FEBS Letters 586 (2012) 905–911

FEBS Letters

journal homepage: www.FEBSLetters.org

Lysine degradation through the saccharopine pathway in bacteria: LKR and SDH in bacteria and its relationship to the plant and animal enzymes

Guilherme Coutinho de Mello Serrano [a,b], Thaís Rezende e Silva Figueira [a], Eduardo Kiyota [a], Natalia Zanata [a], Paulo Arruda [a,b,*]

[a] Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas (UNICAMP), 13.083-970 Campinas, SP, Brazil
[b] Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), 13.083-970 Campinas, SP, Brazil

ARTICLE INFO

Article history:
Received 26 January 2012
Revised 8 February 2012
Accepted 14 February 2012
Available online 22 February 2012

Edited by Renee Tsolis

Keywords:
Lysine metabolism
Lysine-ketoglutarate reductase
Saccharopine dehydrogenase
Bacteria
Mammal
Plant

ABSTRACT

Lysine degradation through the saccharopine pathway has been shown only in plants and animals. Here, we show that bacteria possess the genes encoding lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH). In *Silicibacter*, the contiguous *lkr* and *sdh* genes are interspersed, in another frame, by a polypeptide of unknown function. The bacterial enzyme does not contain the 110-amino-acid interdomain (ID) that intersperses the LKR and SDH domains of the plant enzyme. The ID was found in Cyanobacteria interspersing polypeptides without similarities and activities of LKR and SDH. The LKR/SDH bifunctional polypeptide of animals and plants may have arisen from a α-proteobacterium with a configuration similar to that of *Silicibacter*, whereas the ID in the plant enzyme may have been inherited from Cyanobacteria.

1. Introduction

The aspartate-derived amino acid pathway is the main route for lysine synthesis in bacteria and plants [1–3]. In fungi, lysine is synthesised through the saccharopine pathway [4–6]. In this pathway, α-aminoadipate is converted to α-aminoadipate-δ-semialdehyde, which is then condensed with glutamate to form saccharopine, which is finally hydrolysed to form lysine and α-ketoglutarate [5,6] (Fig. 1). In lysine synthesis, the last two reactions of the saccharopine pathway are catalysed by saccharopine dehydrogenase (SDH, EC 1.5.1.9) and lysine-α-ketoglutarate reductase (LKR, EC1.5.1.8) [5,6]. The saccharopine pathway also exists in plants and animals, but instead of functioning in lysine synthesis, it works in the reverse reaction, leading to lysine degradation (Fig. 1) [7,8]. In the direction of degradation, the first two enzymatic steps can be viewed as an atypical transamination reaction in which the α-amino group of lysine is transferred to α-ketoglutarate to form glutamic acid (Fig. 1).

What causes the saccharopine pathway to function in a given direction is still unknown, but one striking characteristic of the two central enzymes in the pathway is that in fungi, LKR and SDH are monofunctional polypeptides encoded by genes located at separate loci in the genome [5], whereas in plants and animals, LKR and SDH are encoded by a single gene that, after transcription and translation, gives rise to a bifunctional polypeptide that performs both activities [9,10]. A major difference between the plant and the animal enzymes is that although in animals the LKR and SDH domains are contiguously linked, in plants they are separated by a ~110-amino-acid peptide referred to as the interdomain (ID) [11]. The role of the ID is unknown, but it is conserved in all plants and absent in all animals whose genomic information is available in public databases.

We previously investigated the role of the bifunctional architecture of LKR/SDH and the possible function of the ID in plants. The ID could be part of the LKR or the SDH domains, and because the ID does not exist in animals, it could have additional roles related specifically to plant physiology and metabolism. We observed that the SDH domain of the bifunctional maize polypeptide, produced by limited proteolysis, inhibits LKR activity [12]. This inhibitory effect may be associated with the ID linked to the partially hydrolysed SDH domain, as there is no evidence that the SDH domain of animals inhibits LKR activity [12].

In this work, we searched genomic databases for LKR and SDH bacterial homologues; we then asked if there are other proteins in any other organism (whose genomic information is available

* Corresponding author at: Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas (UNICAMP), 13.083-970 Campinas, SP, Brazil. Fax: +55 19 37881089.
E-mail address: parruda@unicamp.br (P. Arruda).

0014-5793/$36.00
doi:10.1016/j.febslet.2012.02.023

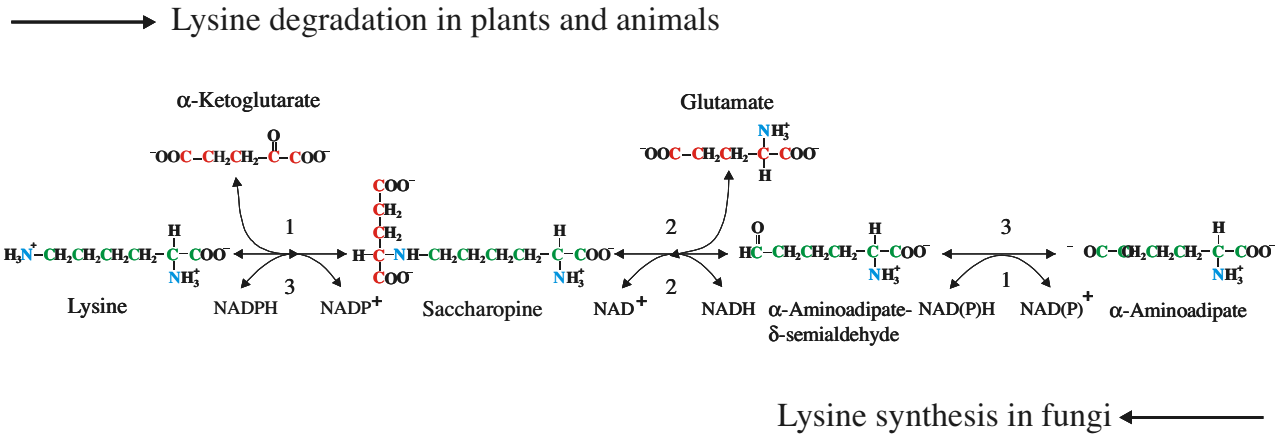

Fig. 1. Schematic representation of the saccharopine pathway. In fungi, the pathway functions in the direction of lysine synthesis whereas, in plants and animals, it functions in the direction of lysine degradation. The enzymes indicated in the direction of lysine degradation are the following: (1) lysine-ketoglutarate reductase (LKR); (2) saccharopine dehydrogenase (SDH); and (3) aminoadipic semialdehyde dehydrogenase (ASADH).

in the public databases) that contain a polypeptide similar to the ID amino acid sequence. We describe the characterisation of bacterial LKR, SDH and ID homologues. The results are discussed in terms of the architecture of LKR, SDH and ID in bacteria and their possible contributions to the LKR/ID/SDH configuration in animals and plants.

2. Materials and methods

2.1. Query selection and BLAST search

The amino acid sequences of LKR and SDH from fungi, plants and animals and the ID sequence from maize were used to query the non-redundant nucleotide and protein databases at National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and the bacterial genome databases. The searches were performed using BLAST algorithms [13]. The sequences with E-values less than e-20 were retrieved and inspected for identity significance.

2.2. Amino acid sequence alignment and phylogenetic analysis

Alignments of the amino acid sequences were performed with ClustalX [14] using the PAM weight matrix [15]. The alignments were visualised using Boxshade suit, where black is the background for identical amino acids and grey is the background for similar amino acids. The following phylogenetic analysis techniques were performed on the ClustalX alignments: 1000 reassemblies by the bootstrap method [16] and the neighbour-joining algorithm [15] using a Poisson distribution model in MEGA 3.1 [17]. The amino acid sequences flanking the ID from Cyanobacteria were used as out-groups in the phylogenetic analysis.

2.3. Bacterial strains

Nostoc punctiforme ATCC 29133 and *Silicibacter pomeroyi* DSS-3 strains were obtained from American Type Culture Collection (ATCC) (www.atcc.org). *N. punctiforme* was plated on solid BG11 medium and grown under a 14-h light period for 15 days. Individual colonies were inoculated in liquid BG11 and grown in a rotary shaker at 100 rpm and 30 °C with a 14-h light period [18]. *S. pomeroyi* was plated on solid Difco Marine broth 2216 containing 0.8% agar and grown for 2 days at 30 °C. Individual colonies were inoculated in liquid Difco Marine Broth 2216 and grown in a rotary shaker at 100 rpm and 30 °C until the OD at 600 nm equalled 0.4.

2.4. Protein extraction and enzyme assays

N. punctiforme and *S. pomeroyi* grown in liquid media were pelleted by centrifugation. The pellets were ground to a powder in a mortar with glass powder and liquid nitrogen. Twenty millilitres of ice-cold extraction buffer (25 mM sodium phosphate pH 7.4, 1 mM DTT, 1 mM EDTA and 5 mM benzamidine [12]) was added to the bacterial powders. The slurries were frozen and thawed several times, followed by six pulses of sonication for 20 s at 70% of the maximum power in a Microson Ultrasonic Cell Disruptor (Misonix Incorporated, NY, USA). The extracts were centrifuged at 20000*g* for 10 min, and the supernatant was used for enzymatic assays. For the kinetic studies, both LKR and SDH were assayed in extracts purified by anion-exchange chromatography.

The activities of LKR and SDH were analysed as described [10], with minor modifications. LKR activity was measured by following the oxidation of NADPH to NADP at 30 °C in a 1-ml reaction containing 20 mM L-lysine, 10 mM α-ketoglutaric acid (neutralised to pH 7.0 with potassium hydroxide), 0.1 mM NADPH, 100 mM Tris/HCl buffer, pH 7.4 and 50–200 μg of protein. SDH activity was assayed by following the reduction of NAD^+ to NADH at 30 °C in a 1 ml reaction containing 2 mM L-saccharopine, 2 mM NAD^+ and 100 mM Tris/HCl, pH 8.5. The oxidation of NADPH and the reduction of NAD^+ were monitored at 340 nm in a Hitachi U-3000 spectrophotometer. One unit of enzyme activity was defined as 1 nmol NADPH oxidised or 1 nmol NAD^+ reduced per min at 30 °C. The protein concentration in the enzyme extracts was determined using the Bio-Rad Protein Assay Dye Reagent.

2.5. Enzyme purification

Total protein extract from a 1-l *S. pomeroyi* culture was applied to a 7-ml Q-Sepharose FF anion-exchange chromatography column (Waters, USA) previously equilibrated with extraction buffer, using the AKTA-FPLC system (GE Healthcare Life Sciences, USA). The enzyme was eluted from the column with a linear gradient of 0–1 M NaCl in extraction buffer. The fractions containing both LKR and SDH activities were combined, brought to 70% saturation with solid ammonium sulphate, and centrifuged at 20000*g* for 10 min. The pellet was resuspended in 200 μl of extraction buffer and applied to a Superdex 200 HR column previously equilibrated with extraction buffer containing 0.3 M NaCl. The enzyme was eluted from the Superdex column with the same buffer, and the fractions containing LKR and SDH activities were stored at −80 °C.

Table 1
LKR and SDH orthologous proteins in selected bacteria. LKR and SDH amino acid sequences from fungi, plants and animals were tBLASTn against sequenced bacteria genome databases and GenBank. The values represent only the best hit with the query sequences.

Bacteria	Group	LKR		SDH	
		Accession Number	% Identity	Accession Number	% Identity
Silicibacter sp.	α-Proteobacteria	ZP_05741167	49 (Ps)	ZP_05741956	30 (Ca)
Roseobacter sp.	α-Proteobacteria	ZP_01901689	48 (Ps)	ZP_01753992	30 (Ca)
Gramella sp.	Bacteriodetes	YP_862590	26 (Gh)	YP_860174	34 (Hs)
Kordia sp.	Bacteriodetes	ZP_02163121	29 (Mm)	ZP_02160476	34 (Mm)
Nostoc sp.	Cyanobacteria	NP_489035	NS	NP_489035	NS
Synechococcus sp.	Cyanobacteria	ZP_05036787	NS	ZP_05036787	NS
Anabaena sp.	Cyanobacteria	YP_322786	NS	YP_322786	NS
Nodularia sp.	Cyanobacteria	ZP_01629339	NS	ZP_01629339	NS

Ps, *Pichis stipitis*; Gh, *Gosipum hirsutum*; Mm, *Mus musculus*; Ca, *Candida albicans*; Hs, *Homo sapiens*; NS, Not significant.

Table 2
ID orthologous polypeptides in bacteria. ID amino acid sequence from maize was tBLASTn against sequenced bacterial genome databases and GenBank. Only sequences with at least 70% sequence coverage along the maize ID amino acid sequence were considered.

Organism	Domain	Group	Accession Number	% Identity
Nostoc sp.	Bacteria	Cyanobacteria	NP_489035	37
Synechococcus sp.	Bacteria	Cyanobacteria	ZP_05036787	39
Anabaena sp.	Bacteria	Cyanobacteria	YP_322786	37
Nodularia spumigena	Bacteria	Cyanobacteria	ZP_01629339	37
Cyanothece sp.	Bacteria	Cyanobacteria	YP_002484532	35
Raphidiopsis brookii	Bacteria	Cyanobacteria	ZP_06304920	36
Cylindrospermopsis raciborskii	Bacteria	Cyanobacteria	ZP_06309200	36
Trichodesmium erythraeum	Bacteria	Cyanobacteria	YP_724101	35
Thermosynechococcus elongatus	Bacteria	Cyanobacteria	NP_681297	34
Microcoleus chthonoplastes	Bacteria	Cyanobacteria	ZP_05024511	34
Arthrospira sp.	Bacteria	Cyanobacteria	ZP_03276286	34
Haloferax volcanii	Archea	Halobacteria	YP_003536554	31
Methanothermobacter marburgensis	Archea	Methanobacteria	YP_003850163	35
Archaeoglobus fulgidus	Archea	Archaeoglobi	NP_070106	35
Haloarcula marismortui	Archea	Halobacteria	YP_134910	36
Ferroglobus placidus	Archea	Archaeoglobi	YP_003436635	33

2.6. Cloning and expression of recombinant LKR and SDH

The sequence spanning the *lkr* and *sdh* genes was amplified from genomic DNA of *S. pomeroyi* DSS-3 strain using the forward primer ATTCCATATGACGCATCTGTGGGTCCGG containing the *Nde*I restriction site and reverse primer CGCGAGCTCTCAGGCGGTGTGATCGACGATTTGC containing the *Sac*I restriction site. The amplified fragment was cloned into pGEM-T Easy vector (Promega, USA), validated by sequencing and than subcloned into the pET28a vector (Novagen, USA) with the His tag fused to the N terminal of the LKR polypeptide. The construct was transformed into BL21 (DE3) *Escherichia coli* strain (Novagen, USA). For protein expression, a single colony was grown for 16 h at 37 °C in 30 ml LB medium containing 50 μg/ml kanamycin. The inoculum was then grown at 37 °C to OD600 = 0.6 in 500 ml LB medium containing 50 μg/ml kanamycin. Recombinant protein was induced overnight at 37 °C with IPTG at 1 mM final concentration. Cells were then pelleted and resuspended in 20 ml of extraction buffer (50 mM sodium phosphate, 5% glycerol, 100 mM NaCl, pH 7.2) containing 80 μg/ml lysozyme. The extract was chilled, sonicated several times in an ice bath and centrifuged for 20 min at 16000*g*. Recombinant proteins were ligated into Ni–NTA agarose (QIAGEN) affinity resin, washed with extraction buffer and eluted with extraction buffer containing 5–500 mM imidazole. Recombinant proteins were verified for purity by SDS–PAGE and enzymatic activities. Fractions containing highest activities were brought to 70% saturation with solid ammonium sulphate and pelleted by centrifugation at 20000*g* for 10 min. The pellet was resuspended in 250 μl extraction buffer containing 1 mM EDTA, 1 mM ditiotreitol, 5 mM benzamidine and 300 mM NaCl and applied to a Superdex 200 HR column previously equilibrated with extraction buffer containing 0.3 M NaCl. The enzyme was eluted from the Superdex column with the same buffer and the fractions analysed for LKR and SDH activities and purity trough SDS–PAGE.

2.7. Electrophoretic methods

SDS–PAGE was performed in 8% gels using ~100 μg protein. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 in methanol/acetic acid/distilled water (54: 12.5:33.5 v/v) and destained with methanol/acetic acid (30:10 v/v). Discontinuous PAGE was performed at pH 7.0 and 4 °C in 8% slab gel. After electrophoresis, the gels were developed for LKR activity as described [10].

3. Results

3.1. Identification of LKR, SDH and ID orthologues in bacteria

Alignment of the amino acid sequences of *Saccharomyces cerevisiae*, *Candida albicans*, maize, *Arabidopsis*, rice, *Caenorhabditis elegans*, mouse and human revealed the general features and domain organisation of LKR and SDH (Supplemental Fig. 1). A clear pattern of conserved domains was revealed among the LKR and SDH polypeptides, indicating the conservation of the catalytic domains of enzymes among distant taxa. There is a ~110-amino-acid domain separating the plant LKR and SDH domains [11] that is conserved among plants but is absent in the bifunctional LKR/SDH of animals (Supplemental Fig. 1).

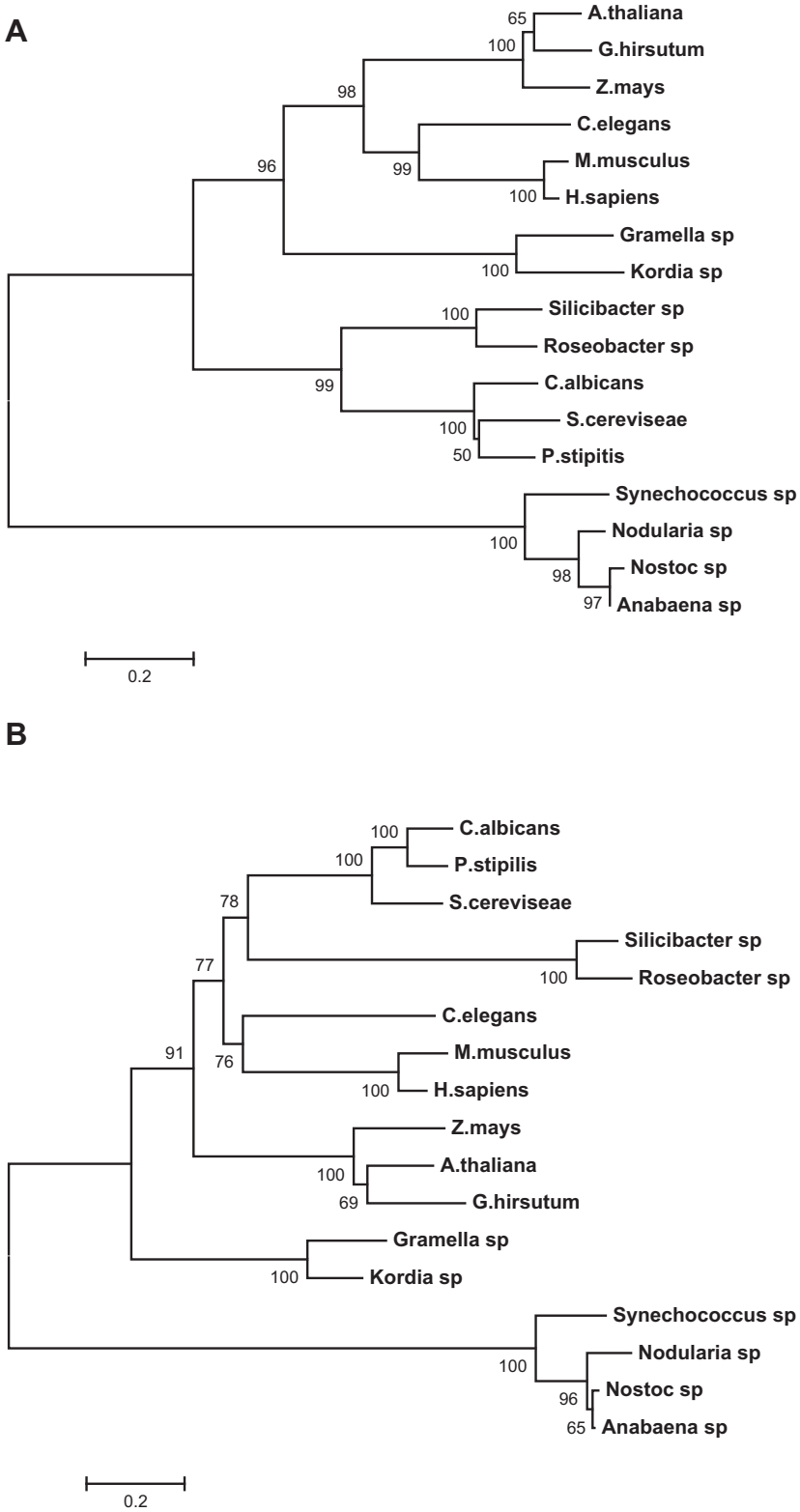

Fig. 2. Dendrograms of the amino acid sequence alignments of LKR (A) and SDH (B) orthologues in mammals, plants, bacteria and fungi. The dendrograms were generated as a consensus of 1000 bootstrap replicates. Cyanobacteria sequences flanking a conserved ID domain were positioned as out-groups in the dendrograms.

The amino acid sequences of LKR and SDH from fungi, plants and animals along with the ID sequence from maize, were used to query orthologous sequences in the bacterial genome databases and in the non-redundant protein database at NCBI (www.ncbi.nlm.nih.gov). tBLASTn searches using LKR and SDH queries returned hits with significant similarity from several bacterial groups; the strongest hits were from Bacteroidetes and Proteobacteria (Table 1), whereas tBLASTn searches using the maize ID query returned significant hits with Cyanobacteria and members of *Archaea* (Table 2). The ID amino acid sequences found Cyanobacteria link

polypeptide sequences without significant similarity to LKR or SDH (Table 1). The alignment of the LKR and SDH amino acid sequences of *Silicibacter* sp., *Roseobacter* sp., *Gramella* sp. and *Kordia* sp. with those from plants, animals and fungi confirmed the polypeptide identity with conserved domains within the amino acid sequences (Supplemental Figs. 2 and 3). The alignment of the amino acid sequences of the polypeptides flanking the ID of Cyanobacteria was poor and did not permit us to annotate them as LKR and SDH (Supplemental Figs. 2 and 3). Phylogenetic analysis of the LKR and SDH amino acid sequences from *Silicibacter* sp., *Roseobacter* sp., *Gramella* sp., *Kordia* sp., plants, fungi and animals grouped bacteria and fungi sequences in a cluster, plant and animal sequences in another cluster, whereas the sequences of the polypeptides flanking the ID in Cyanobacteria clustered as out-groups in the dendrograms (Fig. 2). The alignment of the ID amino acid sequences shows that this polypeptide is highly conserved among distant groups of bacteria and *Archea* (Supplemental Fig. 4).

3.2. LKR and SDH enzymatic activities in S. pomeroyi

To test the functionality of LKR and SDH from bacteria, the enzymes were assayed in crude and purified extracts prepared from *S. pomeroyi* using the methods established for the maize enzymes [10]. LKR and SDH from *S. pomeroyi* showed kinetic behaviours different from the plant and animal enzymes in relation to cofactor specificity. Contrasting to the plant and animal enzymes, the LKR from *S. pomeroyi* showed higher activity with NADH compared to NADPH, whereas SDH used both NAD^+ and $NADP^+$, though with a slightly higher affinity for NAD^+ (Fig. 3). Neither LKR nor SDH showed activity in the reverse direction leading to lysine synthesis.

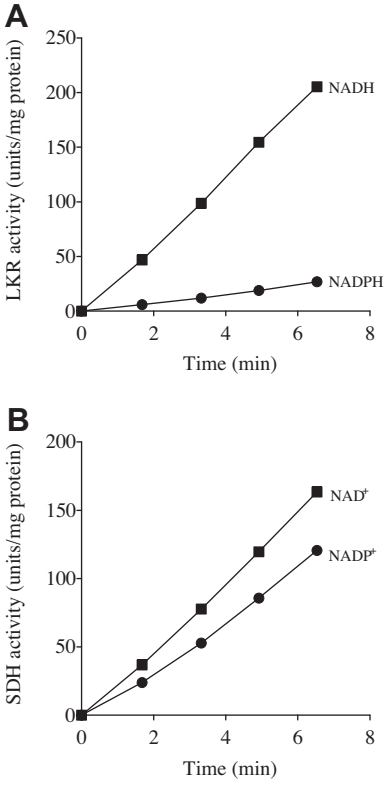

Fig. 3. Cofactor specificity of LKR and SDH from *S. pomeroyi*. (A) LKR extracts after anion-exchange chromatography were assayed with a complete reaction mixture containing either NADH or NADPH as a cofactor. (B) SDH extracts after anion-exchange chromatography were assayed with a complete reaction mixture containing NAD^+ or $NADP^+$. One unit of enzyme activity is defined as 1 nmol of NAD(P)H oxidised or 1 nmol of $NAD(P)^+$ reduced per min at 30 °C.

The cofactor specificity of the *S. pomeroyi* LKR and SDH differed from the plant and animal enzymes, whose SDH presented approximately 10% of the activity when NAD^+ was replaced by $NADP^+$ and whose LKR has a stringent cofactor requirement for NADPH [10,17].

3.3. Partial purification of LKR and SDH from S. pomeroyi

To elucidate whether bacterial LKR and SDH activities are associated in a bifunctional polypeptide, as in plants and animals, the enzyme was partially purified from *S. pomeroyi* using procedures based on that described for the maize enzyme [10]. The elution pattern of enzyme activities from a Q-Sepharose anion-exchange column showed that the peaks of LKR and SDH were superimposed (Fig. 4A). The fractions containing both activities were combined and applied to a Superdex 200 HR gel filtration column. Again, LKR and SDH co-eluted in the same fractions (Fig. 4B). The Superdex 200 HR gel filtration column was calibrated with thyroglobulin (molecular mass = 669 kDa), ferritin (molecular mass = 440 kDa), BSA (molecular mass = 67 kDa) and ovalbumin (molecular mass = 45 kDa), to estimate the molecular mass of the native bacterial enzyme. The co-eluted peaks of LKR and SDH activities from the gel filtration column presented a molecular mass of ~44 kDa (data not shown) indicating the production of separate polypeptides for LKR (estimated molecular mass of 38.5 kDa) and SDH (estimated molecular mass of 44.1 kDa). These results indicate that bacterial LKR and SDH are activities of separate polypeptides and that the native bacterial enzymes are monomers.

3.4. Cloning and expression of recombinant LKR and SDH

A DNA fragment spanning the *lkr* and *sdh* genes was amplified from the *S. pomeroyi* genomic DNA and cloned into the expression vector pET-28a in N-terminal fusion with His-tag. If both enzymes were linked in a single bifunctional polypeptide, the two activities would be co-purified due to the LKR N terminal fused His Tag. If not, only the LKR polypeptide would be purified. The recombinant proteins were purified using the Ni–NTA agarose affinity resin followed by Q-Sepharose column. Both activities were co-purified in exactly the same fraction in the Ni–NTA and anion-exchange columns (Fig. 4C). But when resolved by SDS–PAGE the purified recombinant protein appeared in the gel as a single band of ~44 kDa (Fig. 4D). This experiment was repeated several times, producing exactly the same results. The recombinant LKR and SDH co-eluted from the gel filtration column with a molecular mass of ~44 kDa similar to that of the enzyme purified from the bacterial culture (data not shown). To resolve if the single band of the purified recombinant protein contained both the LKR and the SDH polypeptides, aliquots of the purified protein we electrophoresed in a non-denaturing PAGE gel and revealed for LKR and SDH activities. The recombinant LKR polypeptide with the fused His Tag tail, have and estimated molecular mass of 41 kDa and an estimated isoelectric point of 4.9, while the recombinant SDH polypeptide has and estimated molecular mass of 44 kDa and an estimated isoelectric point of 5.0. The slightly acidic LKR polypeptide migrated faster than the SDH polypeptide (Fig. 4E) allowing us to conclude that both polypeptides interact each other, and this makes than be co-purified in different chromatographic procedures.

3.5. Domain architectures of plant, animal and bacteria LKR and SDH

The amino acid sequence analysis revealed LKR and SDH orthologues in Bacteroidetes and Proteobacteria. ID orthologues were found in Cyanobacteria and *Archaea*, although in these cases, the proteins flanking the ID, could not be annotated as LKR and SDH. In *S. pomeroyi*, the genes encoding LKR and SDH are located in an operon together with genes encoding oxidative stress response

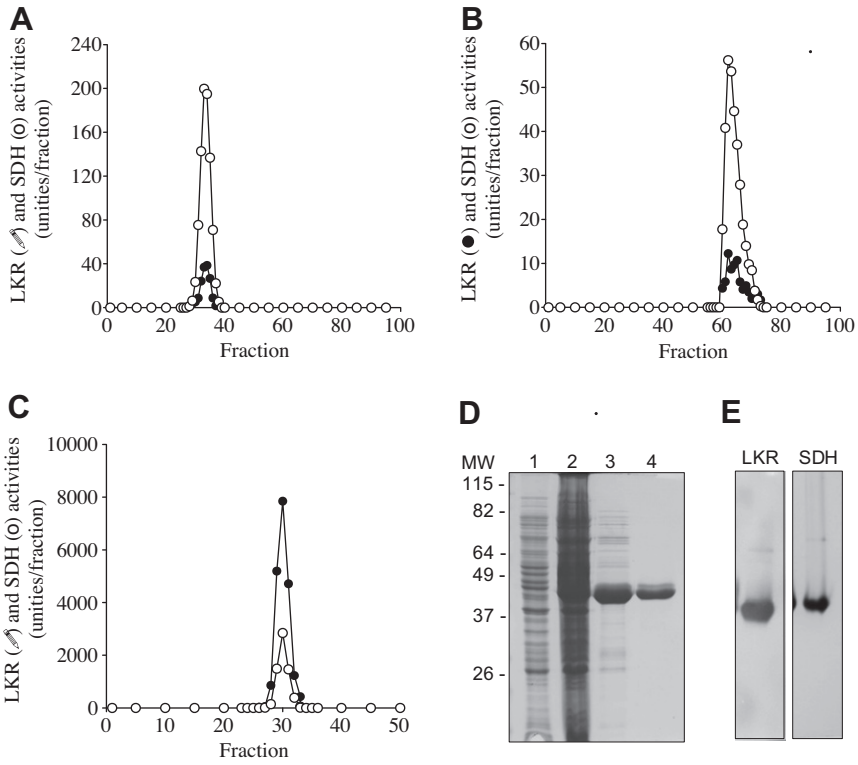

Fig. 4. (A) Elution pattern of *S. pomeroyi* LKR and SDH activities from the Q-Sepharose column. The column was eluted with a 0–1 M NaCl gradient in extraction buffer at a flow rate of 1.5 ml/min. Two-millilitre fractions were collected and assayed for enzyme activity. One unit of enzyme activity is defined as 1 nmol of NADPH oxidised or 1 nmol of NAD^+ reduced per min at 30 °C. (B) Elution pattern of *S. pomeroyi* LKR and SDH activities from the Superdex 200 HR column. The enzymes were eluted with extraction buffer containing 0.3 M NaCl. Fractions of 0.25 ml were collected and assayed for enzyme activities. One unit of enzyme activity is defined as 1 nmol of NADPH oxidised or 1 nmol of NAD^+ reduced per min at 30 °C. (C) Elution pattern of recombinant *S. pomeroyi* LKR and SDH activities from the Q-Sepharose column. The column was eluted with a 0–1 M NaCl gradient in extraction buffer at a flow rate of 1.5 ml/min. Two-millilitre fractions were collected and assayed for enzyme activity. One unit of enzyme activity is defined as 1 nmol of NADPH oxidised or 1 nmol of NAD^+ reduced per min at 30 °C. (D) SDS–PAGE of the recombinant *S. pomeroyi* LKR and SDH. Lane 1, non-induced bacterial extract. Lane 2, IPTG induced bacterial extract. Lane 3, Ni–NTA agarose purified recombinant enzymes. Lane 4, Superdex G200 purified recombinant enzyme. (E) PAGE of the recombinant LKR and SDH protein under non-denaturing tions. Ten mg of protein from the LKR and SDH most active fraction from the Q-Sepharose column were loaded in each well of a 8% slab gel. After electrophoresis individual lanes were developed for LKR and SDH activities.

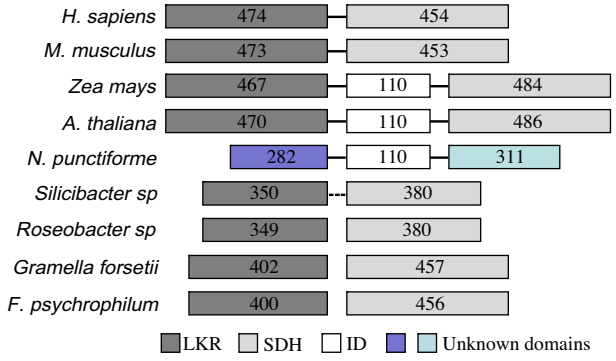

Fig. 5. Schematic representation of the possible architectures of the LKR, SDH and ID found in yeast, animals, plants and bacteria. The numbers inside the boxes indicate the number of amino acids in each domain in the different organisms.

enzymes (data not shown). Sequence analysis of this operon revealed that there is a 84 amino acid polypeptide encoding sequence between the *lkr* and *sdh* genes. This small polypeptide is translated from the frame +3 while LKR and SDH are both translated from the frame +1. Translation from this configuration leads the production of separated LKR and SDH enzymes. Thus, there are four possibilities for the LKR/SDH architecture in plants, fungi, animals and bacteria: (i) the LKR and SDH domains are linked but without the ID; (ii) the LKR and SDH are separated by the ID; (iii) the LKR and SDH domains are separated, as their encoding genes are located at different positions in the genome; (iv) the LKR and SDH domains are contiguous in an operon but are translated into separated polypeptides. Linked LKR and SDH in a bifunctional polypeptide were found only in plants and animals (Fig. 5).

4. Discussion

Lysine is synthesised in plants and bacteria through the aspartate pathway [1–3]. This pathway exists in all plants and bacteria whose genomic information is available in public databases. Fungi do not possess the aspartate pathway; they synthesise lysine through the saccharopine pathway [4]. Animals do not possess the aspartate pathway [1–3], but they do possess the saccharopine pathway, and instead of using it to synthesise lysine, they use this pathway for lysine degradation [9]. Plants also possess the saccharopine pathway and use it for lysine degradation [8]. In this work, we showed the existence of the saccharopine pathway in some bacterial groups, although all of them also possess the aspartate pathway for lysine synthesis. In *S. pomeroyi*, LKR and SDH were shown to reside in an operon in a configuration that enables both enzymes being produce under the same regulatory process. The kinetic characteristics of LKR and SDH suggest that the saccharopine pathway in this bacterium functions in the direction of lysine degradation. LKR from *S. pomeroyi* showed a preference for NADH instead of NADPH that is the preferred cofactor for the plant and mammal enzymes. This could be explained by the amino acid composition of the peptide known to be the NADH binding site [19]. This peptide,

located in *C. albicans* between residues 210 and 225 is identical in *Pichia stipitis* (Supplemental Fig. 2). The peptide is almost fully conserved in *S. pomeroyi and Roseobacter* sp. that differs from that of *C. albicans* by a change of leucine to isoleucine at position 1, and a cysteine to valine at position 9. This peptide in plant and animal is remarkably different conserving only a valine at position 2, a glycine at position 7 and glycine and valine at positions 12 and 13 (Supplemental Fig. 2).

A major difference among the primary structure of LKR/SDH from the diverse organisms we analysed is the presence of an ID polypeptide in plants, Cyanobacteria, *Eubacteria* and *Archaea* (Table 2). The occurrence of ID orthologues with highly conserved amino acid sequences in bacteria of different genera suggests that this peptide could be involved in an important regulatory mechanism. Most surprising was the observation that, in Cyanobacteria, the ID orthologues apparently do not link LKR and SDH. It is reasonable to postulate that if the ID is conserved in bacteria and plants, then the plants inherited the ID from a common ancestor that they share with Cyanobacteria. However, the question of how the ID from both bacteria and plants evolved to link different proteins is intriguing. In the case of the Cyanobacteria, it is possible that the polypeptides flanking the ID could be reminiscent of orthologues of LKR and SDH that diverged in their amino acid sequences and lost both enzymatic activities. The loss of these enzymes during evolution could be possible, as we found an orthologue of LKR in *Cyanothece* sp. but could not find an orthologue of SDH (data not shown).

Some CFB (Bacteroidetes/Chlorobi group) bacteria also possess LKR and SDH orthologues, but the polypeptides are encoded by genes located at different positions in the bacterial genome. Thus, it is possible that an operon configuration such as the one found in *S. pomeroyi* could be the ancestor of the animal and plant LKR/SDH. Yet, this leaves unresolved questions about the origin of the plant ID. One possible scenario is that a member of the α-proteobacteria group with a gene/protein configuration similar to that found in *S. pomeroyi* underwent the endosymbiosis process that gave rise to mitochondria [20,21]. The *lkr* and *sdh* genes present in this bacterium-originated mitochondria were later transferred to the nuclear genome, as was most of the primitive mitochondrial genome [21]. Similarly, the chloroplast, which originated from an endosymbiotic process involving Cyanobacteria, incorporated the ID into the chloroplast genome, and this was later transferred to the plant nuclear genome [22]. During plant evolution, the ancestral Cyanobacteria ID-encoding sequence was inserted between the LKR and SDH domains of the nuclear-incorporated mitochondrial gene.

The presence of LKR and SDH along with genes encoding enzymes involved in oxidative stress response suggests that these lysine-degrading enzymes may interact, in a novel mechanism of oxidative stress response.

Acknowledgements

G.C. Mello Serrano and N. Zanata received fellowships from Fapesp, TR Silva Figueira received a fellowship from the Coordenação de Aperfeiçoamento de professores do Ensino Superior (CAPES). PA is a recipient of a CNPq productivity fellowship. This work was supported by FAPESP grant 10/50114-4.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.02.023.

References

[1] Azevedo, R.A., Arruda, P., Turner, W.L. and Lea, P.J. (1997) The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochemistry 46, 395–419.
[2] Azevedo, R.A. (2002) Analysis of the aspartic acid metabolic pathway using mutant genes. Amino Acids 22, 217–230.
[3] Azevedo, R.A., Lancien, M. and Lea, P.J. (2006) The aspartic acid metabolic pathway, an exciting and essential pathway in plants. Amino Acids 30, 143–162.
[4] Trupin, J.S. and Broquist, H.P. (1963) Lysine biosynthesis in *Neurospora crassa*. Fed. Proc. 22, 243–250.
[5] Bhattacharjee, J.K. (1985) Alpha-aminoadipate pathway for the biosynthesis of lysine in lower eukaryotes. Crit. Rev. Microbiol. 12, 131–151.
[6] Broquist, H.P. and Trupin, J.S. (1966) Amino acid metabolism. Ann. Rev. Biochem. 35, 231–274.
[7] Arruda, P., Sodek, L. and da Silva, W.J. (1982) Lysine-ketoglutarate reductase activity in developing maize endosperm. Plant Physiol. 69, 988–989.
[8] Arruda, P., Kemper, E.L., Papes, F. and Leite, A. (2000) Regulation of lysine catabolism in higher plants. Trends Plant Sci. 5, 324–330.
[9] Markovitz, P.J. and Chuang, D.T. (1987) The bifunctional aminoadipic semialdehyde synthase in lysine degradation. J. Biol. Chem. 262, 9353–9358.
[10] Gonçalves-Butruille, M., Szajner, P., Torigoi, E., Leite, A. and Arruda, P. (1996) Purification and characterization of the bifunctional enzyme lysine-ketoglutarate reductase-saccharopine dehydrogenase from maize. Plant Physiol. 11, 765–771.
[11] Kemper, E.L., Cord Neto, G., Papes, F., Moraes, K.C.M., Leite, A. and Arruda, P. (1999) The role of Opaque2 in the control of lysine-degrading activities in developing maize endosperm. Plant Cell 11, 1981–1993.
[12] Kemper, E.L., Cord-Neto, G., Capella, A.N., Goncalves-Butruile, M., Azevedo, R.A. and Arruda, P. (1998) Structure and regulation of the bifunctional enzyme lysine-oxoglutarate reductase-saccharopine dehydrogenase in maize. Eur. J. Biochem. 253, 720–729.
[13] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
[14] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTALX windows interface. Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
[15] Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4 (406), 425.
[16] Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.
[17] Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: Integrated software for molecular evolutionary genetic analysis and sequence alignment. Brief. Bioinf. 5, 150–163.
[18] Porchia, A.C., Curatti, L. and Salerno, G.L. (1999) Sucrose metabolism in cyanobacteria: sucrose synthase from *Anabaena sp*. Strain PCC 7119 is remarkably different from the plant enzymes with respect to substrate affinity and amino-terminal sequence. Planta 210, 34–40.
[19] Garrad, R., Schmidt, T.M. and Bhattacharjee, J.K. (1994) Molecular and functional analysis of the LYS1 gene of *Candida albicans*. Infec. Immun. 62, 5027–5031.
[20] Emelyanov, V.V. (2001) Rickettsiaceae, rickettsia-like endosymbionts, and the origin of mitochondria. Biosci. Rep. 21, 1–17.
[21] Searcy, D.G. (2003) Metabolic integration during the evolutionary origin of mitochondria. Cell Res. 13, 229–238.
[22] McFadden, G.I. (2001) Chloroplast origin and integration. Plant Physiol. 125, 50–53.