), in human liver extracts employing [$^2H_6$]GHB and 2-KG as substrates. We measured incorporation of ^2H in D-[^2H]2-HG using GC-MS analyses, providing evidence for HOT activity in humans. Kinetic characterization of HOT was undertaken in forward and reverse directions. We employed [$^2H_6$]GHB and [$^2H_4$]2-KG as cosubstrates in order to develop a HOT activity assay in cultured human fibroblasts derived from patients with D-2-hydroxyglutaric aciduria. HOT activity was quantified in this system by the measurement of D-[$^2H_5$]2-HG production. Fibroblasts derived from patients with D-2-hydroxyglutaric aciduria showed normal HOT activities. Our results provide the first demonstration and preliminary kinetic characterization of HOT activity in human tissues.

The isolation and characterization of a hydroxyacid–oxoacid transhydrogenase (HOT; EC 1.1.99.24) from rat kidney mitochondria was described in 1988 (Kaufman et al 1988a). This enzyme was uncovered in experiments related to the oxidation of γ-hydroxybutyrate (GHB) and HOT activity was found in rat liver, kidney and brain (Kaufman et al 1988b). HOT catalyses the reversible oxidation of a hydroxyacid obligatorily coupled to the reduction of an oxoacid, and requires no cofactor. Several hydroxyacids and oxoacids proved to be substrates for HOT, but the main reaction catalysed is the oxidation of GHB in the presence of 2-ketoglutarate (2-KG), resulting in the formation of succinic semialdehyde (SSA) and

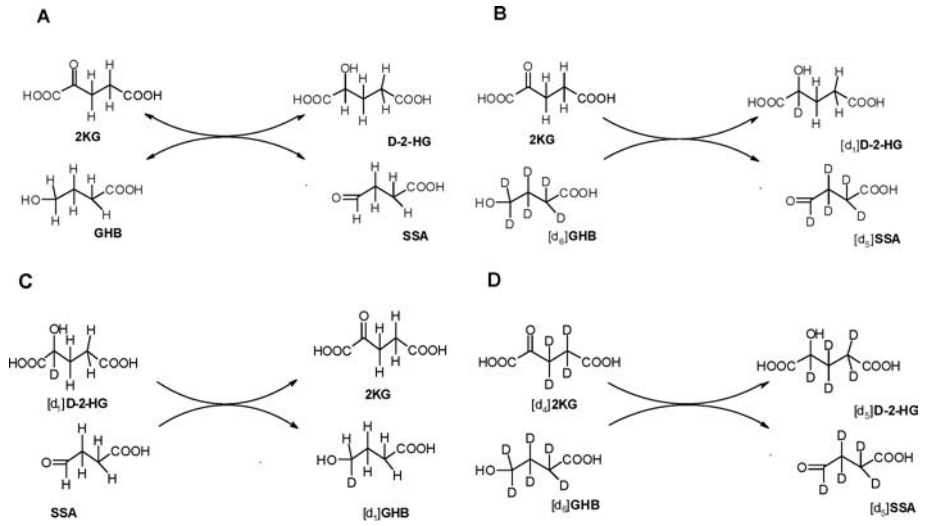

Figure 1 (A) The main reaction of human HOT. (B) The labelled assay for the measurement of HOT activity in human liver homogenates. (C) The labelled assay for the reverse reaction. (D) The double-labelled assay for the determination of HOT activity in homogenates of human fibroblasts

D-2-hydroxyglutarate (D-2-HG) (see Figure 1A). Further studies revealed a postnatal increase of HOT activity in rat brain and kidney (Nelson and Kaufman 1994). This effect was most pronounced in the rat brain, where the increase was 100-fold over the period of birth to the 70th day of life. There was a less than 10-fold increase of HOT in rat kidney during the same period. To date, there are no data available about the amino acid sequence of the *HOT* gene.

It has been postulated that HOT deficiency is a possible cause of the neurometabolic disorder D-2-hydroxyglutaric aciduria (D-2-HGA) (Craigen et al 1994; Gibson et al 1993a). However, the existence of HOT in humans has not been demonstrated. We recently reported pathogenic mutations in the D-2-hydroxyglutarate dehydrogenase gene causing D-2-HGA in two unrelated patients (Struys et al 2005), but for other D-2-HGA patients the genotype remains undefined. We therefore investigated whether a deficiency in HOT activity caused D-2-HGA for this group of D-2-HGA patients. The existence of human HOT was investigated in homogenates of human liver and a functional enzyme assay was developed to measure HOT activity in homogenates of cultured human fibroblasts derived from patients with D-2-HGA and from controls.

MATERIALS AND METHODS

Reagents: The fibroblast culture medium Ham F-10 was obtained from Life Technologies (Grand Islands, NY, USA). 2-KG, Hepes, and dithiothreitol were from Sigma Chemical (St Louis, MO, USA). Stable-isotope-labelled [3,3,4,4-$^2H_4$]2-KG was from Euriso-Top (Gif-sur-Yvette, France). γ-[2,2,3,3,4,4-$^2H_6$]hydroxybutyrate was from CDN isotopes

(Quebec, Canada). (*R*)-(−)-2-butanol and D_2O (99.9% isotopic purity) were purchased from Aldrich (St Louis, MO, USA). Solid-phase extraction cartridges containing 60 mg Oasis HLB were from Waters (Milford, MA, USA). All other chemicals and solvents used were of analytical grade.

D,L-[2-$^2H_1$]2-HG sodium salt was prepared via an approach as described for D,L-[3,3,4,4-$^2H_4$]2-HG (Gibson et al 1993b). Briefly, a solution of 2-KG (0.12 g) in D_2O (1.2 ml) was evaporated in a stream of nitrogen. The deuterated (carboxylic groups) acid was reduced with zinc (0.11 g) in D_2O (2 ml) as described, producing D,L-[2-$^2H_1$]2-HG zinc salt (0.13 g). Treatment with Dowex 50WX8 (sodium form, 5 g) gave the disodium salt (0.12 g). This salt was purified by crystallization from water–methanol, and finally washed with acetone and dried in a stream of nitrogen. Incorporation of deuterium was 99% as determined from the di-(*R*)-(−)-butyl-*O*-acetyl derivative.

Preparation of human liver homogenates: Approximately 100 mg of human liver was obtained by carefully cutting small slices of frozen human liver with a scalpel. The liver sections were placed in a pre-cooled Potter tube and 5 ml of freshly prepared cold assay buffer containing Hepes (200 mmol/L), EDTA (0.2 mmol/L) and dithiothreitol (0.75 mmol/L), pH 7.5, was added. The liver was manually disrupted for 5 min, leaving the Potter tube on ice. The liver preparation was centrifuged for 5 min at 4000*g* at 4°C, and experiments were performed with 250 μl of the supernatant, supplemented with the appropriate substrates, in a final volume of 500 μl for 2 h at 37°C.

Fibroblast cell lines: Fibroblasts were from 12 individual subjects. Six cell lines were from unrelated patients with D-2-HGA. Of these patients, three were affected with the mild phenotype, two with the severe phenotype, and one with an intermediate phenotype (for description of the phenotypes see Van der Knaap et al 1999a, b). One cell line was from a patient with moderate but consistent urinary elevations of D-2-HG. Five cell lines derived from individuals who were not affected with a metabolic disorder were employed as controls.

Cell culture: Fibroblasts were grown on Ham F-10 medium containing 100 ml/L fetal bovine serum and 10 ml/L penicillin–streptomycin at 37°C in a 5% CO_2–95% air incubator. After the cells reached at least 90% confluency, the cell medium was removed, the cells were washed with Hanks buffered saline solution (HBSS) and harvested by the addition of trypsin. After the release of the cells from the bottom surface, trypsin was deactivated by the addition of culture medium. The cell suspension was centrifuged, and the cell pellet was washed twice with HBSS. After the removal of HBSS, the cell pellet was resuspended in 350 μl of freshly prepared assay buffer containing Hepes (200 mmol/L), EDTA (0.2 mmol/L) and dithiothreitol (0.75 mmol/L), pH 7.5. Cells were sonically disrupted (3 × 10 seconds at standard capacity) using a Soniprep 150 Ultrasonic Desintegrator (MSE), and cooled on ice during this procedure. Cell membranes and intact cells were removed by centrifugation at 10 000*g* at 4°C for 5 min. A volume of 250 μl was used for the determination of HOT activity; the remainder was used for protein determination.

Enzyme assays of HOT activity

Human liver: All experiments were performed with 250 µl of the supernatant (see 'Preparation of human liver homogenates'), supplemented with the appropriate substrates, in a capped vial with a final volume of 500 µl for 2 h at 37°C. The measurements of HOT activity in human liver were performed with nonlabelled 2-KG and [$^2H_6$]GHB as substrates, yielding D-[$^2H_1$]2-HG as product (Figure 1B). In the experiments where substrate specificity of HOT relative to GHB was investigated, nonlabelled substrates were used yielding nonlabelled D-2-HG as product. The reverse reaction of HOT was investigated using nonlabelled SSA and D-[$^2H_1$]2-HG as substrates, with [$^2H_1$]GHB as the product (Figure 1C).

Human fibroblasts: The enzyme assay was performed in capped glass vials in a total volume of 500 µl containing 250 µl of cell suspension (see 'Cell culture'), 100 µl of 10 mmol/L [$^2H_6$]GHB and 50 µl of 25 mmol/L [$^2H_4$]2-KG, yielding D-[$^2H_5$]2-HG as product (Figure 1D). The mixture was incubated for 4 h at 37°C.

GC-MS sample preparation

D-2-HG: Prior to the sample preparation, 10 µl of 0.02 mmol/L D,L-[$^2H_1$]2-HG serving as internal standard (fibroblast assay, in which D-[$^2H_5$]2-HG was formed) or 10 µl of 1 mmol/L D,L-[$^2H_4$]2-HG serving as internal standard (liver assay, in which D-[$^2H_1$]2-HG was formed), was added to each vial. The sample preparation has been described previously (Struys et al 2003). Briefly, after the incubation mixture was acidified by the addition of 2 drops of 6 mol/L HCl, it was applied to a solid-phase extraction cartridge that was conditioned with 750 µl of methanol followed by the addition of 750 µl of 0.1 mol/L HCl. The cartridge was washed with 750 µl of 0.1 mol/L HCl and the analytes were eluted from the cartridge with 850 µl of methanol. After the eluate was blown to dryness, 2-HG was derivatized in two steps, to its corresponding di-(*R*)-(−)-butyl-*O*-acetyl derivative (Gibson et al 1993b).

GHB (HOT liver assay): Prior to the sample preparation, 50 µl of 0.2 mmol/L [$^2H_6$]GHB was added to each incubation vial, in which [$^2H_1$]GHB was formed out of SSA and D-[$^2H_1$]2-HG as substrates. After mixing, 25 µl of the mixture was pipetted into a fresh vial, 300 µl of methanol was added to facilitate evaporation, and the sample was evaporated at RT with nitrogen. Di-(trimethylsilyl) derivatives of GHB were formed by the addition of 25 µl of pyridine, 25 µl of BSTFA and 25 µl of acetonitrile, followed by heating for 30 min at 60°C.

Gas chromatography–mass spectrometry: All GC-MS analyses were performed on an Agilent Technologies 6890 N gas chromatograph, equipped with a CP-Sil 88 CB (D-2-HG measurements) or CP-Sil 19 CB (GHB measurements) (Chrompack, The Netherlands) capillary column, and linked to an Agilent Technologies 5973 *inert* mass-selective detector operating in electron impact mode (Santa Clara, CA, USA).

D-[$^2H_5$]2-HG measurement fibroblast HOT assay: Under electron-impact conditions, the di-(*R*)-(−)-butyl-*O*-acetyl derivatives of nonlabelled D,L-2-HG, D,L-[$^2H_1$]2-HG and D,L-[$^2H_4$]2-HG yielded intense fragments of m/z 173, m/z 174 and m/z 177, respectively. This

observation verified that this fragment contained all carbon-bound hydrogen/deuterium atoms from the 2-HG backbone. Measurements of D-2-HG in electron impact mode reduced the contribution of the first natural isotope compared to measurements in the chemical ionization mode. The D-2-HG isotopomers measured were m/z 173 (endogenous D-2-HG), m/z 174 (internal standard (IS)), m/z 177 (D-[$^2H_4$]2-HG nonspecific formed from [$^2H_4$]2-KG), and m/z 178 (D-[$^2H_5$]2-HG). Quantification of D-[$^2H_5$]2-HG was achieved by subtraction of the first natural isotope peak area of the m/z 177 trace (D-[$^2H_4$]2-HG), from the peak area of the m/z 178 trace (D-[$^2H_5$]2-HG). This was also performed for trace m/z 174 (IS), to correct for the first natural isotope peak area of m/z 173 (endogenous 2-HG). The GC-MS performance and calibration were checked by the analysis of nonlabelled D-2-HG. The corrected peak areas of m/z 174 (IS) and m/z 178 (D-[$^2H_5$]2-HG) were employed to determine enzyme activity.

D-*[$^2H_1$]2-HG measurement liver HOT assay:* In the liver experiments, D-2-HG was measured as described for the fibroblasts experiments. The D-2-HG isotopomers measured were m/z 174 (D-[$^2H_1$]2-HG) and m/z 177, (D-[$^2H_4$]2-HG, IS). Since liver HOT activity was significantly higher than that in fibroblasts, no correction for endogenous D-2-HG was necessary.

[$^2H_1$]GHB measurement in liver HOT assay: For the determination of GHB, the same instrumentation as described above for the measurement of D-2-HG was employed. In electron-impact the measured GHB isotopomers were m/z 233 (endogenous GHB), m/z 234 ([$^2H_1$]GHB), and m/z 239 ([$^2H_6$]GHB, IS). When performing incubations with SSA and D-[$^2H_1$]2-HG as substrates, considerable amounts of nonlabelled GHB were formed, making obligatory the correction for the contribution of the first isotope of nonlabelled GHB to the trace of m/z 234.

RESULTS

Experiments with human liver: Preliminary incubations were performed in liver homogenates using [$^2H_6$]GHB and 2-KG as substrates followed by the measurement of D-[$^2H_1$]2-HG as product. In these experiments we measured considerable incorporation of one deuterium into D-[$^2H_1$]2-HG, without measuring nonlabelled D-2-HG, proving the enantiomer-specific transfer of a carbon-bound deuterium from [$^2H_6$]GHB to 2-KG. The enzyme was saturated with 1 mmol/L of [$^2H_6$]GHB and 2 mmol/L of 2-KG. The mathematical treatment of the double-reciprocal plot yielded apparent K_m values of 0.17 mmol/L and 1.2 mmol/L for [$^2H_6$]GHB and 2-KG respectively. The V_{max} of HOT activity in human liver homogenate using [$^2H_6$]GHB and 2-KG as substrates was 16 nmol/h per mg protein. No isotope effect was found in experiments in which [$^2H_6$]GHB was replaced by nonlabelled GHB. HOT activity was also determined using D-[$^2H_1$]2-HG and SSA as substrates followed by determination of [$^2H_1$]GHB. The mathematical treatment of the double-reciprocal plot yielded apparent K_m values of 0.04 mmol/L and 0.12 mmol/L for SSA and D-[$^2H_1$]2-HG respectively. The V_{max} of HOT activity in human liver homogenate using D-[$^2H_1$]2-HG and SSA as substrates was 0.5 nmol/h per mg protein.

We have tested several hydroxyacids to investigate the specificity of HOT relative to GHB, using 2-KG as hydrogen acceptor in these experiments. Incubations with the following

Table 1 Comparison of apparent K_m values (mol/L) found in this study with published values

Substrate	*Human liver*	*Human fibroblasts*	*Rat kidney* [a] *(partially purified)*
GHB	0.2×10^{-3}	0.2×10^{-3}	0.3×10^{-3}
D-2-HG	0.1×10^{-3}	ND[b]	0.4×10^{-3}
L-3-OHB	0.8×10^{-3}	ND	3.0×10^{-3}
2-KG	1.2×10^{-3}	0.8×10^{-3}	1.8×10^{-5}
SSA	0.4×10^{-4}	ND	0.5×10^{-5}

[a] Kaufman et al (1988a)
[b] ND, not determined

hydroxyacids (1 mmol/L in incubation) did not result in formation of D-2-HG: 2-hydroxyisovalerate, 3-hydroxyisovalerate, malate, 3-hydroxyglutarate, L-2-hydroxyglutarate, lactate, glycolate, and 2-hydroxybutyrate. D,L-3-Hydroxyisobutyrate and L-3-hydroxybutyrate (L-3-OHB) were also substrates for HOT with 10-fold lower activities when compared to GHB as substrate. The enzyme was saturated with 2 mmol/L of L-3-OHB and mathematical treatment of the double-reciprocal plot yielded apparent K_m values of 0.8 mmol/L, with V_{max} of 1.8 nmol/h per mg protein. No enantiomerically pure 3-hydroxyisobutyrate is commercially available. Table 1 summarizes the apparent K_m values found in this study in comparison to the K_m values of the same substrates in experiments with partially purified HOT from rat kidney mitochondria (Kaufman et al 1988a).

Fibroblast HOT-enzyme assay optimization: The enzyme assay was optimized and evaluated in relation to the parameters of substrate dependency, time dependency, pH, protein dependency and inhibition by D-2-HG. The enzyme was saturated with 1.5 mmol/L [$^2H_6$]GHB and 2 mmol/L [$^2H_4$]2-KG. The mathematical treatment of the double-reciprocal linear plot yielded apparent K_m values of 0.17 mmol/L and 0.77 mmol/L for [$^2H_6$]GHB and [$^2H_4$]2-KG, respectively. The production of D-[$^2H_5$]2-HG, using 1000 nmol of [$^2H_6$]GHB and 1250 nmol of [$^2H_4$]2-KG, was found to be linear with time up to 5 h, with optimal activity at pH 7.5, and was linear with respect to protein up to 500 μg. No inhibition by D-2-HG was detected, tested to the level of 15 nmol per incubation. This amount was more than 5 times the intracellular concentration of D-2-HG in fibroblasts derived from D-2-HGA patients.

HOT activities for controls and D-2-HGA patients: The results of HOT determinations in fibroblast homogenates are presented in Table 2. The mean activity measured in six control fibroblast homogenates was 15 ± 3 pmol/h per mg protein. The mean activity measured in six D-2-HGA fibroblast homogenates was 18 ± 6 pmol/h per mg protein. The fibroblast homogenates from a patient with moderate urinary D-2-HG elevations displayed HOT activity moderately higher than the control range. A pooled fibroblast homogenate was repetitively analysed ($n = 5$), resulting in an average activity of 9.5 ± 0.4 (SD) pmol/h per mg protein (range 9.1–10.1). An illustrative mass fragmentogram of the 2-HG isotopomers following an incubation of the pooled fibroblast homogenate is shown in Figure 2.

Table 2 HOT activity (pmol/h per mg protein) in homogenates of cultured fibroblast from controls (a–e) and patients (f–l)

	Controls	*D-2-HGA*	*Moderate D-2-HGA*
	(a) 18	(f) 14	(l) 25
	(b) 12	(g) 29	
	(c) 14	(h) 18	
	(d) 13	(i) 13	
	(e) 17	(j) 16	
		(k) 19	
Mean ± SD	15 ± 3	18 ± 6	–

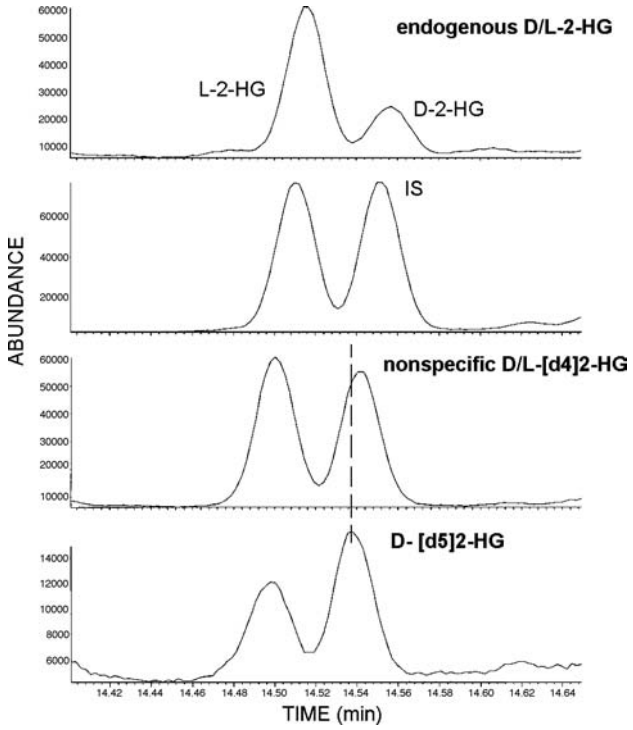

Figure 2 A representative mass fragmentogram of the enzyme assay for HOT activity in a control subject. Note that because of the incorporation of one deuterium into [$^2H_4$]2-KG, yielding D-[$^2H_5$]2-HG, this isotopomer elutes slightly ahead of D-[$^2H_5$]2-HG

DISCUSSION

In 1988, a mitochondrial enzyme responsible for the formation of D-2-HG and SSA from 2-KG and GHB in the rat tissues was described (Kaufman et al 1988a). We investigated the

existence of this enzyme in human liver homogenates and its possible link to D-2-HGA by the measurement of its activity in human liver and fibroblast homogenates from controls and/or D-2-HGA patients.

Experiments with human liver: The results obtained in our studies with homogenates of human liver are consistent with the original description of HOT in the rat (Kaufman et al 1988a). We measured incorporation of one deuterium into D-[$^2H_1$]2-HG when using [$^2H_6$]GHB and 2-KG as substrates, documenting the existence of a human HOT since the reaction is very specific. In agreement with the results of Kaufman and colleagues (1988a), L-3-OHB was also a substrate for HOT when using 2-KG as hydrogen acceptor, resulting in the formation of D-2-HG. In addition, we found that D,L-3-hydroxyisobutyrate was also a substrate for HOT. Comparison of the apparent K_m values found in our study with those observed by Kaufman and colleagues (1988a) showed good agreement for the hydroxyacids GHB, L-3-OHB, and D-2-HG (table 1). In contrast, the apparent K_m values for the oxoacids 2-KG and SSA found in our study were considerably higher. This discrepancy may be due to the fact that we used crude homogenates containing a pool of enzymes acting on 2-KG and SSA, instead of the partially purified enzyme from rat kidney mitochondria in the experiments of Kaufman and colleagues (1988a). The availability of only small frozen portions of human liver precluded the isolation of intact mitochondria from these human samples. We also found a strong difference for HOT activities in the two directions of this reversible enzyme. The V_{max} of the D-2-HG-generating direction using GHB and 2-KG as substrates was 30-fold higher than the V_{max} in the D-2-HG-consuming reaction using D-2-HG and SSA as substrates. This difference is more pronounced than described by Kaufman and colleagues (1988a), in which study the activity in the D-2-HG-generating direction was 5-fold higher than in the opposite direction. Again, the use of crude homogenates in our study may be responsible for this. In the incubations performed with SSA and D-[$^2H_1$]2-HG, we found considerable amounts of nonlabelled GHB formed by a succinic semialdehyde reductase possibly inhibiting HOT. The partially purified HOT from rat kidney mitochondria probably contained less of this succinic semialdehyde reductase.

Experiments with human fibroblasts: In previous studies, we showed that fibroblasts from D-2-HGA patients are suitable for research related to D-2-HGA, since these cells accumulate D-2-HG (Struys et al 2003). HOT is a reversible enzyme and, because of the accumulation of D-2-HG in D-2-HGA, we explored a possible HOT deficiency in these patients, using SSA and D-2-HG as substrates, followed by the measurement of the products formed: 2-KG and GHB. We were unable to detect HOT activity in homogenates of control cultured fibroblasts in this direction. Both our results with this reaction in homogenates of human liver and the original description of HOT (Kaufman et al 1988a), indicate that the reaction velocity in the direction of the formation of 2-KG and GHB using D-2-HG and SSA as substrates was significantly lower than that in the opposite direction, probably explaining the low product formation with fibroblast extracts as tissue source. To our knowledge, no defects have been documented for reversible enzymes that are deficient in only one direction, which should allow us to perform the assay for HOT activity in the D-2-HG-generating direction. Recently an assay of 3-methylglutaconyl-CoA hydratase was described with which the activity of this reversible enzyme was also studied in the reverse direction using 3-hydroxy-3-methylglutaryl-CoA as substrate (Loupatty et al 2004). This finding suggests that it is

valid to measure enzyme activity in the reverse direction for those enzymes that are known to be reversible.

Attempts to measure HOT activity in fibroblast homogenates from D-2-HGA patients, using [$^2H_6$]GHB and nonlabelled 2-KG as substrates were unsuccessful. The considerable levels of accumulated D-2-HG in the cells gave rise to a substantial first natural isotope contribution in the trace of D-[$^2H_1$]2-HG, hampering accurate quantification. To circumvent this problem, we used a double-labelled procedure with [$^2H_6$]GHB and [$^2H_4$]2-KG as substrates, resulting in the formation of D-[$^2H_5$]2-HG representing the HOT activity. This procedure allowed us, for the first time, to measure HOT activity in human fibroblasts from both controls and D-2-HGA patients.

Activities of HOT in fibroblast homogenates from D-2-HGA patients in this study were comparable to those in controls, indicating that HOT cannot be related to D-2-HGA in these cell lines. Nevertheless, after the demonstration of HOT in humans, it is very likely that HOT is co-responsible for the formation of D-2-HG in humans. Recent findings show that, in body fluids of patients with succinic semialdehyde dehydrogenase deficiency, D-2-HG is significantly increased (E.A. Struys, N.M. Verhoeven, C. Jakobs and K.M. Gibson, unpublished findings). The body fluids of those patients contain large amounts of GHB, which is one of the substrates for HOT. Further (animal) studies are underway to obtain more insight into this secondary phenomenon in succinic semialdehyde dehydrogenase deficiency.

For the first time we have demonstrated HOT activity in homogenates of human liver and fibroblasts, and show that HOT is not deficient in homogenates of cultured fibroblasts from patients with D-2-HGA. Further studies are needed to fully understand the biochemical relevance of HOT in humans, while the relatively high activity in human liver illustrates that HOT is an important enzyme. HOT activity in liver may serve as a mechanism for detoxification of GHB, and it will be of interest to examine the induction of HOT following sequential administration of GHB to animals. Such studies will be very relevant with respect to the use of GHB as a recreational drug. However, it is also possible that HOT may have a key role in liver GABA homeostasis, since liver possesses significant concentrations of GABA and GABA-transaminase activity (Jaeken et al 1984). SSA is the product of the GABA-T reaction, and 2-KG is the nitrogen acceptor that replenishes the GABA precursor glutamate. Accordingly, a link between GABA homeostasis and HOT activity in liver would appear feasible. Studies are in progress to explore further these potential regulatory relationships.

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