

Identification of a dehydrogenase acting on D-2-hydroxyglutarate

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Extracts of frozen rat liver were found to catalyse the formation of ³H₂O from DL-2-hydroxy[2-³H]glutarate. Three peaks of enzyme activities were observed on separation by chromatography on DEAE-Sepharose. The first and second peaks corresponded to an enzyme acting on L-2-hydroxyglutarate and the third peak corresponded to an enzyme acting on D-2-hydroxyglutarate, as indicated by competitive inhibition of the detritiation of the racemic radioactive compound by the unlabelled L- and D-isomers respectively. The enzyme acting on the D-form was further characterized. It was independent of NAD or NADP and it converted D-2-hydroxyglutarate into α -ketoglutarate, transferring electrons to artificial electron acceptors. It also oxidized D-lactate, D-malate and *meso*-tartrate and was stimulated by Zn²⁺, Co²⁺ and Mn²⁺, but not by

Mg²⁺ or Ca²⁺. Subcellular fractionation indicated that it was present in the mitochondrial fraction. The enzyme was further purified by chromatography on Blue Trisacryl and phenyl-Sepharose, up to a stage where only a few bands were still visible by SDS/PAGE. Among the four candidate polypeptides that were identified by MS, one corresponded to a predicted mitochondrial protein homologous with FAD-dependent D-lactate dehydrogenase. The corresponding human protein was expressed in HEK-293 cells and it was shown to catalyse the detritiation of DL-2-hydroxy[2-³H]glutarate with similar properties as the purified rat enzyme.

Key words: dehydrogenase, D-2-hydroxyglutarate, D-2-hydroxyglutaric aciduria, D-lactate, FAD.

INTRODUCTION

D-2-Hydroxyglutaric aciduria is a neurometabolic disease characterized by the accumulation of abnormal amounts of D-2-hydroxyglutarate in cerebrospinal fluid, blood and urine [1,2]. D-2-Hydroxyglutarate is formed as a degradation product of L-hydroxylysine [3] and, possibly, also from aminolaevulinic acid [1]. The enzyme that catalyses its utilization is not well identified. Wanders and Mooyer [4] reported the presence of a mitochondrial D-2-hydroxyglutarate dehydrogenase independent of NAD⁺ or NADP⁺, and transferring electrons to the artificial acceptor, iodo-tetrazolium chloride. This enzyme is present mainly in liver and kidney and also in other tissues. Neither the molecular identity of this enzyme nor the product of the reaction it catalyses are known to date. This enzyme is possibly related to D-2-hydroxyacid dehydrogenase, a mitochondrial, FAD-dependent enzyme capable of oxidizing a variety of D-2-hydroxyacids, including D-lactate, D-malate and *meso*-tartrate [5–7], and therefore possibly also D-2-hydroxyglutarate. The purpose of the present study was to characterize and identify the enzyme acting on D-2-hydroxyglutarate.

EXPERIMENTAL

Materials

Blue Trisacryl was obtained from Sigma. Phenyl-Sepharose and tritiated borohydride were obtained from Amersham Biosciences. The source of other materials was as reported previously [8]. DL-2-Hydroxy[2-³H]glutarate was prepared by incubating 1 mM α -ketoglutarate with 3.5×10^8 c.p.m. of sodium [³H]borohydride (15 Ci/mmol) in a final volume of 200 μ l for 90 min at 25 °C.

The remaining borohydride was destroyed by the addition of 0.1 ml of 10% (w/v) HClO₄. After 60 min at 25 °C, the sample was neutralized with 3 M K₂CO₃; the precipitate was removed by centrifugation and the supernatant was brought to 3 ml with water before being applied to a Dowex AG1 \times 8 column. The column was washed first with 5 ml of 50 mM NaCl and then with 5 ml of 500 mM NaCl to elute the radiolabelled DL-2-hydroxy[2-³H]glutarate.

Purification of D-2-hydroxyglutarate dehydrogenase

Frozen rat liver (typically approx. 20 g) was homogenized with 2 vol. (v/w) of buffer A (25 mM Hepes, pH 7.1/1 μ g/ml leupeptin/1 μ g/ml antipain) containing 100 mM KCl. The extract was made to 2% in poly(ethylene glycol) 8000 and centrifuged for 15 min at 15 000 g. The supernatant (33 ml) was diluted 2-fold with buffer A and applied to a DEAE-Sepharose column (1.5 cm \times 20 cm) equilibrated in the same buffer. The column was washed with 30 ml of buffer A and developed with a 0–0.5 M NaCl gradient in 2 \times 100 ml of buffer A. The most active fractions (approx. 13 ml) were pooled, supplemented with 2.5 mM CoCl₂, diluted with 2 vol. of a buffer containing 2.5 mM CoCl₂ and applied to a Blue Trisacryl column (9 cm³) equilibrated in the same buffer. The column was washed with 25 ml of equilibration buffer and developed with a 0–1 M NaCl gradient in 2 \times 75 ml of buffer A containing 2.5 mM CoCl₂. The active fractions from two such preparations were pooled (approx. 30 ml) and supplemented with 0.5 M NaCl. They were then applied to a phenyl-Sepharose column (5 cm³), equilibrated with buffer A containing 2.5 mM CoCl₂ and 0.5 M NaCl. The column was then developed with a stepwise decreasing gradient of NaCl (0.5–0 M), followed by a stepwise increasing gradient of ethylene glycol (to 50%, v/v).

Abbreviations used: ORF, open reading frame; DCIP, dichlorophenol indophenol; ETF, electron transfer flavoprotein; PMS, phenazine methosulphate.

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Protein identification by MS

In-gel tryptic digestions of protein bands and desalting of the peptides were performed as described in [9]. Peptides were analysed by nano-electrospray ionization–tandem MS in an LCQ Deca XP Plus ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, U.S.A.) fitted with a nano-electrospray probe. The results were analysed using the X-calibur software (ThermoFinnigan) and the proteins were identified using TurboSEQUEST of the BioWorks software suite (ThermoFinnigan).

Enzyme assays

In the radiochemical assay, the preparation to be assayed (usually 25 μ l) was incubated for 20 min at 30 °C in the presence of 20 mM Hepes (pH 7.1), 25 mM KCl, 1 mM MgCl₂ and 300 000 c.p.m. DL-2-hydroxy[2-³H]glutarate (approx. 0.1 nmol) in a final volume of 250 μ l. Where indicated, non-labelled D-2-hydroxyglutarate (2 μ M in the experiments shown in Figures 2–4 and 7), salts of bivalent cations or other compounds were also present. The reaction was stopped by the addition of 125 μ l of 10% HClO₄. After neutralization with 3 M K₂CO₃ and elimination of the salt precipitate by centrifugation, the samples were diluted 3-fold with water and applied to 1 ml Dowex AG1 \times 8 columns, which were washed with 2 ml of water to elute ³H₂O. The eluate was mixed with 5 vol. of scintillation cocktail (Ultima Gold; Packard, Zürich, Switzerland) and its radioactivity was counted. The *K_m* value for D-2-hydroxyglutarate was determined by this method using a constant amount of radiolabelled substrate and variable amounts of unlabelled D-2-hydroxyglutarate. It can be demonstrated that the *K_m* value determined in this way corresponds to the *K_m* value for unlabelled D-2-hydroxyglutarate, whereas, due to the primary kinetic isotope effect, the *V_{max}* value corresponds to the *V_{max}* value for unlabelled D-2-hydroxyglutarate divided by a factor corresponding to the ratio of the catalytic efficiencies for unlabelled and tritiated D-2-hydroxyglutarate. It was checked that the activity was linear with time and the concentration of the enzyme at least up to a substrate consumption of approx. 25%.

Enzyme activity was also determined spectrophotometrically at 600 nm via the reduction of DCIP (dichlorophenol indophenol) in the presence of PMS (phenazine methosulphate) [6,7]. The assay mixture (1 ml) comprised 40 μ M DCIP, 11 μ M PMS, 50 mM Tris/HCl (pH 8) and 250 μ M D-2-hydroxyglutarate or other substrates. In some experiments, such incubations were stopped after 180 min by the addition of activated charcoal to a final concentration of 2% to remove PMS and DCIP. The samples were centrifuged and the concentration of α -ketoglutarate in the resulting supernatant was determined with a spectrophotometric assay using glutamate dehydrogenase [10]. H₂O₂ formation was determined spectrophotometrically at 440 nm in an assay mixture containing 50 mM Tris/HCl (pH 8), 0.4 mM *o*-dianisidine, 0.5 mM NaN₃ and 2 units/ml horseradish peroxidase [11]; 1 unit is the amount of enzyme catalysing the oxidation of 1 μ mol of substrate per min under the specified conditions.

Subcellular fractionation

Fresh liver from an overnight fasted rat was homogenized in isotonic sucrose containing 25 mM Hepes (pH 7.1), 1 μ g/ml leupeptin and 1 μ g/ml antipain, and the homogenate was submitted to differential centrifugation [12]. The fractions were stored at –80 °C. D-2-Hydroxyglutarate dehydrogenase was partially purified from 0.5 ml of each fraction by chromatography on 1 cm³ DEAE-Sepharose columns, from which the protein was eluted with a stepwise NaCl gradient (successively, 50, 100, 150, 200, 300 and 500 mM NaCl in 25 mM Hepes, pH 7.1). D-2-Hydroxy-

glutarate dehydrogenase activity was determined by the radiochemical assay in the presence of 2 μ M D-2-hydroxyglutarate and 50 μ M ZnCl₂. It was essentially present in the fractions eluted with 200–500 mM NaCl.

Overexpression of human D-2-hydroxyglutarate dehydrogenase

The open reading frame of D-2-hydroxyglutarate dehydrogenase (accession no. BC036604) was amplified by PCR using *Pwo* DNA polymerase, human liver cDNA as the template and the primers 5'-AGGAGGAGCCCGAGGTCTCC-3' and 5'-CCTTGGCAGC-AGCAGGAGTG-3'. The PCR product was cloned into pBlue-script (restricted with *EcoRV*) and checked by sequencing. The ORF was excised from this plasmid by digestion with *SacI* and *HindIII* and inserted into the *SacI* and *HindIII* sites of pCMV5 (Novagen), a mammalian expression vector.

HEK-293 cells were plated on day 0 in Petri dishes (100 mm diameter) at a cell density of 3.6×10^6 cells/plate in DMEM, supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% (v/v) foetal bovine serum. After 16 h, the medium was replaced by a fresh medium, supplemented with 1% foetal bovine serum. The cells were then transfected with 8 μ g of pCMV5 vector containing the D-2-hydroxyglutarate dehydrogenase ORF or empty vector, using high-molecular-mass polyethyleneimine [13] obtained from Aldrich. The medium was replaced by fresh medium containing 10% foetal bovine serum 16 h after transfection and the culture was continued for 48 h. The cells were then washed with PBS and harvested in 500 μ l of 20 mM Hepes (pH 7.1) containing 5 μ g/ml leupeptin, 5 μ g/ml antipain and 0.5 mM PMSF. Cells were frozen in liquid nitrogen, thawed and lysed by mixing. The lysates were clarified by centrifugation at 2000 g for 5 min.

RESULTS

Separation of enzymes acting on L- and D-2-hydroxyglutarate

Extracts of frozen rat liver catalysed the detritiation of [2-³H]DL-2-hydroxyglutarate, the reaction reaching approx. 40% completion after 20 min incubation of a 1/40 homogenate at 30 °C. This reaction was inhibited by both unlabelled L- and D-2-hydroxyglutarate with an apparent *K_i* value of approx. 20 μ M (results not shown).

The enzymes acting on radioactive DL-2-hydroxy[2-³H]glutarate were separated by chromatography of a frozen liver extract on DEAE-Sepharose. As shown in Figure 1, three peaks were observed. The first peak corresponded to the flow-through fraction; the second one, which was quite small, eluted at approx. 100 mM NaCl; the third one eluted at approx. 250 mM NaCl. Competition experiments indicated that the enzyme in the first and second peaks was inhibited more powerfully by L-2-hydroxyglutarate than by D-2-hydroxyglutarate. In peak 1, 50% inhibition of the formation of radiolabelled water was reached at 3.5 μ M L-2-hydroxyglutarate and 180 μ M D-2-hydroxyglutarate. The opposite was true for the enzyme present in the third peak (50% inhibition at 65 and 4 μ M L- and D-2-hydroxyglutarate respectively). Allowing for small reciprocal contaminations of the two enantiomers, these results indicated that the enzyme(s) present in the first two peaks acted on L-2-hydroxyglutarate, whereas the enzyme of the third peak acted on D-2-hydroxyglutarate. Further work was focused on the characterization of the latter enzyme.

Characterization of the enzyme acting on the D-isomer

As shown in Figure 2, fractions containing activity towards the D-isomer reduced DCIP enzymically in the presence of D-2-hydroxyglutarate, indicating that the enzyme is a D-2-hydroxyglutarate

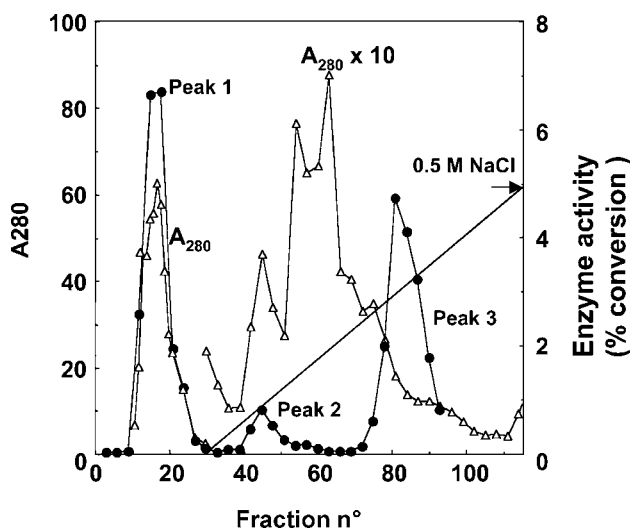


Figure 1 Separation of the enzymes acting on D- and L-2-hydroxyglutarate by chromatography on DEAE-Sephrose

Activity (●) was measured with radiolabelled DL-2-hydroxy[2-³H]glutarate on 25 μ l of each fraction. The absorbance A_{280} was also measured (Δ). The oblique line indicates the NaCl gradient.

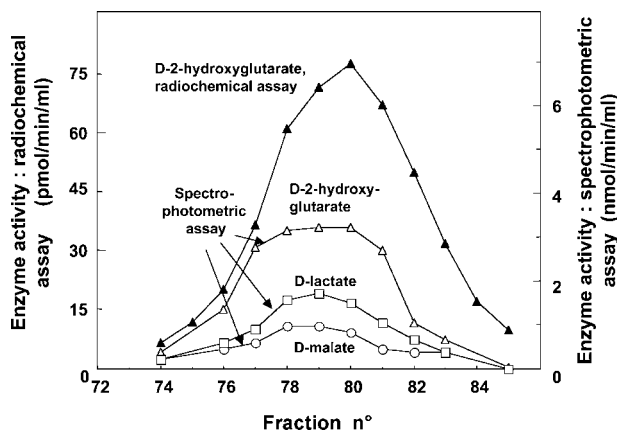


Figure 2 Co-elution of the enzyme utilizing radiolabelled D-2-hydroxyglutarate with a dehydrogenase acting on D-2-hydroxyglutarate, D-malate and D-lactate

A portion of the elution profile of a DEAE-Sephrose column is shown similar to the one shown in Figure 1. The activity was determined by the radiochemical assay in the presence of 2 μ M unlabelled D-2-hydroxyglutarate (\blacktriangle) or through the oxidation of DCIP in the presence of 1 mM D-2-hydroxyglutarate (Δ), D-lactate (\square) or D-malate (\circ). The approx. 50-fold difference in activity between the two conditions is due to a combination of several factors such as the use of subsaturating concentrations of substrate in the radiochemical assay, a primary isotopic effect and the stimulation exerted by electron acceptors (see the Discussion section).

dehydrogenase. The total D-2-hydroxyglutarate dehydrogenase activity recovered in the column amounted to the equivalent of 17 m-units/g liver. The enzyme acted also on D-lactate, D-malate (Figure 2) and *meso*-tartrate (results not shown) with approx. 2-, 4- and 3-fold lower V_{max} values respectively. Assays with different concentrations of these dicarboxylic acids indicated that the enzyme was saturated with 0.25 mM D-2-hydroxyglutarate, D-malate and *meso*-tartrate, and the K_m value for D-lactate was 0.25 mM. Figure 3 shows that *meso*-tartrate, D-malate and D-lactate inhibited the detritiation of DL-2-hydroxy[2-³H]glutarate, indicating that the same enzyme is involved in all cases. The

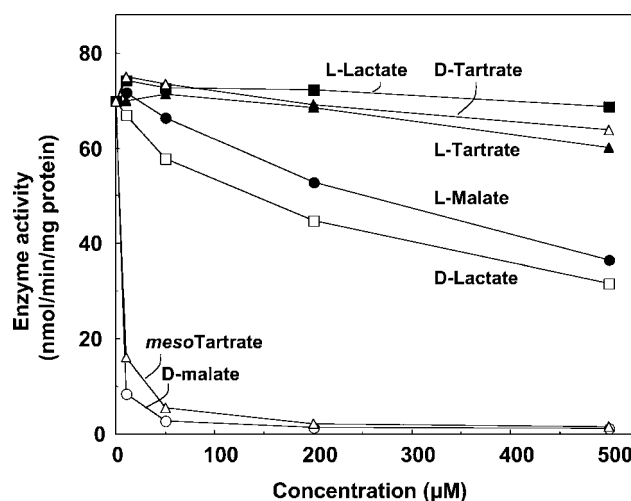


Figure 3 Sensitivity of D-2-hydroxyglutarate dehydrogenase to several 2-hydroxy carboxylic acids

The partially purified enzyme was assayed through the release of tritium from DL-2-hydroxy[2-³H]glutarate in the presence of 2 μ M unlabelled D-2-hydroxyglutarate, 50 μ M CoCl_2 and the indicated concentrations of D-malate (\circ), *meso*tartrate (Δ), D-lactate (\square), L-malate (\bullet), L-tartrate (\blacktriangle), L-lactate (\blacksquare) and D-tartrate (\triangle).

L forms of malate and lactate and both D- and L-tartrate were poor inhibitors. Some activity was observed in the spectrophotometric assay with L-2-hydroxyglutarate, but the change in absorption rapidly levelled off with time, indicating that it was due to the presence of a substrate, presumably D-2-hydroxyglutarate, contaminating the L-2-hydroxyglutarate preparation. This contamination was estimated at approx. 6% by comparing the activity with 'L-2-hydroxyglutarate' with a series of low concentrations of D-2-hydroxyglutarate.

The detritiation of DL-2-hydroxyglutarate by purified D-2-hydroxyglutarate dehydrogenase was unaffected by FAD, FMN, NAD^+ or NADP^+ . However, this does not exclude the possibility that the enzyme has tightly bound FAD, as indeed known for D-2-hydroxyacid dehydrogenase [7]. Since D-lactate dehydrogenase from several species [14] and mammalian D-2-hydroxy acid dehydrogenase [5] appear to be metalloenzymes, we tested the effect of bivalent cations on the activity of D-2-hydroxyglutarate dehydrogenase using the radiochemical assay. As shown in Figure 4, Zn^{2+} , Co^{2+} and Mn^{2+} increased the activity by 2.5–4.5-fold, whereas Ca^{2+} and Mg^{2+} had no effect and EGTA decreased the activity. Other experiments showed that 50 μ M Zn^{2+} or Co^{2+} decreased the K_m value for D-2-hydroxyglutarate from 3.2 ± 0.2 (mean \pm S.E.M.; $n = 3$) to 2.1 ± 0.4 and 1.0 ± 0.2 μ M respectively, whereas 1 mM EGTA increased it to 9.9 ± 0.8 μ M. Zn^{2+} increased the V_{max} value by approx. 4-fold and Co^{2+} increased it by only 35%, whereas EGTA had no significant effect. It should be noted that the effect of metals tended to decrease as the enzyme became older. This loss of effect resulted from both an increase in the basal activity and a decrease in the activity with Zn^{2+} or Co^{2+} . It could be partly restored by preincubating the 'desensitized' enzyme with mercaptoethanol (results not shown).

To compare the radiochemical assay with the spectrophotometric assay, we tested the effect of the electron acceptors used in this assay on the activity as determined by the radiochemical assay. In the absence of metal, V_{max} increased from a value of 0.31 m-unit/ml (i.e. 0.16 m-unit/mg of protein) to 0.91 and 1.07 m-units/ml in the presence of 11 μ M PMS + 40 μ M DCIP at pH 7.0 and 8.0

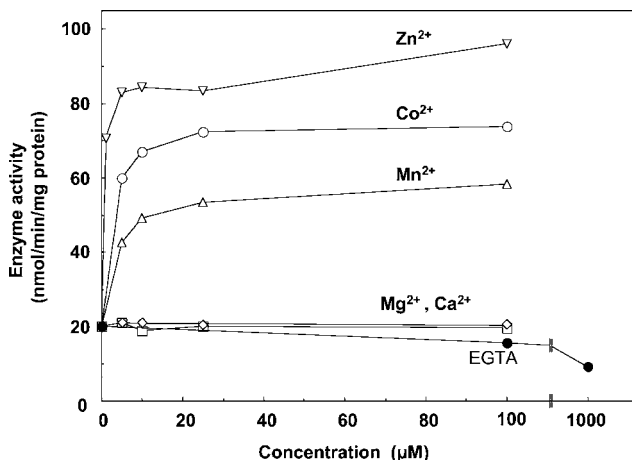


Figure 4 Effect of bivalent cations and EGTA on the activity of D-2-hydroxyglutarate dehydrogenase

Radiochemical assay was performed on the enzyme in the presence of 2 μM unlabelled D-2-hydroxyglutarate and the indicated concentrations of CoCl_2 (\circ), MnCl_2 (Δ), MgCl_2 (\square), ZnCl_2 (∇), CaCl_2 (\diamond) and EGTA (\bullet).

respectively. The latter value corresponded to 22% of the rate of the reaction determined spectrophotometrically in the same fraction and at the same pH (results not shown). As mentioned in the Discussion section, this effect is most probably explained by a primary kinetic isotopic effect. Using the radiochemical assay, we also noted that detritiation of the radiolabelled substrate was stimulated up to 2.5-fold by DCIP (at 300 μM), up to 5-fold by PMS (at 1 mM) and up to 4-fold by potassium ferricyanide (at 1 mM).

We also measured the ability of the enzyme to transfer its reducing equivalent to O_2 by measuring the formation of H_2O_2 in the peak fractions of the DEAE-Sepharose column. This rate was estimated to be approx. 20% of the rate of reduction of DCIP and PMS (results not shown), indicating that the enzyme is capable of transferring its electrons to PMS better than to O_2 .

To determine the identity of the product formed from D-2-hydroxyglutarate, the purified enzyme was incubated in the presence of PMS and DCIP together with 250 μM D-hydroxyglutarate. The amount of α -ketoglutarate formed at the end of the 3 h incubation (91 μM) amounted to 84% of the quantity expected from spectrophotometric assays of the D-2-hydroxyglutarate activity. Allowing for a probable time-dependent decrease in the activity, these results indicate that α -ketoglutarate is the reaction product. No α -ketoglutarate was formed in the absence of D-2-hydroxyglutarate or enzyme.

Subcellular distribution

Fractionation of a rat liver homogenate by differential centrifugation [12] indicated that D-2-hydroxyglutarate dehydrogenase was essentially present in the heavy and light mitochondrial fractions in which specific activities of 2.1 and 0.5 $\text{pmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ were found, compared with 0.1 $\text{pmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ in the microsomal fraction and the final supernatant.

Identification of the sequence encoding D-2-hydroxyglutarate dehydrogenase

D-2-Hydroxyglutarate dehydrogenase was further purified by chromatography on Blue Trisacryl and eluted from the column

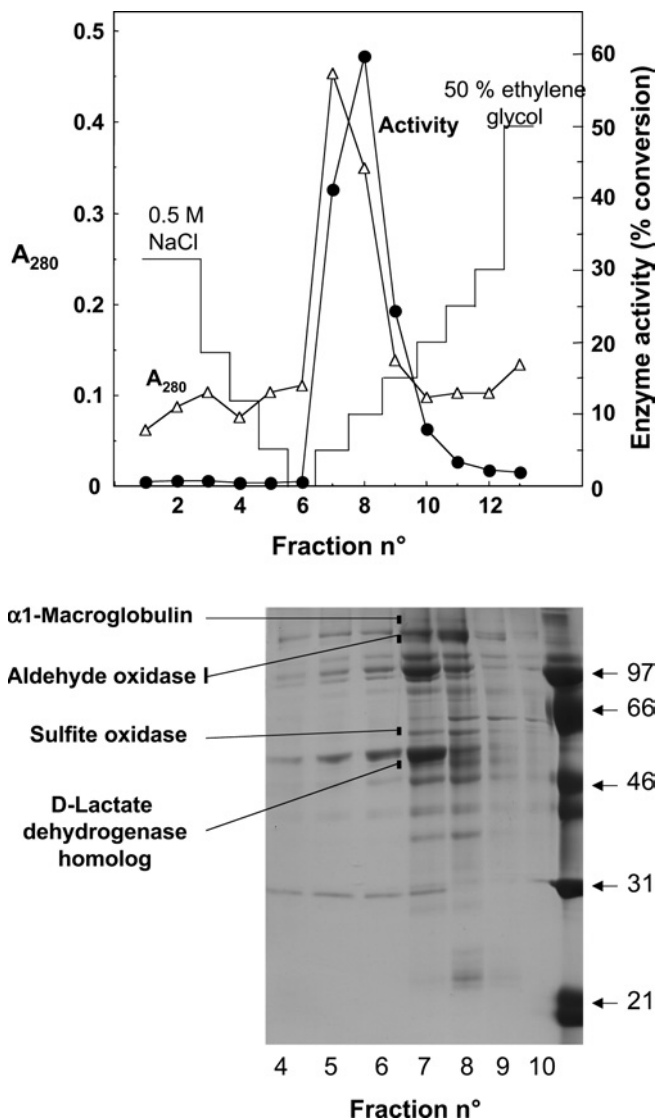


Figure 5 Purification by chromatography on phenyl-Sepharose

Upper panel: a preparation (9 mg of protein) purified by chromatography on DEAE-Sepharose and Blue Trisacryl was applied on to a phenyl-Sepharose column and eluted with a decreasing gradient of NaCl and an increasing gradient of ethylene glycol. Fractions of 30 ml (fraction 1) and 2 ml (fractions 2–13) were collected. D-2-Hydroxyglutarate dehydrogenase (\bullet) was measured by the radiochemical assay on 10 μl of each fraction. Lower panel: SDS/PAGE of the fractions. The indicated bands were found to co-elute with the activity both in the Blue Trisacryl (results not shown) and the phenyl-Sepharose columns. They were cut from the gel, digested with trypsin and their identity was determined by electrospray ionization–tandem MS.

at a concentration of approx. 150 mM NaCl (results not shown). Further purification was obtained by chromatography on phenyl-Sepharose, where the enzyme eluted at the start of the ethylene glycol gradient (Figure 5, upper panel). The overall yield of these two steps was approx. 20% as estimated by the radiochemical assay, and the purification factor over the homogenate was approx. 1000-fold, assuming complete recovery of the enzyme after the DEAE-Sepharose column. SDS/PAGE indicated the presence of about 15 bands in the most purified fractions. Four of these, which co-purified with the D-2-hydroxyglutarate dehydrogenase activity in both the Blue Trisacryl (results not shown) and the phenyl-Sepharose columns (Figure 5), were cut from the gel, digested with trypsin and analysed by electrospray ionization–tandem MS.

HsHd	MLPRRPLAWPAWLLRGAPGAAGSW ----- GRPVGP --- LARRGCCSAPGTPEVP	046
MmHd	MVLPLVSRW SARV LWAS PGWRR TYTQRACVGLKRL GCP RGVYSPLA H RAYSVVAGS PEVT	060
CsD2	MLRNILVRSTGSNFKFAGRYMKSSALLGYRRVNYSTKIQTR	043
HsLd	MARLLRS	007
HsHd	L TRERYP-VQRLP-FSTVSKQDLAAFERIVPGGVV---TDPEALQAPNVDWLRTL RGCSK	101
MmHd	L TPERYP-VQRLP-FSTVSEEDLAAFE CII PGRVI---TDPEQLQTCNVDWLK TVRGCSK	115
CsD2	LT SENYPDVHRDPRFKKLTSDDLNYFKSILSEQEILRASESEDLSEFY NEDW MRKYKGQ SK	103
HsLd	ATWELFPWRG YCSQ KAKGELCRDFVEALKAVVGGSHV STA AVVREQHGR DES VHRCEPPD	067
HsHd	VLLR PRTSEEVSHILRHCHERNLAVNPQGGNTGMVGGSVVFDEIILSTAR MNRVLSFHS	161
MmHd	VLLR PQTSEEV SQIL RHCYKRNLAVNPQGGNTGMVGGSVVFDEVILSTAL MNQVLSFHD	175
CsD2	L VLR PKSV EKVS LILN YCN DEK IAV VPQGGNTGLVGGSV PIF DELIL LSLAN LNKIRDFDP	163
HsLd	AVVWPQN VEQV SR LAAL CYRQGV PII PFGTGTGLEGGVCAVQGGVCVNL THMDRILELNQ	127
HsHd	VSGIL VQAGCVLEELSRV VEERDF FIMPLDLGAKGSCHIGGNVATNAGGLR FLRYGSLHG	221
MmHd	VSGIL VQAGCVLEELSRV QERDF FIMPLDLGAKGSCHIGGNVATNAGGLR FLRYGSLRG	235
CsD2	VSGIL KCDAGVILENANN YVMEQ NYMFPLDLGAKGSCHVGGV VATNAGGLRL RYGSLHG	223
HsLd	EDFSVV VEPG VTRKAL NAHL RD SGLW FPVDFGADAS--LCG MAATG ASGTNA VRYG TMRD	185
HsHd	TVL GLEVVLADGT VLDC LTSL---RKDNTGYDLK QL FIGSEGT LGI ITTVSILCPP KPRA	278
MmHd	TVL GLEVVLADGT ILNCL TSL---RKDNTGYDLK QM FIGSEGT LGI ITAVSIVCPP RPKA	292
CsD2	SVL GLEVVM PNGQ IVNSM HSM ---RKDNTGYDLK QL FIGSEGT IGI ITGV SILT VP KPKA	280
HsLd	NVLN LE VVLP DG RL LHTAGRGR HF RK SAAG YN L T GLF VGSEGT LGL ITATTLRL HPAPEA	245
HsHd	VNV AF LGCPG FAEV LQ -TFSTCK GMLGE IL SAFE FMDAVCM QLVGR HHL AS -PVQ-ESP	335
MmHd	VNV AF LGCPG FAEV LQ -TF RTC R GMLGE IL SAFE FMD TEC M QLV G HLQ LT N -PVQ-ESP	349
CsD2	FNV S YLS VE SFED VQ K -V FVR ARQ ELSE IL SAFE FMD AKS Q VLAK SQ LK DA AF PLE DEHP	339
HsLd	TVA AT CA FP S VQA AVD STV HIL QA AVP--VAR IE F L DEV MDAC NR Y SK L N CL VAP ILFL	303
HsHd	FYV LI ETSG SNAG HDAE KL GH FLE HALG SGLV TDG TM ATD Q RK V KML WAL RERITEA ---	392
MmHd	FYV LV ETSG SAG HDAE KL TNV LE QVL NSGLV TDG TM ATD Q RK V QML WAL RERITEA ---	406
CsD2	FYI LI ETSG SN KD H DSK LE TFL ENV MEGI V TDG V VAQ DE TEL Q NL W KW RE MIPEA ---	396
HsLd	----- EF HGS Q Q ALE - E Q L Q RTE E I V Q Q NG AS DF SWAKE AE ER TR - L W TAR H NA W YA ALA	356
HsHd	LSR DG VY FK YD LS LP VER LYD IV TD LR RL ----- G PHAK HVV --- G Y GHL GD GN LHL N	443
MmHd	LSR DG VY FK YD LS LP VER LYD LV ID LR TRL ----- G PRAK HVV --- G Y GHL GD GN LHL N	457
CsD2	SQ ANG G V Y KY D V SL PL KD LY S LV EAT N ARL SE AE LV GDS PK F V VGA I GY GH V GD GN LHL N	456
HsLd	TR PG CK GY ST D V CV PI S RL PE IV VQ T KED L----- N ASGL TGS --- I V GH V GD GN F HC I	407
HsHd	V -T AE AF S PS LLA LE PH VYEW TAG Q Q -- G SV SAE H G V G FR KRD V LG Y SK PP GA L Q LM Q	500
MmHd	V -T AE AF S RE LL GA LE PY VYA WT AE Q R -- G SV SAE H GL G F K K D V LG Y SK PP AV TL M Q	514
CsD2	V -AV RE YN KNI E K T LE PF VYE F V SS KH -- G SV SAE H GL G F Q K NY IG Y SK SP EE V K MM D	414
HsLd	LL VN PD AAE EL GR V K FA EQ L GRR AL AL H G T CT G EH G IG M G K R Q LL Q EE V G AV G V ET M R Q	467
HsHd	LK ALL DP K GIL NP YK TL PS QA	521
MmHd	LK AM LDP K GIL NP YK TL PA QA	535
CsD2	LK V HY DP NG IL NP Y K K YI	530
HsLd	LK AV LDP Q GL M NP G K VL	484

Figure 6 Sequence alignment of human D-2-hydroxyglutarate dehydrogenase (HsHd) and mouse D-2-hydroxyglutarate dehydrogenase (MmHd) with *S. cerevisiae* actin-interacting protein 2 (CsD2) and human D-lactate dehydrogenase (HsLd)

The peptidic sequences identified in the rat protein are underlined in the mouse sequence. Except for the last peptide (NVLGYSKPPVAVK in the rat sequence), they are identical in the sequences from both species. The predicted cleavage site for the mitochondrial presequence is double underlined.

The upper three bands were identified as rat α 1-macroglobulin, rat aldehyde oxidase and rat sulphite oxidase. The fourth gave a high score (five identical peptides; Figure 6) with a 536-residue mouse putative protein (gi/23271107) that displays approx. 30% sequence identity with D-lactate dehydrogenase from *Kluyveromyces lactis* (results not shown). It also matched (four identical peptides) a rat sequence (gi/27686389) corresponding to the last 150 residues of the above-mentioned mouse protein. As shown in Figure 6, the mouse protein was homologous with human hypothetical protein gi/22477764 (85% identity in the last 480 residues), as well as with *Saccharomyces cerevisiae* 'actin-interacting protein 2' (P46681). These sequences are more

distantly related to D-lactate dehydrogenases, including the human enzyme (gi/29126992) [15].

Using the TargetP prediction program [16], the human and mouse putative D-2-hydroxyglutarate dehydrogenases were predicted to be mitochondrial proteins with scores of 0.781 and 0.916 respectively. This is in agreement with the subcellular fractionation mentioned above. The predicted mitochondrial presequence cleavage sites (RRG/CC in the human sequence, HRAY/S in the mouse sequence) are at similar positions in the two sequences. The N-terminal presequences were found to be poorly conserved (<20% identity) when the mouse and human proteins were compared. Removal of the prepeptide results in polypeptide

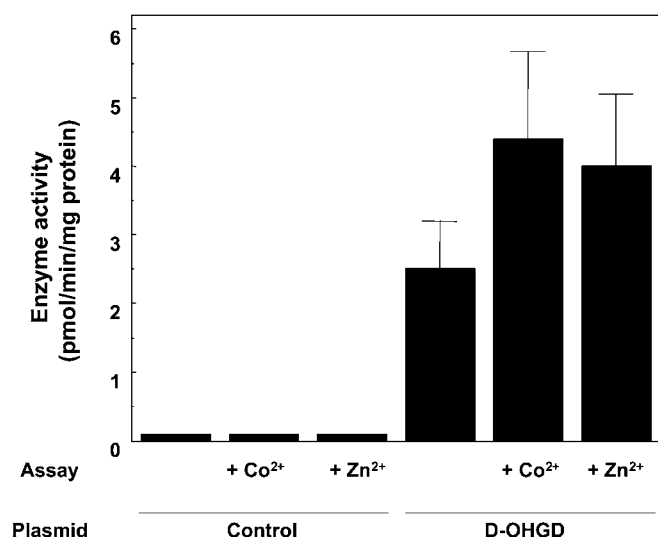


Figure 7 Effect of overexpression of the putative human D-2-hydroxyglutarate on the detritiation of radiolabelled 2-hydroxyglutarate

Cells were transfected with a vector containing the ORF of the putative D-2-hydroxyglutarate dehydrogenase (D-OHGD) or with an empty vector (control). The enzyme activity was determined by the radiochemical assay in the presence of 2 μ M unlabelled D-2-hydroxyglutarate; 50 μ M CoCl₂ or ZnCl₂ was present where indicated. Results are the means \pm S.E.M. for four independent transfections.

chains with a predicted mass of approx. 53 kDa, in agreement with the molecular mass of the purified rat protein as determined by SDS/PAGE.

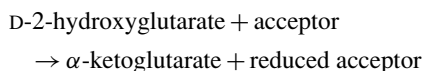
Expression of D-2-hydroxyglutarate dehydrogenase in HEK-293 cells

To confirm the identity of the protein, we overexpressed the human sequence encoding the putative D-2-hydroxyglutarate dehydrogenase in HEK-293 cells. As shown in Figure 7, overexpression of the protein significantly increased the conversion of radiolabelled DL-2-hydroxyglutarate. As expected, the activity on radiolabelled DL-2-hydroxyglutarate was stimulated by Zn²⁺ and Co²⁺ and was inhibited by unlabelled D-2-hydroxyglutarate at concentrations lower than 10-fold when compared with L-2-hydroxyglutarate (50% inhibition of the detritiation at 0.8 and 10 μ M respectively). Allowing for a contamination of L-2-hydroxyglutarate by the D-isomer, this indicated that the sequence encoded D-2-hydroxyglutarate dehydrogenase.

DISCUSSION

Identification of D-2-hydroxyglutarate dehydrogenase

In the present study, we report the identification of an enzyme catalysing the following reaction:



This enzyme also catalyses the oxidation of other D-2-hydroxy acids, particularly D-malate, D-lactate and mesotartartrate, although at lower rates or with lower affinities than observed with D-2-hydroxyglutarate. Therefore this enzyme is best named as 'D-2-hydroxyglutarate dehydrogenase'.

The activity of this enzyme could be measured in several ways, e.g. through the formation of a reduced acceptor or α -ketoglutarate

or through the detritiation of DL-2-hydroxy[2-³H]glutarate. The first two types of assays were not very sensitive and could be used only on a partially purified enzyme, due to interferences in cruder material (presence of compounds reacting spontaneously with artificial electron acceptors; presence of substrates and enzymes, leading to the formation and/or the consumption of α -ketoglutarate).

The principle of the radiochemical assay is based on the fact that the reduced function in FADH₂ [17] or in artificial acceptors (unlike in NAD or NADP) is a nitrogen or an oxygen, which easily exchanges protons with water. Since the tritiated substrate was a racemic mixture, this assay could be used to measure D-2-hydroxyglutarate dehydrogenase only after it had been separated from an enzyme acting on the L-form. This difficulty should be overcome by preparing the pure radiolabelled D-isomer. An advantage of this radiochemical assay is its high sensitivity, allowing us indeed to detect D-2-hydroxyglutarate dehydrogenase and to determine its very low K_m value. The activity was less with DL-2-hydroxy[2-³H]glutarate compared with unlabelled substrate. The value of the primary kinetic isotopic effect (approx. 4.5) indicates that the breaking of the C2-H bond is probably a rate-limiting step in the reaction. This is reminiscent of the situation observed with L-2-hydroxyacid oxidase, for which a large (approx. 8) deuterium isotopic effect has been observed using L-lactate as a substrate [18].

Extensive purification of the enzyme allowed us to obtain sequence information on several candidate polypeptides. Three of them could be excluded because of their function (aldehyde oxidase 1; sulphite oxidase) or extracellular localization (α 1-macroglobulin). A fourth protein, a hypothetical protein of unknown function, homologous with D-lactate dehydrogenase, was a logical candidate. Its identity with D-2-hydroxyglutarate dehydrogenase was confirmed by overexpression in HEK-293 cells. Extracts from these cells indeed catalysed the detritiation of DL-2-hydroxy[2-³H]glutarate with characteristics (activation by Zn²⁺ and Co²⁺, inhibition by unlabelled D-2-hydroxyglutarate at concentrations in the μ mol range) similar to that of the enzyme partially purified from rat liver.

The sequence D-2-hydroxyglutarate dehydrogenase belongs to a family of enzymes (including D-lactate dehydrogenases and vanillyl-alcohol oxidase) that use FAD as a cofactor. Search of the pfam database (<http://www.sanger.ac.Uk/Software/Pfam/>) indicates the presence of a FAD-binding domain type 4 (residues 100–261) and a FAD-oxidase C-terminal domain (residues 275–516). The absence of any detectable effect of FAD on the enzyme activity was presumably due to a tight binding of this prosthetic group to the enzyme, as observed with many flavoproteins. Several D-lactate dehydrogenases appear to be Zn²⁺-dependent enzymes [14,19,20]. Similarly, D-2-hydroxyglutarate dehydrogenase is stimulated by Zn²⁺, Co²⁺ and Mn²⁺, and inhibited by the chelating agent EDTA.

Comparison with previous work

The enzyme that we have identified shares many of the properties of a D-2-hydroxyglutarate dehydrogenase described by Wanders and Mooyer [4]. The latter enzyme is indeed able to transfer electrons from its substrate to artificial acceptors, but not to NAD or NADP. Furthermore, it is found in several tissues, particularly liver and kidney. Our preliminary results indicate a similar tissue distribution for the low- K_m enzyme described in the present study (E. van Schaftingen, unpublished work). However, neither the substrate specificity nor the identity of the reaction product has been reported by Wanders and Mooyer [4]. Furthermore, these authors mention that their enzyme has a K_m value of 15 mM, which

is more than three orders of magnitude higher than our estimate. One explanation for the discrepancy is that there are two distinct enzymes acting on D-2-hydroxyglutarate. The low concentration of D-2-hydroxyglutarate found in the plasma of control subjects ($< 1 \mu\text{M}$ according to Cremona and Singer [20]) indicates that the low- K_m dehydrogenase that we describe would be of more importance to account for the physiological metabolism of this compound.

The D-2-hydroxyglutarate dehydrogenase described in the present study also shares several properties with a rabbit kidney D-2-hydroxyacid dehydrogenase characterized approx. 40 years ago [5–7]. Both enzymes are mitochondrial proteins that contain FAD, are inhibited by metal-chelating agents and reduce O_2 at only a fraction of the rate at which they reduce DCIP in the presence of PMS [6,7]. Both of them act on D-malate, D-lactate and *meso*-tartrate, but either do not act or act poorly on L-lactate and L-malate [6]. Unfortunately, D-2-hydroxyglutarate was not tested on D-2-hydroxyacid dehydrogenase. A major difference between the two enzymes again appears to be in the affinity for the substrates. The K_m value of 1 mM for D-malate found by Tubbs and Greville [6] is much higher than that found for D-2-hydroxyglutarate dehydrogenase, since, using the same type of assay as these authors, we found that the rate was already maximal with 0.25 mM D-malate. Furthermore, the competition experiments (see Figure 3) suggest that the K_m value is probably lower than 10 μM . This large difference again suggests the existence of two different FAD-dependent dehydrogenases acting on D-2-hydroxy acids. One of them, described in the present study, acts best on D-2-hydroxyglutarate. The other would act best on D-lactate [6] and may therefore correspond to the sequence reported as D-lactate dehydrogenase [15].

The enzyme that we have isolated is probably also distinct from the *trans*-hydrogenase identified by Kaufman et al. [21]. This enzyme catalyses the reversible transfer of reducing equivalents from γ -hydroxybutyrate to α -ketoglutarate, leading to the formation of succinate semialdehyde and D-2-hydroxyglutarate. We have indeed observed that the activity of D-2-hydroxyglutarate dehydrogenase, measured by the radiochemical assay, was not affected by up to 5 mM γ -hydroxybutyrate (results not shown).

Physiological and pathophysiological implications

Since D-2-hydroxyglutarate dehydrogenase is most probably the main enzyme that consumes D-2-hydroxyglutarate, mutations in its gene are probably responsible for primary D-2-hydroxyglutaric aciduria [22]. D-2-Hydroxyglutarate also accumulates in glutaric aciduria type II [23] due to a deficiency in ETF (electron transfer flavoprotein) or in ETF dehydrogenase [24]. ETF and ETF dehydrogenase serve to transfer the electrons collected by dehydrogenases that are soluble in the mitochondrial matrix to the respiratory chain. The accumulation of D-2-hydroxyglutarate in glutaric aciduria type II therefore suggests that the ETF–ETF-dehydrogenase system also serves to transfer electrons from D-2-hydroxyglutarate dehydrogenase. It is worth stressing, in this respect, that D-2-hydroxyglutarate dehydrogenase appears to be easily released from mitochondria by freezing–thawing, indicating that it is not tightly bound to membranes but is probably well soluble in the mitochondrial matrix.

Assuming that the activity of the dehydrogenase is the same in both human liver and rat liver (approx. 15 m-units/g) and the activity at 37 °C is two times higher than that at 30 °C, it can be calculated that the liver can metabolize at least 40 mmol of D-2-hydroxyglutarate per day in a 70 kg adult. This value has to be compared with excretions of D-2-hydroxyglutaric acid at the rate of 4 mmol/mmol of creatinine [22], i.e. approx. 40 mmol/day.

Therefore the enzyme that we have identified could account for the rate of disposal of D-2-hydroxyglutarate, particularly if one takes into account the fact that D-2-hydroxyglutarate dehydrogenase is also present in tissues other than liver.

Presence of an enzyme acting on L-2-hydroxyglutarate

Our findings also confirm the existence of an enzyme that utilizes L-2-hydroxyglutarate. Although we have not formally proven that it is a dehydrogenase, it is tempting to speculate that it is responsible for the oxidation of L-2-hydroxyglutarate by tissues observed more than 60 years ago by Weil-Malherbe [25] and its deficiency is responsible for L-2-hydroxyglutaric aciduria [26], a disease for which the enzyme defect is hitherto unknown. Further work will be devoted to its characterization and identification.

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