Lysine degradation through the saccharopine pathway in mammals: involvement of both bifunctional and monofunctional lysine-degrading enzymes in mouse

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Lysine–oxoglutarate reductase and saccharopine dehydrogenase are enzymic activities that catalyse the first two steps of lysine degradation through the saccharopine pathway in upper eukaryotes. This paper describes the isolation and characterization of a cDNA clone encoding a bifunctional enzyme bearing domains corresponding to these two enzymic activities. We partly purified those activities from mouse liver and showed for the first time that both a bifunctional lysine–oxoglutarate reductase/saccharopine dehydrogenase and a monofunctional saccharopine dehydrogenase are likely to be present in this organ. Northern analyses indicate the existence of two mRNA species in liver and kidney. The longest molecule, 3.4 kb in size, corresponds to the isolated cDNA and encodes the bifunctional enzyme. The 2.4 kb short transcript probably codes for the monofunctional dehydrogenase. Sequence analyses show that the bifunctional enzyme is likely to be a mitochondrial protein. Furthermore, enzymic and expression analyses suggest that lysine–oxoglutarate reductase/saccharopine dehydrogenase levels increase in livers of mice under starvation. Lysine-injected mice also show an increase in lysine–oxoglutarate reductase and saccharopine dehydrogenase levels.

Key words: amino acid, catabolism, cDNA, kidney, liver.

INTRODUCTION

The saccharopine pathway is thought to be the main metabolic route for lysine degradation in upper eukaryotes. This pathway has been described in plants [1–4] and mammals [5–10], and its first two reactions are catalysed by enzymic activities known as lysine–oxoglutarate reductase (LOR; EC 1.5.1.8) and saccharopine dehydrogenase (SDH; EC 1.5.1.9). The reductase activity condenses lysine and 2-oxoglutarate to form saccharopine [ϵ-*N*-(L-glutaryl-2)-L-lysine]. Saccharopine is subsequently oxidized by the dehydrogenase activity to produce α-aminoadipic δ-semialdehyde and glutamic acid.

Characterization of LOR activity in the immature maize endosperm [1,11] gave evidence for the operation of this lysine degradation pathway in plants. Both LOR and SDH activities reside on a single bifunctional polypeptide whose native form is a homodimer composed of identical 117 kDa subunits [4]. Recently, cDNA and genomic sequences from maize (E. L. Kemper, G. Cord-Neto, F. Papes, K. C. M. Moraes, A. Leite and P. Arruda, unpublished work) and *Arabidopsis* [12,13] have been obtained. The proteins encoded by these clones bear regions exhibiting similarity to the corresponding separate LOR and SDH from yeast.

In mammals, the saccharopine pathway is important for lysine catabolism in the liver, and it involves LOR and SDH activities that are biochemically similar to those described in plants [6–10,14]. In baboon and bovine livers these two activities reside on a single polypeptide [14,15] and the bifunctional protein purified from these sources has been named aminoadipic semialdehyde synthase. Its native form seems to be a tetramer of molecular mass 468 kDa composed of four identical bifunctional 115 kDa subunits [14]. A tetrameric form has also been observed in human liver and placenta [16,17]. However, non-linked LOR and SDH polypeptides have been isolated from rat liver [18].

Several aspects of the functional relevance of LOR and SDH in mammalian physiology should be considered. Firstly, these enzyme activities are responsible for lysine catabolism in the liver, contributing not only to the general nitrogen balance in the organism but also to the controlled conversion of lysine into ketone bodies [6,7,9,19,20]. Secondly, lysine is an essential amino acid and its supply is therefore required during embryonic development and early childhood. Moreover, lysine is frequently the first limiting amino acid in human and animal diets high in grain [21,22]. Hence, further knowledge about the enzymes and corresponding genes involved in lysine degradation is of special interest from the nutritional point of view. Thirdly, LOR activity has also been detected in rat brain mitochondria during embryonic development [23]. This opens the question of whether the degradation of lysine has any functional significance during brain development and puts a new focus on the nutritional requirements for lysine in gestation and infancy. Fourthly, LOR and/or SDH deficiencies seem to be involved in a human autosomic genetic disorder known as familial hyperlysinaemia, which is characterized by serious defects in the functioning of the nervous system [10,24,25].

Here we describe the isolation and characterization of a mouse liver cDNA clone encoding the bifunctional enzyme LOR/SDH. Northern blot analysis of the corresponding gene in a variety of

Abbreviations used: LOR, lysine–oxoglutarate reductase; PEG, poly(ethylene glycol); SDH, saccharopine dehydrogenase.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AF003551 (maize LOR/SDH and AJ224761) (cDNA-LOR/SDH, encoding the mouse LOR/SDH bifunctional enzyme).

tissues along with the partial purification of LOR and SDH activities suggest that a bifunctional LOR/SDH and a monofunctional SDH are likely to be present in mouse. Furthermore, expression analyses and enzyme assays suggest that LOR/SDH levels increase in the liver of animals under starvation and in the liver of lysine-injected mice.

EXPERIMENTAL

Chemicals

L-Lysine, L-saccharopine, poly(ethylene glycol) (molecular mass 8000 Da) (PEG 8000), NADPH, NAD^+, dithiothreitol, 2-oxoglutaric acid, Nitro Blue Tetrazolium, phenazyne methosulphate, EDTA, paraformaldehyde, phenol, benzamidine, GTP and common buffer salts were purchased from Sigma. DEAE-Sephacel and proteins used in molecular mass calibration were obtained from Pharmacia Biotech.

Library screening and computer analysis

An *Eco*RI/*Not*I 1.2 kb fragment of a mouse liver expressed sequence tag clone (I.M.A.G.E. Consortium; I.D. 738285) was used as a probe to screen a normal liver cDNA library (6-week-old C57Bl6/CBA females; Stratagene). Screening procedures were performed as recommended in the manufacturer's protocol.

Nucleotide and predicted protein sequences were analysed on DNASIS Sequence Analysis software (Pharmacia/LKB). Amino acid sequence alignments were performed with CLUSTAL W software [26] and the BOXSHADE program, used remotely at the ISREC Bioinformatics Group WWW server (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland).

Subcellular targeting computational analysis was performed remotely with the Claros and Vincens MitoProt II program [27] at the Ludwig-Maximillians University WWW server (Munich, Germany). Subcellular localization prediction was also performed at the PSORT II server at the University of Tokyo (Tokyo, Japan). First this engine runs a subprogram to predict the presence of signal sequences by the McGeoch method [28]. Next, PSORT applies von Heijne's method for signal sequence recognition [29]. Lastly, PSORT employs a discriminant method (called 'MITDISC') to recognize mitochondrial targeting [30].

DNA and RNA gel blot analyses

Genomic DNA from C57 Black 6 mice was extracted from tail tips, by the method of Hogan et al. [31]. DNA (10 μg) was digested with *Eco*RI and *Bam*HI and analysed by electrophoresis in 0.8 % agarose gel. Digests were transferred to Hybond N^+ membrane (Amersham) with the use of standard procedures [32]. The membrane was hybridized with LOR- and SDH-specific probes, corresponding to nt 1–864 and 1410–2139 respectively of the isolated cDNA clone. Hybridization was performed at 65 °C in 5 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate)/-5 × Denhardt's (Denhardt's solution is 0.02 % Ficoll/0.02 % polyvinylpyrrolidone/0.02 % BSA)/1 % (w/v) SDS. The blot was washed twice for 10 min at 65 °C in SSPE [0.15 M NaCl/-10 mM sodium phosphate/1 mM EDTA (pH 7.5)]/0.1 % SDS, followed by two additional washes for 10 min at 65 °C in 0.1 × SSPE/0.1 % SDS. Before hybridization with a different probe, the blot was stripped in boiling 0.5 % SDS solution. Radioactive bands were detected by autoradiography.

For analysis of the tissue expression pattern, Northern blotting was performed with the Mouse Multiple Tissue Northern Blot (Clontech) by using the LOR- and SDH-specific probes. Hybridization was performed at 65 °C with the ExpressHyb hybridization buffer (Clontech). Radioactive bands were detected by autoradiography. For the remaining Northern blot procedures, 20 μg of total RNA was analysed in formaldehyde-containing 1 % (w/v) agarose gels as described by Sambrook et al. [32]. Hybridization conditions were the same as for the Southern blots, with the SDH-specific probe. Radioactive bands were quantified on a Storm 840 PhosphorImager (Molecular Dynamics). RNA from each animal was processed and analysed separately.

Partial purification of LOR and SDH activities from mouse liver

LOR and SDH activities were partly purified from mouse liver (C57 Black 6) by using the method described by Gonçalves-Butruile et al. [4], with some modifications. Livers were removed surgically, fragmented and washed thoroughly in PBS cold solution (137 mM NaCl/2.7 mM KCl/4.3 mM Na_2HPO_4/-1.4 mM KH_2PO_4) to remove excess blood and were frozen immediately under liquid nitrogen. The following steps were performed at 4 °C. Semi-thawed tissue (15 g) was homogenized in 35 ml of buffer A [25 mM sodium phosphate (pH 7.4)/1 mM EDTA/5 mM dithiothreitol] containing 5 mM benzamidine and 100 μM leupeptin. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 10 min at 19000 ***g*** in an SS-34 rotor (Sorvall Instruments). The pH of the supernatant was adjusted to 5.6 by the addition of solid NaH_2PO_4; PEG 8000 was then added to a 7.5 % final concentration. The sample was mixed gently but thoroughly for 20 min and then centrifuged for 10 min at 19000 ***g***. The supernatant was brought to a final concentration of 15 % PEG 8000 and centrifuged again for 10 min at 19000 ***g***. The pellet was resuspended in 9 ml of buffer A containing 100 μM leupeptin and dialysed overnight at 4 °C in 1 litre of buffer A. The dialysed sample was applied to a DEAE-Sephacel column (1.2 cm × 15 cm), previously equilibrated with buffer A. The column was washed with 30 ml of buffer A and the enzyme was eluted with a 50 ml linear NaCl gradient (0–500 mM) in buffer A. Fractions containing LOR and SDH activities were pooled, brought to 70 % satn. with solid $(NH_4)_2SO_4$ and centrifuged at 4 °C for 15 min at 19000 ***g***. The pellet was resuspended in a small volume of buffer B (buffer A containing 300 mM NaCl) and applied to a Superdex-200 HR column (Pharmacia Biotech) previously equilibrated with buffer B.

Enzyme assays

LOR activity was measured spectrophotometrically in the direction of NAPDH to $NADP^+$ at 37 °C. The reaction mixture had a final volume of 0.8 ml and contained 20 mM L-lysine, 0.1 mM NADPH, 10 mM 2-oxoglutaric acid (neutralized to pH 7.0 with KOH), 175 mM Tris/HCl, pH 7.4, and approx. 0.04 mg of total protein. SDH activity was also measured spectrophotometrically in the direction of NAD^+ to NADH at 37 °C in 0.8 ml of reaction mixture containing 1 mM L-saccharopine, 2 mM NAD^+, 0.1 M Tris/HCl, pH 8.5, and approx. 0.04 mg of total protein. One enzyme unit was defined as 1 mmol NADPH oxidized or NAD^+ reduced at 37 °C. Protein concentrations were determined by the method of Bradford [33] with the Bio-Rad protein dye reagent.

PAGE

Discontinuous PAGE was performed at 4 °C on 6 % (w/v) slab gels. After electrophoresis, the gels were developed for SDH activity in 4 mM saccharopine (absent from the control gel)/-2 mM NAD^+/0.1 % Nitro Blue Tetrazolium/0.02 mM phenazyne methosulphate/0.1 M Tris/HCl (pH 8.5)/15 mM 2-oxoglutarate/1 μM GTP at 37 °C for 30 min.

Histochemical detection of SDH activity in sectioned kidneys

Kidneys from 10-week-old C57 Black 6 animals were removed surgically and washed in cold PBS to remove excess blood. The organs were fixed rapidly in 0.4% paraformaldehyde, pH 7.0, for 30 min at 4 °C and then sectioned longitudinally with a razor blade. Sections were incubated in PBS at 4 °C for 3 days to remove endogenous substrates. Subsequently, sections were equilibrated in 0.1 M Tris/HCl, pH 8.5, before being stained for 1 h in the SDH-staining solution described above, except that 2-oxoglutarate and GTP were not used. Control sections were incubated in the absence of saccharopine. Reaction was stopped by rinsing the organ in doubly distilled water.

Starving conditions and lysine and saccharopine injection protocols

For experiments involving starvation, 10-week-old C57 Black 6 animals were starved for 1 or 2 days. Livers were removed surgically, washed thoroughly in PBS to remove excess blood, then frozen under liquid nitrogen. For enzymic measurements of LOR or SDH, 0.5 g of semi-thawed tissue was homogenized in 5 ml of cold buffer A, centrifuged at 39000 ***g*** for 45 min at 4 °C in an SS-34 rotor to obtain a very clear extract. Approx. 1 mg of total protein was used for enzymic assays. For RNA extraction, 0.2 g of tissue was processed by the method of Chomczinsky and Sacchi [34].

For the injection experiments, L-lysine (0.5 ml of 1 M solution) or L-saccharopine (0.5 ml of 20 mM solution) were injected intraperitoneally and after 1 or 2 days, during which the animals were given free access to a laboratory chow diet, livers were processed as for the starvation experiments. Livers were always collected at the same time during the day so as to avoid influences of diurnal variations in LOR and SDH activities [35].

RESULTS

Isolation of a mouse liver cDNA encoding an LOR/SDH bifunctional protein

In yeast, the saccharopine pathway is used for lysine biosynthesis and involves reactions similar to those described for lysine degradation in mammals and plants [36]. The two final steps of the yeast pathway are catalysed by LOR and SDH encoded by separate genes, namely *Lys1* and *Lys9* [37,38]. We searched the GenBank looking for expressed sequence tags presenting similarity either to these yeast enzymes or to the maize LOR/SDH, whose gene had been cloned recently in our laboratory. Clone no. 738285, sequenced from a mouse normal liver cDNA library, showed significant similarity to the 3′ end of the yeast SDH and to the SDH domain of the maize enzyme.

A restriction fragment of this clone was then used to screen a C57 Black 6 mouse liver cDNA library. Seven independent positive clones were obtained from a total of 10^6. Three clones exhibiting inserts larger than 3.0 kb were sequenced and proved to represent the same transcript. The longest clone (cDNA-LOR/SDH), 3.3 kb in size, was chosen for further characterization; 5′ rapid amplification of cDNA ends experiments confirmed that this cDNA was full-length (results not shown). cDNA-LOR/SDH has a single open reading frame encoding a protein of 926 amino acid residues with an expected molecular mass of 109 kDa, which is consistent with the 115 kDa determined for the bifunctional polypeptide purified from bovine and baboon livers [14]. Multiple amino acid sequence alignments of the obtained clone and the yeast LOR and SDH enzymes revealed that the cDNA-LOR/SDH clone predicts a protein similar to both LOR and SDH (results not shown). Regions

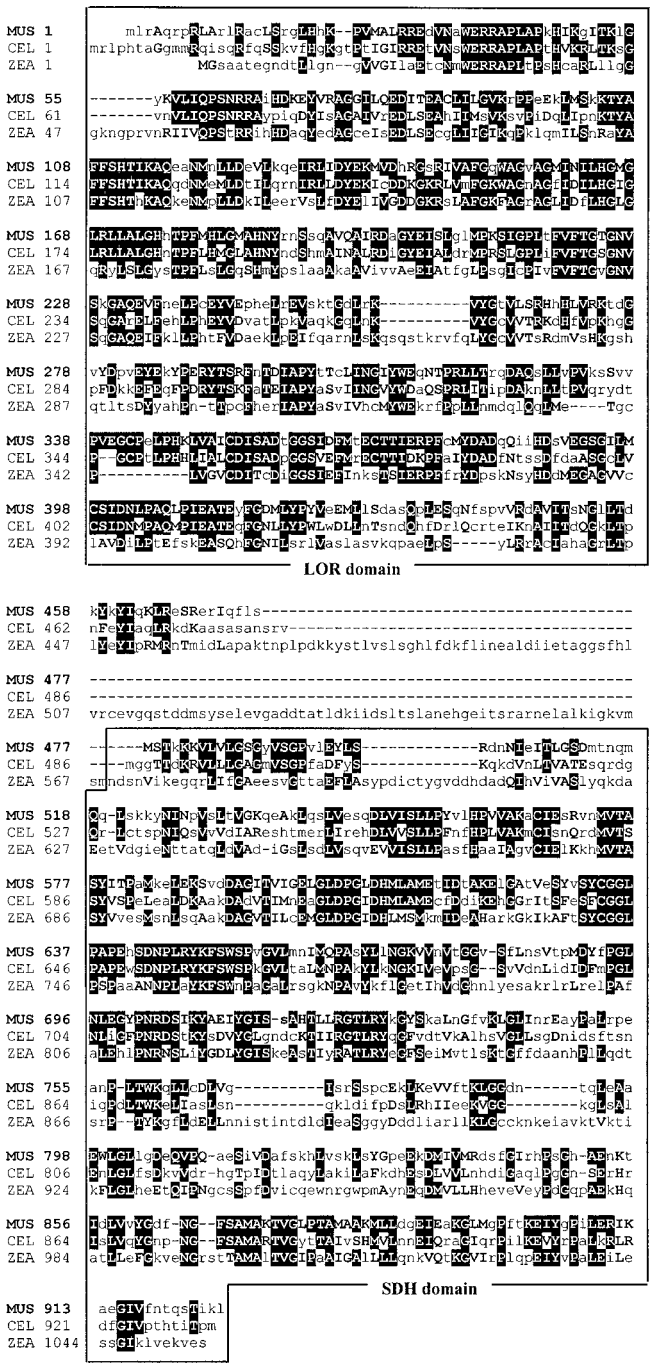

Figure 1 Alignment of various bifunctional LOR/SDH proteins

The mouse bifunctional enzyme LOR/SDH, predicted by translation of the isolated cDNA clone, is aligned with the maize and *C. elegans* proteins. Black boxes indicate amino acids that are identical between mouse (MUS) and maize (ZEA) or *C. elegans* (CEL). Capital bold letters indicate amino acids similar between mouse and maize or *C. elegans*. Lower case letters denote residues that are neither similar to nor identical with the aligned residues in other sequences. The LOR and SDH domains, identified by sequence comparisons with the yeast enzymes (results not shown), are delimited by large boxes.

spanning residues 1–455 and 477–926 are similar to those in yeast LOR and SDH respectively (indicated by boxes in Figure 1). Moreover, the predicted protein exhibits 41.6% similarity to maize LOR/SDH, 62.3% similarity to the putative *Caenor-*

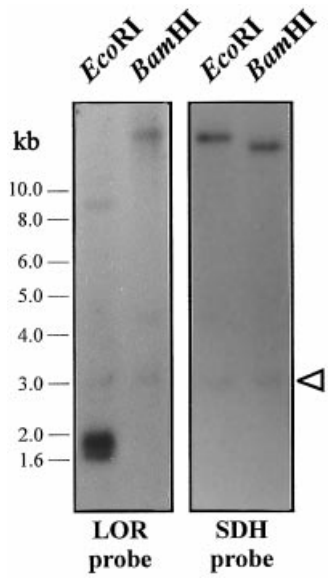

Figure 2 Genomic DNA gel blot analysis

A Southern blot of *Bam*HI- and *Eco*RI-digested mouse genomic DNA was hybridized with probes restricted either to the LOR domain or to the SDH domain. The open arrowhead denotes a 3.0 kb band that hybridized to both probes. The migration of size markers is indicated at the left.

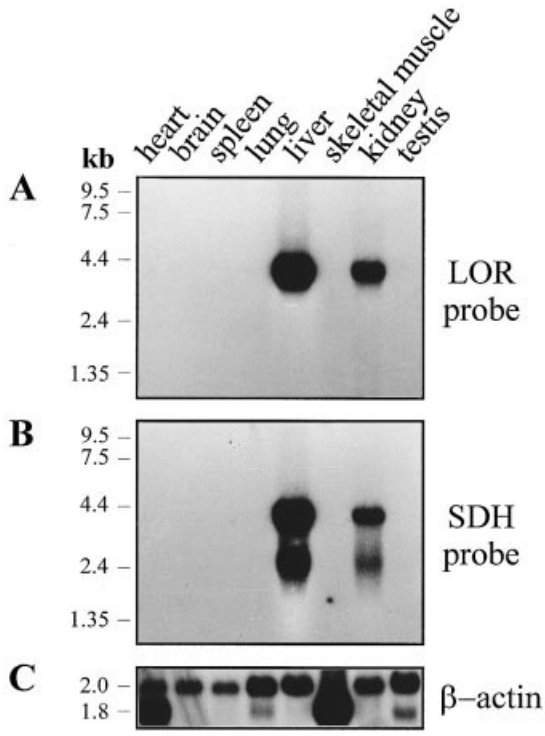

Figure 3 *Lor/Sdh* gene expression in mouse tissues

Expression of the *Mus-Lor/Sdh* gene was analysed in various mouse tissues. A commercial multiple-tissue Northern blot was sequentially hybridized with the LOR-specific probe (**A**) and the SDH-specific probe (**B**), and also a β-actin probe (**C**) as a loading control. The tissues of origin of the RNA are indicated at the top; the migration of size markers is shown at the left.

habditis elegans LOR/SDH counterpart (Figure 1) and 44% similarity to the *Arabidopsis* LOR/SDH ([13], not shown). On the basis of these sequence similarities, we concluded that the isolated mouse cDNA codes for a bifunctional LOR/SDH protein.

The existence of a cDNA clone encoding a bifunctional protein strongly suggests that, as in plants, the *Lor* and *Sdh* genes are in fact fused in mammals.

DNA gel blot analysis

To analyse the organization of the gene(s) encoding LOR and SDH, genomic DNA from C57 Black 6 mice was digested with *Eco*RI and *Bam*HI and hybridized to probes corresponding either to the LOR or to the SDH domain of the cDNA-LOR/SDH clone (Figure 2). Both digests showed single strongly hybridizing bands with both probes, suggesting that *Lor* and *Sdh* are likely to be present as single-copy gene(s) in the mouse genome. Moreover, the digest produced with *Bam*HI exhibits an approx. 3.0 kb band when hybridized with both probes (Figure 2, open arrowhead). This indicates that this restriction fragment contains LOR and SDH regions, supporting the hypothesis that the *Lor* and the *Sdh* genes are fused in the mouse. We have recently obtained the genomic sequence of this gene (F. Papes, E. L. Kemper, K. C. M. de Moraes, F. R. da Silva, A. Vettore, A. Leite and P. Arruda, unpublished work) and concluded that it in fact represents a fused *Lor*/*Sdh* gene coding for a bifunctional LOR/SDH. We shall hereafter name this gene *Mus-Lor*/*Sdh*, solely to distinguish it from the isolated cDNA clone.

Mus-Lor/Sdh is expressed in adult mouse liver and kidney

The expression of *Mus-Lor*/*Sdh* was analysed by RNA gel blot hybridization of mRNA samples from a number of mouse adult tissues, with the LOR- and SDH-specific probes described for the Southern blot analysis. Hybridization with the LOR-specific probe revealed a strongly hybridizing band in the liver and kidney RNA samples (Figure 3). Heart, brain, spleen, lung, skeletal muscle and testis showed only very faint bands after a prolonged exposure. The transcript detected by the LOR-specific probe was approx. 3.4 kb in size, in good agreement with the size of the isolated cDNA-LOR/SDH clone (3.3 kb).

Hybridization with the SDH-specific probe produced identical tissue expression patterns. However, this probe detected not only the 3.4 kb transcript but also a smaller 2.4 kb mRNA (Figure 3). This band was not as intense as the 3.4 kb main transcript band but the intensity ratio between the two bands was maintained in the liver and kidney. The 2.4 kb mRNA was not detected by the LOR-specific probe, even with exposure times exceeding 1 week, indicating that it contained only SDH-specific sequences.

Partial purification of LOR and SDH activities from mouse liver

The existence of a 2.4 kb SDH-only transcript in our Northern analyses opened the question of whether it encoded a monofunctional SDH enzyme in the mouse. In contrast, it was not clear yet whether the mouse liver contained a bifunctional LOR/SDH encoded by the cDNA-LOR/SDH clone. Other authors had found only non-linked LOR and SDH enzymes in the rat liver [18].

To determine whether a bifunctional LOR/SDH and monofunctional LOR or SDH polypeptides are present in mouse, both activities were partly purified from liver tissue. A single peak exhibiting both LOR and SDH activities was obtained after chromatography on a DEAE-Sephacel column (Figure 4A). The most active fractions from this peak were pooled, concentrated and dialysed against buffer A overnight. The dialysed sample was

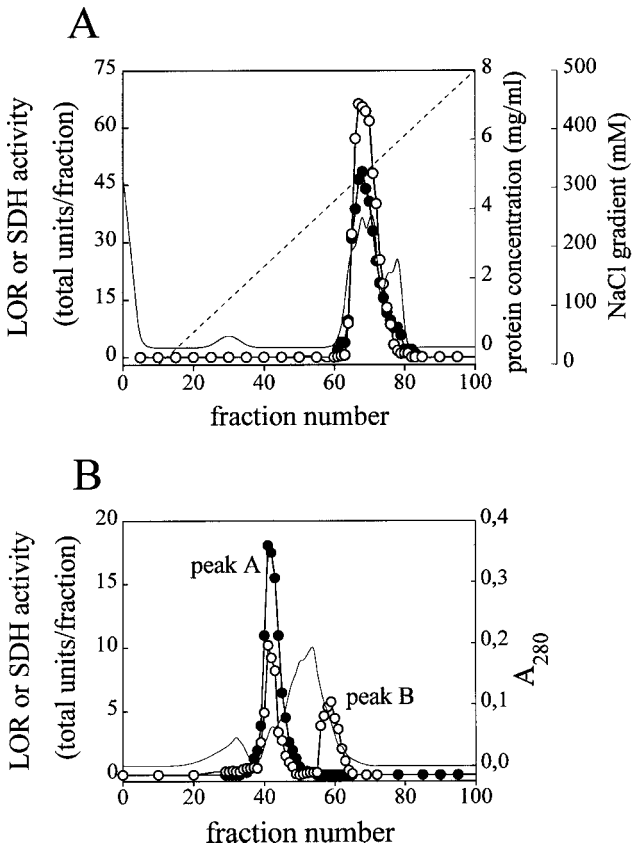

Figure 4 Chromatographic elution profiles of the partial purification of LOR and SDH activities from mouse liver

(**A**) Elution pattern of mouse LOR and SDH enzymic activities from the DEAE-Sephacel column. The column was eluted with a gradient of 0–500 mM NaCl in buffer A (broken line). Fractions of 0.75 ml were collected; assays for enzyme activities were performed on 50 μl of each fraction. The protein concentration profile is indicated by the solid line. (**B**) Elution profile of LOR and SDH enzymic activities from the Superdex-200 HR column. The enzyme was eluted in 0.25 ml fractions; 50 μl of each was used for enzyme assays. Protein concentration as measured by A_{280} is indicated by the solid line. Prominent peaks (A and B) are indicated. Symbols: ●, LOR; ○, SDH.

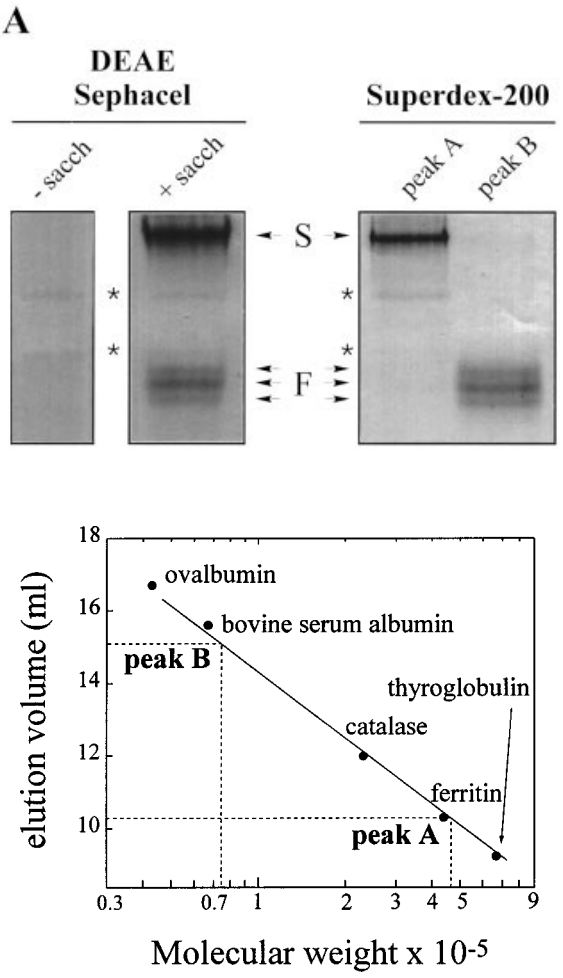

Figure 5 Gel analysis and molecular mass determination of partly purified LOR and SDH from mouse liver

(**A**) Pooled fractions from the DEAE-Sephacel column and from both peaks (A and B) of the Superdex-200 HR column (see Figure 4) were separated by native discontinuous gel electrophoresis and subsequently stained for SDH activity. The control gel, incubated in the absence of saccharopine, is labelled '—sacch'. The slowly migrating band is denoted S; the three fast-moving bands are indicated with F. Two very faint and clearly distinguishable bands were detected simultaneously in the saccharopine-containing and control gels (marked by asterisks); it was concluded that they represented persistent non-specific dehydrogenases (not SDH). (**B**) Molecular mass determination for LOR and SDH from peaks A and B of the Superdex-200 HR column. The column was calibrated with the following molecular mass standards: ovalbumin (43 kDa), BSA (67 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (668 kDa). Elution volumes for these markers were monitored by measuring A_{280}. Broken lines indicate elution volumes corresponding to molecular masses of approx. 474 kDa (peak A; combined LOR and SDH activities) and 73 kDa (peak B; SDH-only activity).

separated by non-denaturing gel electrophoresis and submitted to an in-gel staining procedure to detect SDH activity. GTP and 2-oxoglutarate were included in the staining solution (see the Experimental section) to inhibit glutamate dehydrogenases, which are abundant NAD^+-utilizing enzymes in the partly purified liver extract (results not shown). Four specific SDH bands were detected in the sample from the DEAE column (Figure 5A, arrows), three of them migrating faster (F) and one showing slow migration (S) in the saccharopine-containing gel.

The combined DEAE-Sephacel fractions (Figure 4) were precipitated with 70%-satd. $(NH_4)_2SO_4$ and applied to a Superdex-200 HR gel-filtration column. Two peaks exhibiting LOR or SDH activities were recovered from this step, one presenting both activities (peak A) and the other possessing only the SDH activity (peak B; Figure 4B). The most active fractions of both peaks were combined separately, subjected to non-denaturing PAGE and stained for SDH activity, as done previously for the DEAE-Sephacel sample. The same set of four bands were specifically detected in this gel: peak A corresponded to the S band, whereas peak B yielded the three F bands (Figure 5A).

The exclusion volumes from the Superdex-200 column (Figure 5B) enabled us to calculate the molecular masses of both peaks recovered from this column. Peak A corresponds to approx. 474 kDa, which is in good agreement with the 468 kDa of the tetrameric form of the bovine liver and human placenta bifunctional LOR/SDH enzymes [14,16]. Peak B has an estimated molecular mass of 73 kDa (Figure 5B). The fractions from both peaks were separately combined, concentrated and reapplied to the Superdex-200 column to confirm their previously attained elution volumes. Peaks A and B were eluted at the same volumes as in the first chromatographic run, strengthening the molecular mass assignments given before (results not shown).

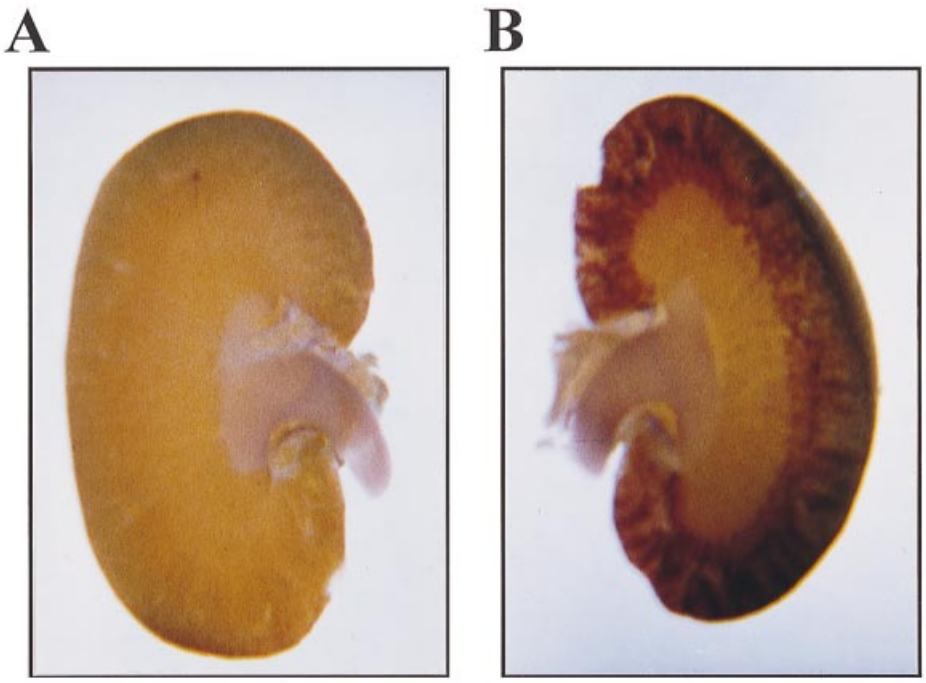

Figure 6 Histochemical detection of SDH activity in the kidney

Kidneys sectioned longitudinally were incubated in an SDH-developing reaction mixture as described in the Experimental section. (**A**) Control section incubated in the absence of saccharopine; (**B**) positive section, in which the SDH-specific staining appears as the darkened area in the periphery of the organ.

It is unlikely that the 73 kDa peak corresponds to a bifunctional monomer presenting only SDH activity, because its molecular mass is not similar either to that of the bifunctional monomer isolated from baboon and bovine livers (115 kDa) [14] or to that predicted for the LOR/SDH enzyme encoded by the cDNA clone isolated here (109 kDa). It is more reasonable that peak B corresponds to a monofunctional SDH monomer similar to the yeast monofunctional SDH, which in fact has a similar molecular mass (73 kDa) [38].

We were unable to separate the three F bands by standard chromatographic procedures. When run on a 7% (w/v) gel, these three bands separated as two bands. This distinct migration pattern in a more concentrated gel is likely to imply that the three isoforms have slightly distinct shapes. Nevertheless, given their nearly identical exclusion times from the Superdex column (results not shown), we concluded that the F bands have approximately the same molecular mass. Why these isoforms should migrate as three distinct bands is not known but it could be due to covalent modifications such as protein phosphorylation, as will be discussed later.

Taken together, the molecular and biochemical data presented in Figures 3, 4 and 5 support the existence of both bifunctional LOR/SDH and monofunctional SDH enzymes in the mouse liver.

Spatial distribution of SDH activity in the kidney

Northern analyses indicated that the *Mus-Lor/Sdh* gene is expressed in liver and kidney. To determine the role of LOR/SDH in the kidney, the enzyme location in this organ was analysed by histochemical staining for SDH activity *in situ*. As indicated in Figure 6, SDH activity was detected only in the cortical region of the kidney. The staining is not present in the control section incubated in the absence of saccharopine, indicating that it is specific for SDH and not due to spurious detection of other NAD^+-using dehydrogenases (Figure 6). This cortical distribution suggests that the enzyme could be located in the proximal and/or distal renal tubules, where most reabsorption processes take place.

LOR/SDH is induced by starvation or exogenous lysine administration

Lysine is a ketogenic amino acid and so its degradation can be coupled with the energetic balance in situations of limited carbon supply, such as starvation. Hence LOR and SDH activities along with *Mus-Lor/Sdh* gene expression were analysed to determine whether the lysine degradation was activated in the liver during starvation. In starved mice, LOR and SDH activities were 17.29 ± 0.64 and 9.39 ± 0.91 units/mg of protein respectively (means $\pm$ S.E.M. for four animals). These values represent statistically significant 52% and 47% increases in LOR and SDH activities in comparison with activity values in control animals (11.08 ± 0.04 and 6.48 ± 0.46 units/mg of protein respectively). These increases in LOR and SDH enzymic activities were paralleled by an 80% increase in *Mus-Lor/Sdh* mRNA levels (Figure 7). Increases in mRNA levels have also been observed during starvation for a number of amino-acid degrading enzymes, and transcriptional regulation of gene expression is likely to occur in these cases [39].

Not only starvation is able to affect lysine catabolism. Lysine and saccharopine have been shown to influence lysine degradation in yeast, plants and mammals. In tobacco, the injection of lysine *in vivo* into developing seeds promotes an increase in LOR activity [3]. The same occurs in lysine-overproducing plants, suggesting that the accumulation of this substrate leads to an

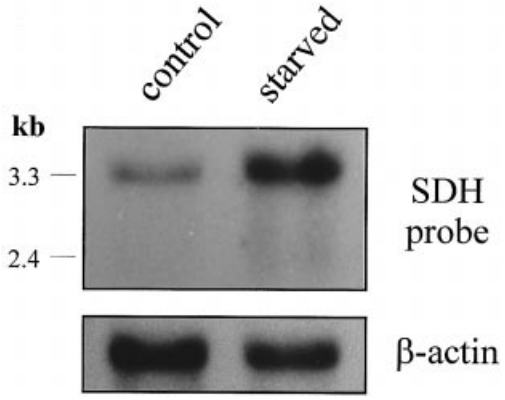

Figure 7 Effect of starvation on LOR/SDH mRNA levels

Mus-Lor/Sdh gene expression was analysed in starved and control mice by using RNA extracted from the same animals as were used for measuring enzymic activities (results given in the text). A representative Northern blot is shown. It was hybridized sequentially with the SDH-specific probe and with the β-actin probe (as a loading control). The sizes of the two prominent mRNA species are shown at left.

increase in the activity of its degrading enzyme [3]. In yeast, *Lys1* and *Lys9* genes, encoding the enzymes LOR and SDH, are transcriptionally regulated by the LYS14 activator, which in turn is modulated by a saccharopine derivative (aminoadipate semialdehyde) [37,38]. In particular, high-lysine or high-protein diets seem to promote increases in LOR and SDH activities in the rat liver [40–43].

To study the effects of these substances in LOR/SDH enzymic activities and gene expression in mammals seems relevant because physiological or pathological lysine-accumulating or limiting conditions might seriously affect the lysine degradation pathway, leading to restricted or enhanced catabolism. To study those effects on the mammalian LOR/SDH, enzymic activity and mRNA levels were assayed in livers collected from C57 Black 6 mice injected with lysine or saccharopine.

Liver LOR activity was 20.09 ± 0.62 units/mg of protein in lysine-injected animals, representing a significant increase in comparison with control values (17.17 ± 0.15 units/mg of protein). In contrast, mice injected with a low dose of saccharopine exhibited a 25% decrease in hepatic LOR activity after 48 h (12.61 ± 0.73 units/mg of protein). No significant increase was observed after a shorter period (24 h) (results not shown). SDH activity also increased after lysine administration (7.19 ± 0.52 units/mg of protein, in comparison with 5.57 ± 0.15 units/mg of protein in the control animals) but did not vary significantly between control and test in saccharopine-injected animals (5.08 ± 0.44 units/mg of protein).

Mus-Lor/Sdh mRNA levels increased in the liver of lysine-injected mice (Figure 8). This finding matched the induction of LOR activity in the same animals. Conversely, the decrease in LOR activity in saccharopine-injected mice was not paralleled by a decrease in mRNA levels (Figure 8). It is therefore likely that this alteration in LOR activity resulted from post-transcriptional modification. Indeed, protein phosphorylation has been shown to modulate LOR activity in maize (E. L. Kemper, F. Papes and P. Arruda, unpublished work) and *Arabidopsis* [44,45]. Alternately, the alteration observed in LOR activity in animals treated with saccharopine could be due to the inhibitory effect *in vitro* of endogenous saccharopine on the measurement of LOR activity [4,46]. However, we obtained the same decrease in LOR activity when endogenous saccharopine was eliminated from the sample by dialysis or precipitation with $(NH_4)_2SO_4$ before the enzyme assay (results not shown). Additionally, this decrease was not observed if the sample was collected from animals killed after a short (24 h) post-injection period. Taken together, these results indicate that the LOR activity alteration in saccharopine-injected animals is the result of processes *in vivo*, probably protein phosphorylation, rather than of enzyme inhibition *in vitro*.

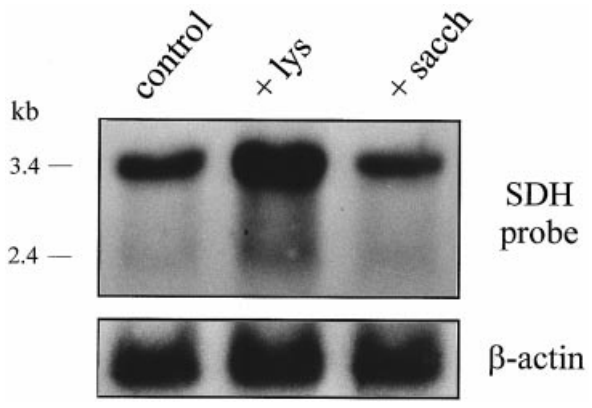

Figure 8 Effects of injections of lysine and saccharopine on *Mus-Lor/Sdh* gene expression

Mus-Lor/Sdh gene expression was analysed in the lysine-injected, saccharopine-injected and PBS-injected animals that were used for measuring enzymic activities (results given in the text). A representative Northern blot was hybridized sequentially with the SDH-specific probe and the β-actin probe (as a loading control). The sizes of the two prominent mRNA species are shown at left.

DISCUSSION

A previous study has described the existence of monofunctional, separate LOR and SDH (enzymes catalysing the first two steps of lysine degradation through the saccharopine pathway) in rat liver mitochondria [18]. Even so, other studies reported the isolation of a bifunctional LOR/SDH enzyme from the liver of other mammals [14,16]. In the present paper we provide biochemical and molecular evidence reconciling these apparently conflicting results, showing for the first time that both a bifunctional LOR/SDH and a monofunctional SDH are likely to be present in the murine liver.

We have isolated a cDNA clone from mouse adult liver that encodes a bifunctional polypeptide bearing domains similar to the yeast LOR and SDH enzymes (Figure 1). The bifunctional protein predicted to be encoded by this cDNA possesses 926 amino acid residues, divided into three distinct regions: an N-terminal domain, presenting similarity to LOR; a C-terminal domain similar to SDH; and an interposed short region connecting both domains that presents no similarity to other known protein sequences in the databases (Figure 1). This interposed domain is a distinctive feature of LOR/SDH bifunctional proteins from animals and plants because in mouse and *Caenorhabditis elegans* it is short (Figure 1), whereas in maize (Figure 1) and *Arabidopsis* (comparison not shown) it is longer. Its role in enzyme function or structure remains unknown as yet but the difference in its length between animal and plant sources might give rise to structural differences, eventually explaining why the

native form of the animal bifunctional enzyme is a tetramer ([14,16], and the present study) rather than a dimer as in plants [4].

The LOR and SDH domains of the predicted protein present strong similarity not only to the yeast enzymes but also to the LOR and SDH domains of bifunctional proteins from maize and *C. elegans* (Figure 1) and *Arabidopsis* (result not shown). The domains of the predicted protein from *Arabidopsis* have been expressed separately in bacteria and shown to represent fully active reductase and dehydrogenase domains [13]. Taken together, these results indicate that the protein encoded by cDNA-LOR/SDH represents a truly active bifunctional enzyme.

The predicted molecular mass of this protein is 109 kDa, which is close to the 115 kDa reported for the enzyme purified from bovine liver [14]. The partial purification of LOR and SDH activities from mouse liver led to the isolation of a protein with an estimated molecular mass of 474 kDa possessing both LOR and SDH activities (Figure 4B, peak A). This value matches that found for the tetrameric native enzyme isolated from other mammals (468 kDa) [14,16]. It is therefore likely that the 474 kDa protein is a tetramer of four bifunctional 109 kDa polypeptides, encoded by the isolated cDNA-LOR/SDH clone.

A second, less prominent, protein peak, presenting only SDH activity, was observed in the gel-filtration purification step (Figure 4B, peak B). Because this second peak has an estimated molecular mass of 73 kDa, which is similar to that of the SDH monofunctional enzyme from yeast, we hypothesize that it probably represents a monofunctional SDH. Our results agree in part with Noda and Ichihara [18], who showed the existence of non-linked LOR and SDH activities in rat liver. Nevertheless, we demonstrated that both bifunctional LOR/SDH and monofunctional SDH are likely to be present in this tissue, instead of completely separate activities as suggested by those authors.

We did not verify the precise subcellular localization of the isolated LOR/SDH and SDH. Perhaps these protein variants are differentially located in the cell. LOR activity can be detected in both mitochondrial and cytoplasmic fractions of mouse liver extracts [7]; this finding supports the idea of a widespread cellular distribution. Other amino acid-degrading enzymes exhibit this wide subcellular distribution, such as the branched-chain aminotransferases, which are present both in mitochondria and cytoplasm ([47], and references therein). Because we purified the enzyme from whole liver extracts, it is possible that both the cytoplasmic and the mitochondrial enzyme forms were isolated in our procedure. Most of the LOR and SDH activities in the liver have been ascribed to mitochondria [48]. We performed detailed analyses with the protein sequence deduced from the cDNA-LOR/SDH clone to predict its subcellular targeting. A mitochondrial localization was predicted by two computational methods, at a high probability (see the Experimental section for details). Both programs predicted a mitochondrial targeting sequence at the N-terminus, with a putative cleavage site at residue 32. Whether there is also a bifunctional cytosolic isoform or whether the monofunctional SDH is cytosolic is not known at present. We also predicted the subcellular localization of the *C. elegans* and maize enzymes by using the same programs. The nematode enzyme was also predicted to be a mitochondrial protein. Interestingly, the maize counterpart was assigned to the cytosol, in keeping with results obtained in our laboratory showing that the plant LOR/SDH is a cytosolic enzyme (E. L. Kemper, G. Cord-Neto, F. Papes, K. C. M. Moraes, A. Leite and P. Arruda, unpublished work).

The existence of both a bifunctional LOR/SDH and a monofunctional SDH in the mouse is supported by the Northern blot analyses presented in Figure 3. Two mRNA transcripts were detected, 3.4 and 2.4 kb in size. Both transcripts were detected with a probe restricted to the SDH region of the cDNA-LOR/SDH clone but the shorter message was not detected by an LOR-specific probe, suggesting that it contains only SDH sequences (Figure 3). The 3.4 kb mRNA is likely to code for the bifunctional LOR/SDH because its length is in good agreement with one of the cDNA species described here. We have not sequenced the shorter (2.4 kb) transcript from mouse but have done so for the corresponding 2.4 kb mRNA from *Drosophila* (results not shown). This mRNA encodes a protein with a distinct N-terminal sequence with no similarity to any other protein in the databases and a C-terminal portion corresponding to a short part of the LOR domain, the interposed domain and the whole SDH domain of the LOR/SDH bifunctional enzyme. Because most of the LOR domain is absent from this predicted protein variant, it is likely that it encodes a monofunctional SDH. A similar short mRNA species (1.7 kb) encoding a monofunctional SDH protein has also been observed in *Arabidopsis* [13], but the SDH mRNA shown in this study codes for a protein comprising only the SDH domain and presents complete collinear identity with the longest LOR/SDH mRNA. It is more reasonable that this mRNA is an incomplete cDNA clone rather than a coding SDH-only message. Conversely, the size of 2.4 kb observed for the SDH mRNA described here is more compatible with the cited *Drosophila* short mRNA and with the molecular mass attained for the monofunctional SDH protein (73 kDa) isolated from mouse liver (Figures 3 and 5B).

The *Mus-Lor/Sdh* gene is likely to be present as a single-copy gene in the mouse genome (Figure 2) and so it is possible that the 2.4 kb mRNA is generated by alternative splicing or by the use of an alternative 5′ exon from the same gene that encodes the 3.4 kb mRNA.

The *Mus-Lor/Sdh* gene is expressed conspicuously in liver and kidney (Figure 3) and this expression profile correlates well with the distribution of LOR and SDH enzymic activities in various human tissues [9]. In particular, the high expression level observed in the liver is consistent with the activity observed in this organ, not only in human but also in bovine, baboon and rat livers [14,18]. In the kidney, mRNA levels seem to be lower than in the liver (Figure 3); this parallels the LOR enzymic activity observed in rat kidney, which is only 10 % lower than that found in liver [20].

In the liver, LOR/SDH is thought to participate in the nitrogen and energetic balances, by controlling the degradation of lysine into α-aminoadipic semialdehyde, which is then converted into ketone bodies by several reactions. These energetic compounds can then be used in situations of limited carbon supply. We found that the enzymic activities of both LOR and SDH increased in starved mice in comparison with control non-starved animals. These increases in enzymic activity were accompanied by a significant increase in *Lor/Sdh* mRNA levels (Figure 7), suggesting that the induction in LOR/SDH under starvation might, at least in part, be the result of an increased transcription rate, as with a number of amino acid-degrading enzymes [39]. A previous paper showed a marked induction in rat liver LOR activity after the administration of glucagon [49]. This finding, together with our results on starved mice, points to an important involvement of LOR/SDH and lysine degradation itself in the energetic balance.

Increases in LOR/SDH activities and mRNA levels were also observed in animals given an intraperitoneal injection of lysine (Figure 8). A similar increase in rat liver LOR activity was previously reported in rats fed with a high-lysine diet [43]. Thus free lysine levels might reasonably be a signal to which the liver cells respond by modulating the LOR/SDH activities so as to

cope properly with the available circulating lysine. Moreover, high-protein diets result in increased lysine oxidation and LOR/SDH activity [40–42]. Blemings et al. [35] even showed an important diurnal variation in LOR and SDH activities and lysine oxidation after a protein-rich meal. Once again, the free lysine levels, derived from the degradation of available meal protein, might signal to the liver the need for enhanced degradation of lysine. In brief, the findings that both starvation and the injection of lysine alter LOR and SDH activities suggest that the lysine degradation pathway can respond promptly to and influence both the nitrogen and carbon balances in the organism.

Although the role of the liver enzyme is well understood, its function in the kidney is still unclear. Reports on the role of the kidneys in lysine degradation are in disagreement because some suggest a little participation [19], whereas others suggest an important role [50]. Our work supports the latter view. LOR/SDH might be involved in metabolic processes similar to those occurring in the liver but some distinct role in the kidney cannot be ruled out. One interesting observation favouring the first view is that LOR activity and *Lor/Sdh* mRNA levels also increase in kidneys of starved or lysine-injected animals (results not shown). Another possible role for LOR/SDH in the kidney is to influence the lysine reabsorption process in the tubule cells in some way. The precise tissue localization of SDH activity in this organ (Figure 6) can be seen as a good evidence in support of such a hypothesis.

This study therefore represents the starting point from which to assess the role of lysine-degrading activities in mammalian tissues and of the gene(s) encoding them. We focused mainly on liver and kidney but a number of aspects regarding the function of LOR and SDH in other tissues should be explored hereafter, mainly those concerning their involvement in genetic disorders such as familial hyperlysinaemias. Two types of familial hyperlysinaemia have been described so far: type I is associated with a combined deficiency of the two enzyme activities, LOR and SDH, whereas in familial hyperlysinaemia type II only the dehydrogenase activity is impaired [6,24,25]. A deficiency in the bifunctional LOR/SDH mRNA levels might explain, for example, why there is a combined deficiency in type I disease; a mutation in only the SDH domain might explain the type II disease. The present work therefore stands as the starting point of the characterization and molecular dissection of this inherited genetic disorder and the understanding of the coupling between the lysine degradation pathway and the carbon and nitrogen balances in the body.

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