The Activity of the *Arabidopsis* Bifunctional Lysine-ketoglutarate Reductase/Saccharopine Dehydrogenase Enzyme of Lysine Catabolism Is Regulated by Functional Interaction between Its Two Enzyme Domains*

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Lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) is a bifunctional enzyme catalyzing the first two steps of lysine catabolism in animals and plants. To elucidate the biochemical significance of the linkage between the two enzymes of LKR/SDH, namely lysine ketoglutarate and saccharopine dehydrogenase, we employed various truncated and mutated *Arabidopsis* LKR/SDH polypeptides expressed in yeast. Activity analyses of the different recombinant polypeptides under conditions of varying NaCl levels implied that LKR, but not SDH activity, is regulated by functional interaction between the LKR and SDH domains, which is mediated by the structural conformation of the linker region connecting them. Because LKR activity of plant LKR/SDH enzymes is also regulated by casein kinase 2 phosphorylation, we searched for such potential regulatory phosphorylation sites using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and site-directed mutagenesis. This analysis identified Ser-458 as a potential candidate for regulating LKR activity by casein kinase 2. Moreover, our results also suggest that assembly of LKR/SDH into a homodimer is not mediated by the N-terminal part of the LKR domain functions in a calcium-dependent assembly of LKR/SDH into a homodimer. We found that this region is essential for LKR activity but that it does not control a calcium-dependent assembly of LKR/SDH. The relevance of our results to the in vivo function of LKR/SDH in lysine catabolism in plants is discussed. In addition, because the linker region between LKR and SDH exists only in plants but not in animal LKR/SDH enzymes, our results suggest that the regulatory properties of LKR/SDH and, hence, the regulation of lysine catabolism are different between plants and animals.

Many metabolic pathways in prokaryotes and eukaryotes include bifunctional enzymes containing two different enzymes that are linked on a single polypeptide encoded by a single gene. The regulatory significance of such linkages is still not clearly understood. One member of this group of bifunctional enzymes is LKR/SDH, containing the lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH) enzymes of the α-amino adipic acid pathway of lysine catabolism, a pathway that operates both in animals and plants (1, 2). Defects in the LKR/SDH gene in humans are associated with a severe genetic disorder called familial hyperlysinemias, which is associated in some patients with mental retardation (3, 4). It has also been suggested that in mammals LKR/SDH participates in the metabolism of glutamate needed for nerve signaling via glutamate receptors (5). Two documented functions of the α-amino adipic acid pathway in plants are to balance lysine levels and also to regulate carbon/nitrogen partition in response to abiotic stresses (2, 6–9).

The significance of the bifunctional nature of LKR/SDH is still not known, mostly because its two enzymes were so far studied as single entities. The activity of LKR, which resides on the N-terminal part of LKR/SDH, but not SDH, was demonstrated to be subject to a complex regulatory control in plants. LKR activity in developing tobacco seeds is stimulated by lysine in vivo via an intracellular signaling cascade requiring Ca²⁺ and protein phosphorylation/dephosphorylation (10). Moreover, plant LKR/SDH polypeptides can be phosphorylated in vitro by casein kinase 2 (1, 11) and this in vitro phosphorylation stimulates LKR activity in a lysine-dependent manner (1). The in vitro LKR activities of the maize and rice LKR/SDH enzymes were also shown to be stimulated by salts, including calcium (12, 13).

In the present report, we have expressed a full-length *Arabidopsis* LKR/SDH enzyme as well as several deletion and mutation versions of this protein in yeast to study whether and how LKR activity is regulated by the bifunctional nature of LKR/SDH. We found that LKR activity is modulated by functional interaction with the SDH domain, which is mediated by the linker region linking these two enzymes. We also identified Ser-458 as a potential candidate for regulating LKR activity by casein kinase 2. Moreover, our results also suggest that assembly of the *Arabidopsis* LKR/SDH into a homodimer is not mediated by calcium, as was previously hypothesized for plant LKR/SDH enzymes (1).

**EXPERIMENTAL PROCEDURES**

Materials—*Arabidopsis* and maize plants were grown in a controlled greenhouse (16-h photo period at 25 ± 5 °C). High Fidelity PCR system (Roche Diagnostics) was used for generating point mutation and constructs. The high molecular weight gel filtration calibration kit was purchased from Amersham Biosciences.

Plasmid Constructs—The full-length *Arabidopsis* bifunctional LKR/SDH polypeptides were transformed into the yeast strains. A variety of truncated and mutant enzyme constructs were analyzed for LKR and SDH activities.

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The abbreviations used are: LKR, lysine-ketoglutarate reductase; SDH, saccharopine dehydrogenase; LR, linker region; MALDI/TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
SDH and nonfunctional LKR and SDH cDNAs fused with a tag of six histidines (His tag) in the yeast expression vector pVT102u were previously described (14, 15). To construct the LKR domain plus the linker region (LKR-LR), a PCR amplification was performed to introduce a stop codon and an EcoRI site at the end of the linker region. The PCR product was digested by XhoI and EcoRI, replacing the XhoI/EcoRI fragment to produce SK-nHis-LKR (IEGR1). The PCR1 product was cloned into SK as a fragment of LKR domain (PCR1) or at the beginning of SDH domain (PCR2), replacing the XhoI fragment to produce SK-nHis-LKR (IEGR1/T3). The PCR products were digested with EcoRI and NheI, blunt-ended, and cloned into SK-nHis-LKR (IEGR1). The PCR2 product was digested by XhoI and NheI, was cloned into SK-nHis-LKR/SDH (IEGR1). The resulting plasmids were named as SK-nHis-LKR/SDH (IEGR1/T3), SK-PCR2 (IEGR1/T7), SK-PCR3 (IEGR2/T3), and SK-PCR4 (IEGR2/T7) (Table I).

Site-directed Mutagenesis—To generate point mutations in the EF-hand-like region of the LKR domain, primers Pala369 and Pasp369 (Table I) were used for PCR on SK-nHis-LKR/SDH as a template. Two PCR amplifications were performed with Pala369 and T3 as well as Pasp369 and T3, respectively. The PCR products were digested with PflI and PstI and cloned into SK-nHis-LKR (IEGR1). The PCR2 product was digested by XhoI and SpeI and cloned into SK-nHis-LKR/SDH (IEGR1). The PCR2 product was digested by PstI and XhoI and cloned into SK-nHis-LKR/SDH (IEGR1/T3) to obtain SK-nHis-LKR/SDH (IEGR2). All constructs were subcloned into the yeast expression vector pVT102u (16). The DNA sequences of all PCR products were confirmed by sequencing.

**Table I**

| DNA sequences of oligonucleotides used in the present study |
|-------------------------------------------------------------|
| SC-IEGR1/T3 5'-ctcctgtgcttggtagcatggagaagaggca-3' |
| SC-IEGR1/T7 5'-ctcctgtgcttggtagcatggagaagaggca-3' |
| SC-IEGR2/T3 5'-ctcctgtgcttggtagcatggagaagaggca-3' |
| SC-IEGR2/T7 5'-ctcctgtgcttggtagcatggagaagaggca-3' |

**Protein Expression in Yeast, Purification, SDS-PAGE, and Western Blot Analysis**—The expression of the recombinant His-tagged Arabidopsis LKR/SDH constructs in yeast cells and purification of their encoded proteins on a nickel column as well as SDS-PAGE and Western blot analyses were performed as previously described (9).

**Gel Filtration Chromatography**—Floral organs of Arabidopsis and developing grains of maize were ground in liquid nitrogen and resuspended in extraction buffer (50 mM sodium phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and a protease inhibitor mixture with or without 10 mM EGTA). The extract was centrifuged at 10,000 g for 10 min, and the supernatant was filtered through a 0.2-μm syringe filter. Total protein (300 μg) from plant extracts or 30 μg of purified protein from yeast cells was loaded into a Superdex 200 (Amersham Biosciences) gel filtration column pre-equilibrated with elution buffer (50 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl with or without 10 mM EGTA). Elution was performed with elution buffer at 0.5 ml/min. Fractions of 0.5 ml were collected. High molecular weight protein marker mixture was run under the same conditions and detected by measuring the absorbance of each collected fraction at 280 nm. To monitor the eluted LKR or SDH polypeptides, individual fractions were subjected to Western blot analysis using anti-LKR or anti-SDH monoclonal antibodies.

**Analysis of LKR Activity**—The kinetics of LKR activity was measured spectrophotometrically by determining the rate of NADPH oxidation at 340 nm for 10 min at 30 °C. The activity assays included ~0.1 μg of nearly purified protein in 0.3 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 20 mM Lys, 14 mM potassium phosphate, 0.4 mM and 0.4 mM NADH. In the experiments analyzing the NaCl effect on LKR activity, the 0.1 mM potassium phosphate buffer was replaced with 25 mM Tris-HCl, pH 7.5. One unit of LKR was defined as the amount of enzyme that catalyzes the oxidation of 1 nmol of NADPH per min at 30 °C.

The kinetics of SDH activity was measured spectrophotometrically by determining the rate of NADH reduction at 340 nm for 10 min at 30 °C. The activity assays included ~0.1 μg of protein from the nearly pure recombinant yeast enzyme in 0.3 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 2 mM isocitrate and 2 mM NADH. One unit of SDH was defined as the amount of enzyme that catalyzes the reduction 1 nmol of NADH per min at 30 °C.

**RESULTS**

**LKR Activity of the Arabidopsis Bifunctional LKR/SDH Is Modulated by Functional Interaction between the LKR and SDH Domains**—To test whether the structural conformation of LKR/SDH is affected by interactions between different domains of this bifunctional protein, we have used yeast as an expression system to produce recombinant wild type and mutant forms of the Arabidopsis LKR/SDH enzyme, fused to a His tag. We have previously shown that yeast represents a highly suitable system for this study and that the recombinant LKR/SDH constructs can be fused to various epitope tags without affecting LKR and SDH activities (14, 15). Because in vitro LKR activity of the maize LKR/SDH was previously shown to be stimulated by NaCl (13), we used different NaCl concentrations as a means to alter the structural conformation of the recombinant Arabidopsis LKR/SDH-derived proteins and tested their effects on LKR activity. In addition, because we have used LKR and SDH activities as functional probes to identify interactions between the different domains of LKR/SDH, we have termed the observed interactions as “functional interactions.”

To study whether the NaCl-mediated stimulation of LKR activity is dependent on the LKR domain itself or on a functional interaction of the LKR domain with other domains of LKR/SDH, we used five different constructs. These encoded the full-length LKR/SDH (Fig. 1a) as well as four truncated versions of LKR/SDH. These truncated versions included the LKR domain alone (LKR-), the SDH domain plus the linker region (SDH-LR), the SDH domain alone (SDH), and an LKR/SDH lacking the linker region (LKR/SDH-ΔLR) (Fig. 1, b–f, respectively). As shown in Fig. 2A, LKR activity of the full-length LKR/SDH
The Linker Region Regulates the Functional Interaction between the LKR and SDH Domains of LKR/SDH

To clarify whether the linker region plays any regulatory role in mediating the functional interaction between the LKR and SDH domains we expressed in yeast another construct in which the linker region was deleted and the LKR domain was directly fused to each other. As opposed to LKR activity, no significant change in SDH activity was observed in any of these polypeptides as well as in any of the proteins containing these IEGR1 (cf. Fig. 3A; IEGR1 and IEGR2) and did not alter the overall structure, implying also the structure of the linker region is important for mediating the functional interaction between the LKR and SDH domains of LKR/SDH.

Because the results of Fig. 2A suggested some functional interaction between the linker region and the LKR domain, we wished also to test whether the structural conformation of the linker region is important for mediating the functional interaction between the LKR and SDH domains of LKR/SDH. To this end, we mutated the linker region of the LKR/SDH at two different places, introducing IEGR cleavage sites for the FactorX protease (Fig. 3A; IEGR1 and IEGR2). These mutations included replacement of three amino acids at each site (IANG to IEGR1 and NEDY to IEGR2) and did not alter the overall length of the linker region. In addition, these mutations were easily confirmed by cleavage with FactorX. As shown in Fig. 3B, the IEGR1 mutation did not significantly affect the activity of LKR and its modulation by NaCl (cf. Fig. 3B with Fig. 2A). In contrast, the IEGR2 mutation abolished the NaCl-mediated stimulation of LKR activity, a trait related to the functional interaction between the LKR and SDH domains. This supported the results of Fig. 2B and implied that the structural conformation of some parts of the linker region is important for the functional interaction between the LKR and SDH domains.

We also analyzed whether SDH activity was altered in the truncated polypeptides LKR/SDH, LKR/SDH-LR, SDH-LR, and SDH (Fig. 1) as well as in the two polypeptides with the linker region mutations (Fig. 3) compared with the wild type LKR/SDH. As opposed to LKR activity, no significant change in SDH activity was observed in any of these polypeptides as well as in any of the different NaCl concentrations used (data not shown).

The effect of NaCl on LKR activity of various recombinant Arabidopsis LKR/SDH enzymes expressed in yeast cells. The recombinant proteins were purified from yeast on a nickel column and analyzed for LKR activity under conditions of excess concentrations of all LKR substrates in solutions containing increasing NaCl concentrations. A, the recombinant full-length LKR/SDH (black squares), LKR domain only (black diamonds), and LKR-LR (black triangles). B, the recombinant full-length LKR/SDH (black squares) and the LKR/SDH-LR polypeptides (black circles). The recombinant constructs are as shown schematically in Fig. 1. Bars represent the S.D. of four different experiments.

The effect of mutations in the linker region on the response of LKR activity to NaCl. A, schematic depiction of the locations where the IEGR1 and IEGR2 mutations were inserted within the linker region of the LKR/SDH polypeptide. B, the recombinant proteins containing these IEGR1 (black diamonds) and IEGR2 (black squares) mutations were purified from yeast on a nickel column and analyzed for LKR activity under conditions of excess concentrations of all LKR substrates in solutions containing increasing NaCl concentrations. Bars represent the S.D. of four different experiments.

Figure 1. Schematic depiction of the polypeptide encoded by the different Arabidopsis LKR/SDH-derived constructs. a, the full-length LKR/SDH; b, LKR + linker region; c, LKR domain only; d, SDH domain + linker region; e, SDH domain only; f, LKR/SDH from which the linker region was deleted and the LKR and SDH domains are directly fused to each other.

Figure 2. Effect of NaCl on LKR activity of various recombinant Arabidopsis LKR/SDH enzymes expressed in yeast cells. The recombinant proteins were purified from yeast on a nickel column and analyzed for LKR activity under conditions of excess concentrations of all LKR substrates in solutions containing increasing NaCl concentrations. A, the recombinant full-length LKR/SDH (black squares), LKR domain only (black diamonds), and LKR-LR (black triangles). B, the recombinant full-length LKR/SDH (black squares) and the LKR/SDH-LR polypeptides (black circles). The recombinant constructs are as shown schematically in Fig. 1. Bars represent the S.D. of four different experiments.
SDH and LKR Do Not Functionally Interact with Each Other When Present on Separated Polypeptides—The linker region was shown to be important for the functional interaction between the LKR and SDH domains. However, it was still unknown whether the interaction itself occurs by direct affinity between these two domains or whether the linker region forces these domains to functionally interact. To test this issue we expressed separately in yeast cells either the SDH-LR, LKR-LR, SDH, or LKR domains of the Arabidopsis LKR/SDH (Fig. 1, d, b, e, and c, respectively). The proteins were purified using a nickel column and brought to similar concentrations, as deduced from Coomassie Blue staining of their bands in SDS gel (Fig. 4A). We then mixed the LKR polypeptide with increasing amounts of either the SDH or SDH-LR polypeptides and the LKR-LR polypeptide with increasing amounts of the SDH polypeptide. The addition of up to an 8-fold excess molar amount of the SDH-LR to the LKR polypeptide (Fig. 4) produced from Coomassie Blue staining of their bands in SDS gel (Fig. 4A). We then mixed the LKR polypeptide with increasing amounts of either the SDH or SDH-LR polypeptides and the LKR-LR polypeptide with increasing amounts of the SDH polypeptide. The addition of up to an 8-fold excess molar amount of the SDH-LR to the LKR polypeptide (Fig. 4B) as well as the SDH to the LKR (Fig. 4C) or to the LKR-LR polypeptides (Fig. 4D) had no effect on LKR activity. This suggests that the functional interaction between the LKR and SDH domains does not stem from affinity interaction between these two domains or between the LKR domain and the linker region.

Although the results of Fig. 4 imply that the fully folded LKR and SDH domains do not interact, it is still possible that these domains can interact if allowed to fold together. To address this, mixtures containing increasing concentrations of the SDH or SDH-LR domains with the LKR or LKR-LR domains shown in Fig. 4 were unfolded with urea and then refolded in vitro using conditions that will optimally preserve the activities of both enzymes. In all of these unfolding/refolding cases, the SDH or SDH-LR polypeptides had no effect on LKR activity (data not shown). We also tested whether mixing of the LKR and SDH and LKR-LR and SDH as well as LKR and SDH-LR domains affected SDH activity. In all mixing experiments including those followed by unfolding and refolding no significant difference in SDH activity was observed (data not shown).

Assembly of LKR/SDH into a Homodimer Is Mediated by More Than One Protein Domain—Plant LKR/SDH enzymes are homodimers (1). We therefore wished to test whether assembly of this bifunctional enzyme into a homodimer is mediated specifically by one of its three domains, namely the LKR, linker region, or SDH domains. To this end, purified recombinant polypeptides containing the full-length LKR/SDH, the monofunctional LKR domain, the monofunctional SDH domain, and the LKR/SDHΔLR (Fig. 1, a, c, e, and f, respectively) as well as molecular mass protein markers were each fractionated in parallel on a size exclusion column. Individual fractions were tested for the presence of the different LKR/SDH-related polypeptides. As shown in Fig. 5, a–d, all polypeptides fractionated with the expected sizes of homodimers (nearly double the mass of the single subunit), implying that assembly is mediated by more than one domain of the LKR/SDH polypeptide.

Does the EF-hand-like Sequence in the C-terminal Part of the LKR Domain Regulate LKR Activity and, if Yes, by What Mechanism?—LKR activity of the maize and rice LKR/SDH enzymes is stimulated by calcium (12, 13). However, the amino acid sequence and mechanism responsible for this regulation is not clear. Kemper et al. (13) identified a conserved EF-hand-like domain in the C-terminal part of plant LKR/SDH proteins (VDILPTEFAKEASQHFG) and suggest that this domain may regulate the calcium-dependent stimulation of LKR activity in the maize LKR/SDH enzyme. In addition, in a recent review Arruda et al. (1) present a model in which this EF-hand-like domain operates via mediating the assembly of LKR/SDH into a homodimer. Yet another interesting property of this EF-hand-like region is that it contains a Ser residue (Ser-396) that is situated in a putative consensus site for phosphorylation by a calcium-dependent protein kinase. This is of particular interest, because we have previously shown that LKR activity in tobacco seeds is modulated by protein phosphorylation/dephosphorylation in a calcium-dependent manner (10). So far our attempts to detect a phosphorylation at this EF-hand-like region by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis were unsuccessful (see details under “Discussion”). Nevertheless, we

Fig. 4. The monofunctional SDH does not affect LKR activity when present on a different polypeptide. Purified proteins encoding the SDH-LR, LKR-LR, SDH domain only and LKR domain only (Fig. 1, constructs d, b, e, and c, respectively) were diluted into nearly equal concentrations, as determined by fractionation of SDS-PAGE and staining with Coomassie Blue (panel A). Panels B–D, reaction mixtures containing 6 μl of the LKR (panels B and C) or 6 μl of the LKR-LR (panel D) polypeptide were supplemented with increasing volumes of the SDH-LR (panel B) or SDH (panels C and D) polypeptides. LKR activity was then assayed under conditions of excess concentrations of all LKR substrates in the presence of 0 mM NaCl (black squares, black diamonds, or black circles in panels B, C, and D, respectively) or 100 mM NaCl (open squares, open diamonds, or open circles in panels B, C, and D, respectively). Bars represent the S.D. of four different experiments.

2 X. Zhu and G. Galli, unpublished information.
decided to test the effects of mutations of Ser-396 in this region to Asp or to Ala (mimicking a phosphorylation effect) on LKR activity and homodimer formation of the full-length LKR/SDH. As shown in Fig. 6A, replacement of Ser-396 by Asp completely abolished LKR activity, whereas its replacement by Ala had no effect on LKR activity. In contrast, none of these mutations significantly affected SDH activity (Fig. 6B). In addition, as shown in Fig. 6C, both the wild type and the two mutated polypeptides were homodimers as deduced from fractionation on size exclusion column.

Because the results of Fig. 6A did not support a regulatory role of the EF-hand-like sequence in the assembly of LKR/SDH into homodimer, we also tested whether this assembly is mediated by calcium as was previously proposed (1). To this end, extracts from floral organs of Arabidopsis or developing maize grains were fractionated on a size exclusion column in the presence or absence of 10 mM calcium chelator EGTA, and individual fractions were reacted with anti-Arabidopsis LKR monoclonal antibodies. As shown in Fig. 7, a–d, the Arabidopsis and maize LKR/SDH were eluted as homodimers both in the absence or presence of the EGTA.

Identification of Putative Casein Kinase 2 Phosphorylation Sites That Regulate the Activity of LKR—Previous studies show that the soybean and maize LKR/SDH polypeptides are phosphorylated in vitro by casein kinase 2 (1, 11). Moreover, the casein kinase 2-dependent phosphorylation of the maize LKR/SDH stimulated LKR activity in a lysine-dependent manner (1). To identify potential phosphorylation sites on the LKR domain of the Arabidopsis LKR polypeptides, a His-tagged-purified LKR polypeptide band (see Fig. 4A) was subjected to proteolysis followed by MALDI-TOF-MS analysis. This analysis identified two peptides containing a phosphorylated Thr-238 and Ser-458, which are both situated inside consensus casein kinase 2 phosphorylation sites (TFVE and SNPE, respectively). To test further whether any of these two amino acids regulate LKR activity, the wild type LKR/SDH cDNA was mutated by site-directed mutagenesis to replace each of these two amino acids by Ala or Asp, and the mutants were expressed in yeast cells. As shown in Fig. 8A, replacement of Thr-238 with either Ala or Asp had no significant effect on LKR activity. In contrast, replacement of Ser-458 with Ala significantly inhibited LKR activity, whereas its replacement with Asp had no significant effect of LKR activity. These results are consistent with the presence of a regulatory phosphorylation site on Ser-458. We also tested whether the mutations in Thr-238 and Ser-458 affect SDH activity of the LKR/SDH polypeptide. As shown in Fig. 8B, none of the mutations significantly affected SDH activity.

**DISCUSSION**

The LKR and SDH Domains of the Arabidopsis Bifunctional LKR/SDH Functionally Interact to Regulate LKR Activity—Although bifunctional polypeptides containing two linked enzymes are quite common in metabolic pathways, the significance of these phenomena is not clear. Such a linkage may enable more efficient substrate channeling between the two linked enzymes. However, this is clearly not the sole advantage because a number of the bifunctional enzymes, such as aspartate kinase/homoserine dehydrogenase of the aspartate-family pathway (6), do not contain two consecutive enzymes of a given metabolic pathway. LKR and SDH are two consecutive enzymes in the pathway of lysine catabolism (2), but our previous studies did not support substrate channeling between them (17), suggesting that this linkage serves other
Ser-458 and Thr-238 were purified from yeast cells on a nickel column. Polypeptide. Ser-458 and Thr-238 on LKR and SDH activities of the LKR/SDH floral organs of Arabidopsis Proteins were extracted from of different molecular masses is indicated on top of the panels, whereas the subunit molecular mass of each of LKR/SDH-related polypeptide is indicated on the right.

Fig. 7. Effect of EGTA on the assembly of Arabidopsis and maize LKR/SDH into homodimers. Proteins were extracted from floral organs of Arabidopsis (a and b) or developing maize grains (c and d) in a buffer containing (a and c) or lacking (b and d) 10 mM EGTA and were fractionated on a size exclusion column (see “Experimental Procedures”). Individual fractions were reacted in Western blots with the monoclonal anti-LKR antibodies. The elution profile of marker proteins of different molecular masses is indicated on top of the panels, whereas the subunit molecular mass of each of LKR/SDH-related polypeptide is indicated on the right.

Fig. 8. Effects of the Ser to Ala and Ser to Asp replacements in Ser-458 and Thr-238 on LKR and SDH activities of the LKR/SDH polypeptide. Recombinant full-length LKR/SDH and the four LKR/SDH mutants with the Ser to Asp and Ser to Ala substitutions of Ser-458 and Thr-238 were purified from yeast cells on a nickel column. The purified proteins were analyzed for LKR (A) or SDH (B) activities under conditions of excess concentrations of all LKR substrates or all SDH substrates, respectively. Bars on top of the histograms represent the S.D. of four different experiments.

Regulatory purposes. Using the sensitivity of LKR activity to NaCl as a probe, we indeed demonstrated in the present report that LKR activity in LKR/SDH is modulated by functional interaction between the LKR and SDH domains and to some extent also between the LKR domain and the linker region. In contrast, SDH activity is relatively constant and is not modulated by any of the potential structural interactions that we tested.

The Functional Interaction between the LKR and SDH Domains Is Mediated by the Linker Region and Not by Specific Affinities between These Domains—To test whether the functional interaction between the LKR and SDH domains of LKR/SDH is mediated by specific affinity between these domains, we have expressed each of these domains on a separate polypeptide and mixed them in the test tube. The addition of more than 8-fold higher molar amounts of the SDH polypeptide to the LKR polypeptide did not inhibit LKR activity in incubation medium lacking NaCl, implying that the NaCl effect on LKR activity of LKR/SDH does not result from an affinity-mediated interaction between the LKR and SDH domains. In contrast, two lines of evidence implied that the linker region mediates the effect of the SDH domain on the activity of its linked LKR domain. First, deletion of the linker region, linking the LKR to the SDH domain, completely abolished the reduction of LKR activity under low NaCl conditions, and this polypeptide possessed nearly identical LKR activity to the polypeptide containing only the LKR domain. Second, mutation in one place of the linker region (Fig. 3; IEGR2) abolished the stimulation of LKR activity by NaCl. We therefore hypothesize that the functional interaction between the LKR and SDH domains of LKR/SDH is mediated by the structural conformation of the linker region. Alterations in the structural conformation of this linker region as a result of the NaCl concentration may enable the LKR and SDH domains to become either proximal or distal to each other, causing either an increase or decrease in LKR activity, respectively. The location of the SDH domain in proximal position to the LKR domain may interfere with LKR activity, perhaps by masking its ability to bind one of its substrates. In the IEGR2 mutant, the LKR and SDH domains may remain proximal to each other both in the low or high NaCl concentrations, hence rendering LKR relatively inactive. Our suggestion that the linker region is not inert but can modulate the structural conformation of the LKR/SDH polypeptide is also supported by the differential sensitivity of LKR activity to NaCl in the monofunctional LKR and LKR-LR polypeptides (Fig. 2A).

Our results extend the previous results of Kemper et al. (13), who find that upon progressive proteolysis of a partially purified maize extract with elastase, LKR activity was first decreased and later started to increase, reaching near the LKR activity before the elastase treatment. After column fractionation of the elastase-treated extracts, the addition of excess amounts of fractions containing SDH activity to those containing LKR activity inhibited the activity of LKR. Although the nature of the protease-digested products was not identified, Kemper et al. (13) conclude that LKR activity was inhibited by peptides derived from the SDH domain or the linker region, which were apparently present in the extract. As mentioned above, our results clearly show that the SDH domain, when present on a separate polypeptide, does not inhibit LKR activity.

Role of the EF-hand-like Domain and Calcium in the Assembly of LKR/SDH and LKR Activity—LKR activity of the maize LKR/SDH enzymes is stimulated by calcium (12, 13). Arruda and co-workers (1, 13) identify a conserved EF-hand-like domain in the C-terminal region of the LKR domain of plant LKR/SDH enzymes and hypothesized that this domain promotes a calcium-dependent assembly of LKR/SDH into a homodimer and by that also stimulates LKR activity. We found that assembly of the Arabidopsis and maize LKR/SDH enzymes into homodimers is not calcium-dependent and that an Arabidopsis LKR/SDH polypeptide whose activity was lost due to a mutation in this EF-hand-like domain still assembled into a homodimer. Thus, our results imply that the EF-hand-like domain is essential for LKR activity but do not support the suggested function of this region in a calcium-mediated assembly of LKR/SDH into a homodimer.

Does the EF-hand-like domain contain a phosphorylation site for a calcium-dependent protein kinase? The fact that
replacement of the Ser-396 residue in this region with Asp, but not Ala, abolished LKR activity supports such a potential site (Asp has similar properties to a phosphorylated Ser). However, in an independent research, we have so far been unable to detect any phosphorylation of this Ser residue by MALDI-TOF-MS analysis. Nevertheless, we cannot eliminate the possibility that a minor undetectable fraction of the protein may have been phosphorylated at this site. In any event, if a phosphorylation occurs at this site, its inhibitory effect on LKR activity (as deduced from the Ser to Asp substitution) is certainly different from previously studied phosphorylations of LKR/SDH (1, 10, 11), which caused an induction of LKR activity. Additional studies are needed to solve this interesting observation.

**Regulation of LKR Activity by Casein Kinase 2 Occurs Likely via Phosphorylation of Ser-458**—Previous studies indicate that LKR activity of plant LKR/SDH enzymes is regulated by phosphorylation with casein kinase 2 (1). We therefore search for such potential regulatory sites in the LKR domain using MALDI-TOF-MS analysis followed by site-directed mutagenesis. This analysis identified two phosphorylated amino acids, namely Thr-238 and Ser-458, both situated in a consensus casein kinase 2 phosphorylation site. However, although replacement of Ser-458 by Ala but not by Asp nearly eliminated LKR activity, similar mutations in Thr-238 had no effect. The inhibitory effect of replacing Ser-458 with Ala but not with Asp on LKR activity supports a positive regulatory role of Ser-458 phosphorylation on LKR activity. Thr-238 is apparently also phosphorylated in vivo, but our results do not support any regulatory function for such phosphorylation. Our results also do not rule out the presence of additional regulatory phosphorylation sites in the Arabidopsis LKR/SDH polypeptide.

**The Relevance of the Present Studies to the in Vivo Function of LKR/SDH**—Although not studied directly, our results suggest that LKR activity of the LKR/SDH polypeptide is modulated by its linked SDH domain in vivo. Whether LKR activity is modulated by the ionic composition of the cytosol (the compartment in which LKR/SDH is localized in plant cells) (14, 18) is not known. However, the ionic composition can vary significantly under abiotic stress conditions, in which expression of the LKR/SDH gene is up-regulated (8). In addition, functional interactions between the different domains of LKR/SDH may be responsible for the stimulation of LKR/SDH by lysine and its modulation by protein phosphorylation and dephosphorylation, apparently of Ser-458 (1, 7, 10).

The central role of the linker region in the functional interaction between the LKR and SDH domains and the inability of the LKR and SDH domains to functionally interact when present on different polypeptides may also be relevant to the regulation of lysine catabolism in animal and plant cells.

First, amino acid sequence comparison of LKR/SDH polypeptides from various plants and animals show that the animal LKR/SDH lacks the linker region and that the LKR and SDH enzymes are directly linked to each other (1, 2, 18). This suggests that the linker region has been evolved specifically in plants to acquire special regulatory properties of the LKR/SDH enzymes, which are not needed in animals. Because plants are sessile organisms that cannot escape stress, it may be possible that the linker region enables a fine regulation of lysine catabolism under stress conditions.

Second, we have previously shown that the LKR/SDH gene of plants is a composite locus, which under certain conditions can also encode a monofunctional LKR and a monofunctional SDH enzymes as separate polypeptides (2, 6, 19, 20). The monofunctional SDH is encoded by an internal promoter, whereas the monofunctional LKR is encoded by an intron-localized polyadenylation site within the coding region of the LKR/SDH gene. Our present results suggest that these two enzymes cannot functionally interact with each other and, hence, are not subjected to the same biochemical regulation of the bifunctional LKR/SDH locus.

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