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Identification of the gene encoding hydroxyacid-oxoacid transhydrogenase, an enzyme that metabolizes 4-hydroxybutyrate

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Abstract To identify the sequence of hydroxyacid-oxoacid transhydrogenase (HOT), responsible for the oxidation of 4-hydroxybutyrate in mammalian tissues, we have purified this enzyme from rat liver and obtained partial sequences of proteins coeluting with the enzymatic activity in the last purification step. One of the identified proteins was 'iron-dependent alcohol dehydrogenase', an enzyme encoded by a gene present on human chromosome 8q 13.1 and distantly related to bacterial 4-hydroxybutyrate dehydrogenases. The identification of this protein as HOT was confirmed by showing that overexpression of the mouse homologue in HEK cells resulted in the appearance of an enzyme catalyzing the α-ketoglutarate-dependent oxidation of 4-hydroxybutyrate to succinate semialdehyde.

Keywords: D-2-Hydroxyglutarate; γ-Hydroxybutyrate; Organic acidurias; α-Ketoglutarate; Transhydrogenase

1. Introduction

4-Hydroxybutyrate (γ-hydroxybutyrate), a compound known for its anesthetic properties and an abuse drug, is used as a sleep regulator in the treatment of catalepsy [1]. 4-Hydroxybutyrate is endogenously produced in mammals through reduction of succinate semialdehyde, a product of γ-aminobutyrate deamination [2]. It is also formed by enzymatic oxidation of 1,4-butanediol, an industrial solvent, or through hydrolysis of γ-butyrolactone. Unlike many other alcohols, which are oxidized by NAD-linked dehydrogenases, 4-hydroxybutyrate is metabolized to succinate semialdehyde by an enzyme (hydroxyacid-oxoacid transhydrogenase, HOT) that does not use free NAD or NADP, but α-ketoglutarate as an acceptor, converting it to D-2-hydroxyglutarate [3]. D-2-Hydroxyglutarate is oxidized by a mitochondrial, FAD-linked D-2-hydroxyglutarate dehydrogenase, which shows a low K_m (≈2 μM) for its substrate and thereby normally maintains the D-2-hydroxyglutarate concentration at a low level [4,5]. Despite the critical role played by HOT in the formation of D-2-hydroxyglutarate and the metabolism of 4-hydroxybutyrate and L-3-hydroxybutyrate (another substrate on which it acts much more slowly [3,6]), the sequence encoding this enzyme is not known. This identification is reported in the present paper.

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Abbreviation: HOT, hydroxyacid-oxoacid transhydrogenase

2. Materials and methods

2.1. Materials

NADP-linked succinate semialdehyde dehydrogenase [7] (used as a coupling enzyme in the HOT assay) was overexpressed at 37 °C in Bl21 cells harboring a pET15b vector driving the expression of this enzyme. The enzyme was extracted and purified on DEAE–Sepharose according to previously described procedures [8]. About 130 U with a specific activity of 6.2 U/mg protein were obtained from a 200 ml culture.

2.2. Partial purification of HOT from rat liver

Frozen rat livers (15 g) were homogenized with 2 volumes (v/w) of buffer A (20 mM HEPES, pH 7.1, 1 μg/ml leupeptin and 1 μg/ml antipain) containing 100 mM KCl. After addition of 2% poly(ethyleneglycol) 6000, the extract was centrifuged for 15 min at 15000×*g*. The supernatant (32 ml) was diluted 3-fold with buffer A and applied onto a DEAE–Sepharose column (1.5 × 20 cm) equilibrated in the same buffer. The column was washed with 30 ml buffer A and the retained proteins were eluted with a 0–0.75 M NaCl gradient in 2 × 150 ml of buffer A. The most active fractions (≈15 ml) from two such columns were pooled, supplemented with 1 M NaCl and applied onto a phenyl sepharose column (6 ml) equilibrated with buffer A containing 1 M NaCl. Proteins were eluted with a stepwise, decreasing gradient of NaCl (1, 0.5, 0.3, 0.2, 0.1 to 0 M; 5 ml/fraction). The active fractions (15 ml) were pooled and concentrated to 2 ml on an Amicon YM10 filter. This preparation, which was free from succinate semialdehyde reductase, succinate semialdehyde dehydrogenase and glutamate dehydrogenase, was used for the determination of the kinetic properties. A portion (0.3 ml) of the preparation was gel-filtered on a 24 cm^3 Superdex™ 200 column equilibrated with 20 mM HEPES, pH 7.1, 100 mM NaCl, 1 μg/ml leupeptin and 1 μg/ml antipain.

For tissue distribution studies, frozen rat tissues (300–400 mg) were homogenized in 4 vol. of buffer A containing 25 mM KCl. Triton X-100 and poly(ethyleneglycol) 6000 were added to final concentrations of 0.1% and 2%, respectively, and the homogenates were centrifuged for 15 min at 13000×*g*. The supernatants were diluted with 2 vol. of buffer A and 2-ml portions were applied on 0.5 ml DEAE–Sepharose columns (equilibrated with buffer A). The columns were washed with this buffer and the retained proteins were eluted with a stepwise NaCl gradient (50, 100, 150, 200, 500 mM in buffer A; 1 ml per fraction). HOT activity measurements were performed on 25 or 50 μl samples of the fractions.

2.3. Overexpression of mouse HOT in HEK cells

The open reading frame of mouse HOT (NM-175236.2) was PCR-amplified using *Pwo* DNA polymerase, mouse liver cDNA as a template, and the following primers: 5′ GAATTCGCCACCATGGCTGCAGCTGCACGC 3′ (which contains an *Eco*RI site and a perfect Kozak consensus sequence) and 5′ AGATCTTAGTACAGTTTCATGGATGCTTCA 3′ (containing a *Bam*HI site). The PCR product was inserted between the *Eco*RI and *Bam*HI sites of pCMV5 (Novagen). Transfections of human embryonic kidney (HEK 293) cells were performed as previously described [9]. 48 h after transfection, the cells were washed with phosphate buffer saline and harvested in 500 μl of buffer B (20 mM HEPES, pH 7.1, 5 μg/ml leupeptin, 5 μg/ml antipain). They were frozen in liquid nitrogen, thawed, and after addition of 0.1% Triton X-100, the cell extracts were centrifuged for 20 s at 2000×*g*. The

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supernatant of two dishes (1.5 ml) was diluted 2-fold with buffer B and applied on a 0.5 ml DEAE–Sepharose column equilibrated in the same buffer. The column was washed with this buffer and retained proteins were eluted with a stepwise NaCl gradient (see above). The enzymatic activity was mainly present (>85%) in the fraction eluted with 100 mM NaCl.

2.4. Enzymatic assays

All enzyme assays were carried out at 30 °C in 1 ml assay mixtures. Unless otherwise stated, HOT activity was determined spectrophotometrically at 340 nm in an assay mixture containing 20 mM HEPES, pH 7.1, 25 mM KCl, 0.5 mM $NADP^+$, 20 mU of *Escherichia coli* succinate semialdehyde dehydrogenase, 0.2 mM α-ketoglutarate, 0.2 mM 4-hydroxybutyrate and the enzyme preparation to be tested. One unit of enzyme is the amount catalyzing the formation of 1 μmol/min under these assay conditions. HOT assays in which the formation of α-ketoglutarate was followed contained unless otherwise indicated 50 mM HEPES, pH 7.1, 10 mM NH_4Cl, 0.2 mM NADH, 1 μl glutamate dehydrogenase (from Roche), 0.4 mM D-2-hydroxyglutarate and 25 μM succinate semialdehyde. Succinate semialdehyde dehydrogenase was assayed in a mixture containing 20 mM HEPES, pH 7.1, 25 mM KCl, 0.5 mM $NADP^+$ and 50 μM succinate semialdehyde.

3. Results

3.1. Purification and partial characterization of the transhydrogenase from rat liver

HOT was purified from rat liver by chromatography on DEAE–Sepharose, phenyl sepharose and gel filtration on Superdex. During this purification, HOT was assayed by monitoring the 4-hydroxybutyrate- and α-ketoglutarate-dependent formation of succinate semialdehyde with purified *E. coli* NADP-linked succinate semialdehyde dehydrogenase as coupling enzyme. Due to the presence of interfering dehydrogenases, this assay could not be used in crude liver extracts, but well after the first chromatographic step. Assuming that the yield of enzyme was 100% in this step, the overall purification factor of the enzyme was ≈10-fold after the first step, ≈200-fold after the second and ≈1300-fold after the third step (not shown). The yields through the second and third steps were 53% and 60%, respectively.

The preparation obtained after the second chromatographic step was free from succinate semialdehyde reductase, succinate semialdehyde dehydrogenase and glutamate dehydrogenase and therefore suitable for kinetic studies. When monitoring succinate semialdehyde formation, we found that the enzyme activity was totally dependent on the presence of both 4-hydroxybutyrate and α-ketoglutarate for which K_m values of 60 ± 6 and 85 ± 7 μM were obtained, respectively. The enzymatic activity was unaffected by 1 mM EDTA or EGTA, but was inhibited by phenanthroline and cyanide, which caused 50% inhibition at 0.75 mM and 75 μM, respectively. However, no stimulation of the enzymatic activity was observed with 10–50 μM Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, or 1 mM Mg^{2+} or Ca^{2+} (not shown).

The activity was also measured in the opposite direction, by following the formation of α-ketoglutarate in the presence of succinate semialdehyde and D-2-hydroxyglutarate. The K_m value was <10 μM for succinate semialdehyde and 4.5 mM for D-2-hydroxyglutarate. No activity was observed when the L-isomer of 2-hydroxyglutarate was used instead of the D-isomer. Using a radiochemical assay in which radiolabeled 4-hydroxybutyrate was produced in the presence of [2-^{3}H] D-2-hydroxyglutarate and succinate semialdehyde, we confirmed that the enzymatic activity is unaffected by NAD^+ or $NADP^+$ (tested at up to 1 mM; results not shown).

3.2. Identification of the transhydrogenase sequence

As shown in Fig. 1, the HOT activity coeluted with two bands of ≈50 and 45 kDa in the last purification step. These were in-gel digested with trypsin and sequenced by mass spectrometry [4]. Both bands comprised mixtures of proteins: 2-phosphoglycerate enolase (4 peptides), 'iron-dependent alcohol dehydrogenase' (2 peptides) and HMG-CoA synthase (1 peptide) in the upper band; aminoadipate aminotransferase (3 peptides), aminoacylase (3 peptides) and again 'iron-dependent alcohol dehydrogenase' (4 peptides) in the lower band.

BLAST searches indicated that human 'iron-dependent alcohol dehydrogenase', which is encoded by the ADHFE1 gene on chromosome 8q 13.1, is homologous to proteins of unknown function from fishes (70–90% identity), insects (≈58–61%), *Caenorhabditis elegans* (48%) and several fungi (46–54% sequence identity). It is more distantly related to bacterial NAD(P)-linked dehydrogenases of diverse functions, including *Clostridium kluyveri* 4-hydroxybutyrate dehydrogenase [10], with which it shares 27% sequence identity and *E. coli* lactaldehyde reductase, an iron-dependent enzyme with known 3 D structure [11]. Fig. 2 shows that mammalian 'iron-dependent alcohol dehydrogenase' shares several of the residues that bind the divalent metal cation in lactaldehyde dehydrogenase. Analysis of both mouse and human 'iron-dependent alcohol dehydrogenase' sequences with the Target P [12] program indi-

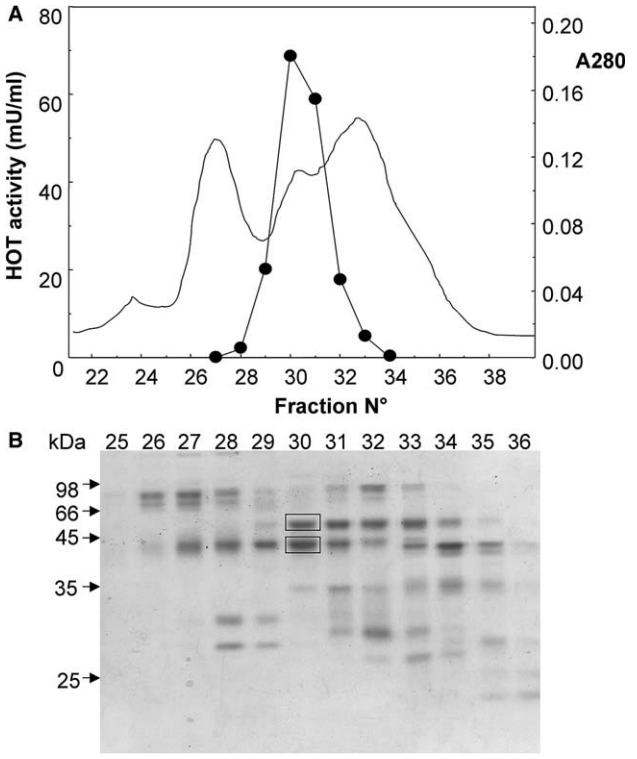

Fig. 1. Coelution of the transhydrogenase with 50 and 55 kDa proteins in the Superose step. A portion (0.3 ml) of the preparation was gel-filtered on Superdex equilibrated with 20 mM HEPES, pH 7.1, 100 mM NaCl, 1 μg/ml leupeptin and 1 μg/ml antipain. Fractions of 0.5 ml were collected and the activity measured (closed circles). Fractions were also analyzed by SDS–PAGE. The indicated bands were cut out from the gel and analyzed by mass spectrometry after trypsin digestion.

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Hosa MAAAARARVAYLLRQLQRAACQCPTHSHTYSQAPGLSPSGKTTDYAFEMAV-  51
Mumu MAAAARARVTHLLRHLQSTACQCPTHSHTYSQAPG--PSGKTADYAFEMAV-  49
Drps    MSRKNVLNLINTIVANSCKCPAHSHNYGSAAPTGVQTGQKEYAFEANSA  49
LcRe                                          MMANRMILN   9

Hosa SNIRYGAAVTKEVGMDLKNMGAKNVCLMTDKNLSKLPPVQVAMDSLVKNGIP 103
Mumu SNIRYGAGVTKEVGMDLQNMGAKNVCLMTDKNLSQLPPVQIVMDSLSKNGIS 101
Drps STVRFGPGVSVEVGADLRNLGAKKVCLVTDRNVAKLPSVKVALDSLARHGIN 101
LcRe ETAWFGRGAVGALTDEVKRRGYQKALIVTDKTLVQCGVVAKVTDKMDAAGLA  61
                              *
Hosa FTVYDNVRVEPTDSSFMEAIEFAQKGAFDAYVAVGGGSTMDTCKAANLYASS 155
Mumu FQVYDDVRVEPTDGSFMDAIEFAKKGAFDAYVAVGGGSTMDTCKAANLYASS 153
Drps YEVYDETRVEPTDASLWHAADYARQNEFDAFLAIGGGSVMDTAKAANLFSSD 153
LcRe WAIYDGVVPNPTITVVKEGLGVFQNSGADYLIAIGGGSPQDTCKAIGIISNN 113

Hosa PHSDFLDYVSAPIGKGKPVSVPLKPLIAVPTTSGTGSETTGVAIFDYEHLKV 207
MuMu PHSEFLDYVNAPIGKGKPVTVPLKPLIAVPTTSGTGSETTGVAIFDYEHLKV 205
Drps REAEFLDYVNCPIGKGKEISVKLKPLIAMPTTSGTGSETTGVAIFDYKRLHA 205
LcRe P--EFADVRS--LEGLSPTNKPSVPILAIPTTAGTAAEVTINYVITDEEKRR 161

Hosa KIGITSRAIKPTLGLIDPLHTLHMPARVVANSGFDVLCHALESYTTLPYHLR 259
Mumu KTGIASRAIKPTLGLVDPLHTLHMPCQVVANSGFDVLCHALESYTAIPYSMR 256
Drps KTGISSKYLKPTLAIIDPLHTLSQPERVMAFAGFDVFCHALESFTAVDYRER 256
LcRe KFVCVDPHDIPQVAFIDADMMDGMPPALKAATGVDALTHAIEGYIT------ 207
                                      *   *
Hosa SPCPSNPITRPAYQGSNPISDIWAIHALRIVAKYLKRAVRNPDDLEARSHMH 311
Mumu SPCPSNPIQRPAYQGSNPISDIWAVHALQIVAKYLKRAVRNPDDLEARSKMH 309
Drps GPAPSDPSLRPTYQGRNPISDVWARFALETIRKNFINAIYEPGNLEARSQMH 309
LcRe -------------RGAWALTDALHIKAIEIIAGALRGSVAG--DKDAGEEMA 244

Hosa LASAFAGIGFGNAGVHLCHGMSYPISGLVKMYKAKDYNVDHPLVPHGLSVVL 363
Mumu LASAFAGIGFGNAGVHLCHGMSYPISGLVKTYKAKEYNVDHPLVPHGLSVVL 361
Drps LASTMAGVGFGNAGVHLCHGLSYPISGNVRNYKPAGYTADHALIPHGLSVVI 361
LcRe LGQYVAGMGFSNVGLGLVHGMAHPLGAFYNT-------------PHGVANAI 283
                        *                           *
Hosa TSPAVFTFTAQMFPERHLEMAEILGADTRTARIQDAGLVLADTLRKFLFDLD 415
Mumu TSPAVFTFTAQMFPERHLETAGILGANIRTARIQDAGLVLADALRKFLFDLN 413
Drps SAPAVFEFTAPACPDRHLEAAKLLGAKVDGVRASDAGRLLADTVRGFMQRAG 413
LcRe LLPHVMRYNADFTGEKYRDIARVMGVKVEGMSLEEARNAAVEAVFALNRDVG 335

Hosa VDDGLAAVGYSKADIPALVKGTLPQERVTKLAPCPQSEEDLAALFEASMKLY 467
Mumu VDDGLAALGYSKDDIPSLVKGTLPQERVTKLAPRAQSEEDLSALFEASMKLY 465
Drps IENGLRELGFSSSDVPNLVDGTLPQERITKLAPRAQTQENLAQLFENSMVAY 465
LcRe IPPHLRDVGVRKEDIPALAQAAL-DDVCTGGNPREATLEDIVELYHTAW    383
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Fig. 2. Sequence alignment of the putative HOT with *E. coli* lactaldehyde reductase. Putative HOT sequences from *Homo sapiens* (Hosa; GI25989126), *Mus musculus* (Mumu, GI37589962) and *Drosophila pseudoobscura* (Drps; GI54636830) are aligned with *E. coli* lactaldehyde reductase (LcRe; GI26249204). The conserved residues are in bold. Asterisks indicate the positions of Asp39 (which discriminates against NADP binding), Asp196, His200, His263 and His277 (metal binding residues) in lactaldehyde reductase. The underlined residues in the mouse sequence correspond to the peptides identified by mass spectrometry in the enzyme purified from rat liver.

cated that these proteins are most likely targeted to mitochondria, the known subcellular localization of HOT [3]. Taken together, these findings suggested that mammalian 'iron-dependent alcohol dehydrogenase' corresponded to HOT.

This identification was confirmed by overexpressing the mouse putative transhydrogenase in HEK cells. Extracts of cells transfected with a pCMV5 plasmid encoding this protein displayed a HOT activity of 1.33 ± 0.44 mU/mg of soluble proteins (mean ± S.E.M. of three independent transfections), whereas the activity was undetectable (<0.05 mU/mg protein) in cells transfected with a control plasmid. We confirmed that the enzymatic activity was dependent on the presence of both 4-hydroxybutyrate and α-ketoglutarate. The K_m values were 77 ± 9 μM for α-ketoglutarate (measured in the presence of 0.1 mM 4-hydroxybutyrate) and 67 ± 12 μM for 4-hydroxybutyrate (measured with 0.5 mM α-ketoglutarate), being therefore similar to the values obtained with the rat liver enzyme.

3.3. Tissue distribution

As shown in Fig. 3, the highest HOT activities were observed in liver and kidneys and an intermediate activity was found in heart, whereas no activity could be detected in brain, skeletal muscle and lung. A similar tissue distribution was observed

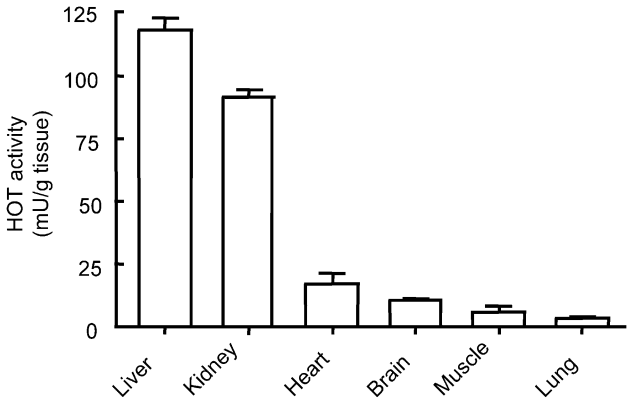

Fig. 3. Tissue distribution of HOT. The enzymatic activity was determined after partial purification on small DEAE–Sepharose columns.

for D-2-hydroxyglutarate dehydrogenase ([4] and unpublished results).

4. Discussion

Our findings indicate that mammalian 'iron-dependent alcohol dehydrogenase' [13], which had never been characterized functionally until now, corresponds to HOT, an enzyme responsible for 4-hydroxybutyrate metabolism in mammalian cells. This identification rests on several findings, most particularly the observation that transfection of the corresponding protein in HEK cells induces HOT activity. The finding that HOT is inhibited by phenanthroline and cyanide suggests that it uses a divalent or trivalent cation as a cofactor. However, none of the divalent or trivalent cations that we tested stimulated the enzymatic activity, presumably because the catalytic site of the enzyme as purified from rat liver is fully occupied by a tightly bound cation. The alignment of the HOT sequence with *E. coli* lactaldehyde reductase indicates that the residues that bind the metal ion (Asp196, His200, His263 and His277) in the *E. coli* enzyme [11] are conserved in mammalian HOT. Interestingly, this is not the case for His267. As mutation of this residue in *E. coli* lactaldehyde reductase favors Zn^{2+} binding over Fe^{2+} binding [14], its lack of conservation in HOT suggests that a metal ion different from iron may be present in the catalytic site of this enzyme.

Kaufman et al. [3] hypothesized that a tightly bound cofactor in HOT served as an intermediate acceptor for the hydride that is transferred from the reduced substrate to the acceptor. The finding that the enzyme is homologous to NAD(P)-dependent dehydrogenases suggests that this cofactor is a pyridine nucleotide, most likely NAD since the residue (Asp39) that discriminates against NADP binding in *E. coli* lactaldehyde reductase [11] is conserved in HOT. The use of a pyridine nucleotide by HOT is consistent with the finding that this enzyme does not catalyze an exchange of deuteride from deuterated substrates with protons of water [3]. Such an exchange would be expected with hydride acceptors in which the hydrogens are transferred onto N (e.g., FAD) or O (quinones) atoms. We conclude therefore that the reaction mechanism of HOT is similar to the one described for malate/pyruvate transhydrogenase a microbial enzyme that was shown to use tightly bound NAD as an intermediate acceptor [15].

The K_m values of purified rat liver HOT are in fair agreement with those reported for the purified rat kidney enzyme [3], to the exception of the value for D-2-hydroxyglutarate, which differs by about 10-fold. This is possibly due to differences in assay conditions. Much higher K_m values for α-ketoglutarate and succinate semialdehyde have been observed with human HOT [6]. However, this enzyme was assayed in crude tissue extracts, with potential interference of other enzymatic activities.

HOT is unique in using the metabolite couple α-ketoglutarate/D-2-hydroxyglutarate as an electron acceptor/donor. Thanks to the presence of D-2-hydroxyglutarate dehydrogenase, a low K_m, FAD-linked enzyme catalysing an irreversible reaction under physiological conditions [4,5], the α-ketoglutarate/D-2-hydroxyglutarate ratio is normally maintained at an elevated value, thus favoring the oxidation of 4-hydroxybutyrate to succinate semialdehyde by HOT rather than the opposite conversion. This functional coupling of the two enzymes is consistent with their similar tissue distribution (this paper), and with the accumulation of D-2-hydroxyglutarate when succinate semialdehyde dehydrogenase is deficient [16,17]. In the last condition, succinate semialdehyde is presumably constantly reduced by NADPH-linked reductases to 4-hydroxybutyrate, which is reconverted to succinate semialdehyde by HOT. This futile cycle results in the transfer of reducing equivalents from NADPH to α-ketoglutarate and to a formation of D-2-hydroxyglutarate that may exceed the capacity of D-2-hydroxyglutarate dehydrogenase. The latter is indeed quite low (≈15 nmol/min/g in liver [4]) as compared to that of HOT (≈120 nmol/min/g, see Fig. 3).

The availability of the HOT sequence opens new perspectives in the study of 4-hydroxybutyrate. Some of the effects of this compound may be due to its conversion to γ-aminobutyrate, via succinate semialdehyde. Animal models in which HOT expression would be decreased or increased would be invaluable to distinguish the pharmacological effects that are contributed by 4-hydroxybutyrate per se from those that are mediated by a metabolite (e.g., γ-aminobutyrate). In the latter case, inhibitors of the transhydrogenase might be helpful in the treatment of acute 4-hydroxybutyrate intoxications. Another interesting perspective relates to inherited metabolic diseases. HOT deficiency would likely lead to the accumulation of 4-hydroxybutyrate in tissues and could therefore be, besides succinate semialdehyde dehydrogenase deficiency [18], a distinct cause of 4-hydroxybutyric aciduria. Studies aimed at testing this possibility will be facilitated by the identification of the sequence encoding HOT.

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