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Isolation and Characterization of Dihydrodipicolinate Synthase from Maize[1]

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ABSTRACT

Dihydrodipicolinate synthase (EC 4.2.1.52), the first enzyme specific to lysine biosynthesis in plants, was purified from maize (*Zea mays* L.) cell suspension cultures and leaves. The subunit molecular weight of maize dihydrodipicolinate synthase was estimated to be 38,000 based on SDS-PAGE. The condensation of L-aspartate semialdehyde and pyruvate by highly purified dihydrodipicolinate synthase exhibited kinetics characteristic of a Ping Pong Bi Bi ordered reaction in which pyruvate binds first to the enzyme. Substrate inhibition evident at higher concentrations of L-aspartate semialdehyde was partially alleviated by increasing concentrations of pyruvate. Pyruvate binding exhibited cooperativity with an apparent number of 2 and 1.86 millimolar concentration required for 50% of maximal activity. The K_m for aspartate semialdehyde was estimated to be 0.6 millimolar concentration. Lysine was an allosteric cooperative inhibitor of dihydrodipicolinate synthase with an estimated Hill number of 4 and 23 micromolar concentration required for 50% inhibition. The physical and kinetic data are consistent with a homotetramer model for the native enzyme.

In plants and bacteria, DHPS[2] catalyzes the first step specific to lysine synthesis in the general pathway for biosynthesis of aspartate-derived amino acids including threonine, isoleucine, and methionine (2). DHPS isolated from plants is feedback inhibited by relatively low concentrations of lysine, indicating that subcellular end product concentrations contribute to regulation of DHPS activity and metabolite flux through the lysine-specific branch. This potential regulatory role in lysine synthesis has attracted interest to DHPS as a target for selection of feedback-resistant mutants (17) and for gene isolation (8, 13); consequently, additional characterization of the physical and kinetic properties of DHPS also is of interest.

DHPS has been characterized in extracts of spinach (*Spinacia oleracea*) leaves (23), wheat (*Triticum aestivum* L.) germ (16), and maize (*Zea mays* L.) seedlings (3) and has been highly purified from wheat cell suspension cultures (14, 15) and tobacco (*Nicotiana sylvestris*) leaves (9). Wheat and maize DHPS cDNA clones have been isolated and sequenced (8, 13) as has the corresponding gene from *Escherichia coli* (18). The native form of DHPS is considered to be a homotetramer with mol wt estimates ranging from 108,000 to 164,000. The DNA sequences predict subunit mol wts of 35,737 to 35,854 for wheat (13) and maize (8), respectively, and 31,372 for *E. coli* (18). The homotetramer has four lysine-binding sites, presumably one per subunit, although it is not known whether the dissociated monomers bind lysine or are catalytically active.

This study was initiated to obtain purified maize DHPS for physical characterization and for kinetic and inhibitor studies. We obtained highly purified DHPS from fully expanded maize leaf blades and cell suspension cultures with 23 to 25% recovery. The native enzyme is composed of four subunits with mol wt of approximately 38,000 and is subject to substrate inhibition by high ASA concentrations (>2 mM) and to feedback inhibition by low lysine concentrations (<100 μM). A partial N-terminal amino acid sequence of the purified DHPS was also used to corroborate the nucleotide sequence of a cDNA clone for maize DHPS (8).

MATERIALS AND METHODS

Plant Material

Black Mexican Sweet maize (*Zea mays* L.) cell suspension cultures were maintained on a modified Murashige and Skoog medium by a 1:20 dilution into fresh liquid medium every 7 d (5). Cells were harvested during mid-log phase 5 d after subculture. Plants of inbred line A619 and two homozygous mutant lines, *Ask-LT19* (12) and *Ask2-LT20* (4), were grown in the field nursery at St. Paul, MN, in 1989. The *Ask-LT19* and *Ask2-LT20* alleles are altered forms of the aspartate kinase structural genes (*Ask* and *Ask2*) which result in reduced feedback inhibition of aspartate kinase by lysine (7). The two or three youngest, fully expanded leaves were harvested from plants at the five-leaf stage.

Chemicals

S-(2-Aminoethyl)-L-cysteine, δ-hydroxy lysine, lysine ethyl ester, diaminopimelic acid, ε-amino caproic acid, norleucine, D-lysine, DL-allylglycine, pyruvate, and buffers were obtained

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[2] Abbreviations: DHPS, dihydrodipicolinate synthase; ASA, aspartate semialdehyde; n_{app}, apparent number; $[S]_{0.5}$, substrate concentration required for 50% of maximal activity; $[I]_{0.5}$, inhibitor concentration required for 50% inhibition of maximal activity.

from Sigma Chemical Co. *threo*hydroxy lysine was from Fluka Chemical Co. and *o*-aminobenzaldehyde was obtained from Aldrich Chemical Co. DL-ASA was synthesized by ozonolysis of DL-allylglycine and purified according to the method of Black and Wright (1). DL-ASA was eluted from a cation exchange resin (AG50W-X8, Bio-Rad) with 4 N HCl and stored at −70°C until use. The L-ASA concentration (140 mM) in the stock preparation was determined in a spectrophotometric assay using a crude extract of maize homoserine dehydrogenase (24).

DHPS Purification from Cell Suspension Cultures

All extraction and column buffers contained 0.01% (w/v) sodium azide. Freshly harvested cells were frozen and ground in liquid nitrogen using a Waring blender. Frozen powder was either stored at −70°C or transferred to 1.5 to 2.0 volumes of extraction buffer (150 mM potassium phosphate [pH 7.0], 10 mM pyruvate, 2% [w/v] polyvinylpolypyrrolidone, 0.1% [v/v] β-mercaptoethanol) and allowed to thaw. The material was homogenized using a Polytron at high speed for 2 min. Cell debris was pelleted by centrifugation at 17,000*g* for 15 min and the supernatant loaded onto a G-25 column (5 × 50 cm) equilibrated with 5 mM potassium phosphate (pH 7.0) and 50 mM pyruvate. The active fractions were collected and heated to 60°C for 5 min and quickly cooled. Denatured proteins were removed by centrifugation at 17,000*g* for 15 min and the supernatant loaded onto a hydroxylapatite column (2.5 × 18 cm) equilibrated with buffer A (5 mM potassium phosphate [pH 7.0], 10 mM pyruvate). The column was washed with buffer A, and DHPS was eluted in one step with 100 mL of buffer A containing 150 mM potassium phosphate (pH 7.0). The active fractions were applied to a phenyl Sepharose column (2.5 × 18 cm; Pharmacia/LKB) equilibrated with buffer B (20 mM bis-tris propane [pH 7.0], 10 mM pyruvate) containing 1.2 M ammonium sulfate. The column was washed with buffer B (pH 8.0) containing 0.6 M ammonium sulfate until absorbance (280 nm) of the eluate returned to the initial baseline. DHPS was eluted with 100 mL of buffer B (pH 8.0), loaded onto a Sephacryl S-300HR column (2.5 × 100 cm; Pharmacia/LKB) equilibrated, and then eluted with buffer B (pH 8.0). Fractions with activity were loaded onto a Mono Q (Pharmacia/LKB) anion exchange column equilibrated with 20 mM bis-tris propane (pH 8.0), and DHPS activity was eluted with a 30-mL, 0 to 1 M NaCl linear gradient.

DHPS Purification from Leaves

Leaf blades were frozen and homogenized in extraction buffer as above. The 17,000*g* supernatant was decanted through two layers of Miracloth and then brought to 20% (w/v) PEG (mol wt 4000). When the PEG was dissolved, the precipitated material was removed by centrifugation at 17,000*g* for 15 min. Solid ammonium sulfate (0.75 mol/L) was added to the supernatant. When the ammonium sulfate was completely dissolved, the solution was centrifuged at 10,000*g* for 10 min. Ammonium sulfate and PEG formed a two-phase system; the upper phase was primarily PEG and the lower, aqueous phase was enriched for protein. The lower phase was applied to a phenyl Sepharose column equilibrated with buffer B (pH 7.0) containing 1.2 M ammonium sulfate and then washed with buffer B containing 0.6 M ammonium sulfate. DHPS was eluted with 100 mL of buffer A (pH 7.0). Fractions with DHPS activity were loaded onto a hydroxylapatite column equilibrated and washed with buffer A until the original absorbance at 280 nm was obtained. DHPS was eluted with 150 mM potassium phosphate (pH 7.0) and 10 mM pyruvate. Fractions with activity were loaded onto a Mono Q anion exchange column equilibrated with 20 mM bis-tris propane (pH 8.0) and eluted with a 30-mL, 0 to 1 M NaCl linear gradient.

Enzyme Assay

Standard assays (22) were conducted in 1.5-mL centrifuge tubes containing 25 μL of 1 M Tris buffer (pH 8.0), 25 μL of 100 mM pyruvate, 25 μL of 14 mM ASA (140 mM ASA stock in 4.0 N HCl neutralized just before use with 10 volumes of 0.4 N NaOH), 5 to 100 μL of enzyme, and water to bring the volume to 250 μL. The tubes were incubated at 37°C for 30 or 60 min, and the reaction was stopped by the addition of 1 mL of stop buffer (0.22 M citric acid, 0.55 M sodium phosphate [pH 5.0]) containing 0.25 mg/mL *o*-aminobenzaldehyde. The color was allowed to develop for 3 to 6 h at 37°C. Maximal color formation occurred after 3 h at 37°C, and the color remained stable for an additional 10 h. After color formation, the samples were spun at 10,000*g* for 5 min, and the absorbance was read at 520 nm. An extinction coefficient has not been estimated for the colored product formed between *o*-aminobenzaldehyde and dihydrodipicolinate produced by DHPS. Therefore, 1 unit of activity was defined as an absorbance increase of 0.001/min at 520 nm. The SE of replicate assays was routinely <1% of the mean. Protein concentrations were determined by a dye-binding assay (Bio-Rad) using BSA as a protein standard according to the manufacturer's directions.

Gel Electrophoresis

For activity and native protein analysis, samples were run on 8 to 25% gradient Phast gels (Pharmacia/LKB) using native buffer strips according to the manufacturer's instructions. Samples were brought to 50 mM pyruvate before loading. Gels were stained for activity by placing filter paper soaked with incubation buffer (100 mM Tris [pH 8.5], 100 mM pyruvate, 1.4 mM ASA, and 0.5 mg/mL *o*-aminobenzaldehyde) on the gel and incubating for 60 min at 37°C. Stop buffer (0.22 M citric acid, 0.55 M sodium phosphate [pH 5.0]) was added to the filter paper while attached to the gel and placed at 37°C for 30 min for color development. The native enzyme sample also was electrophoresed on an isoelectric focusing Phast gel (pH 5–8) according to the manufacturer's instructions. The gel strip was then run in the second dimension on a denaturing 8 to 25% SDS-PAGE Phast gel. Size markers for native and SDS gels were from Pharmacia/LKB and proteins were visualized by silver staining (11).

Table I. *Purification of DHPS from Maize Cell Suspension Cultures and Leaves*

Purification Step	Total Protein	Total Activity	Specific Activity	Fold Purification	Recovery
	mg	*units*[a]			%
Suspension culture cells (100 g fr wt)					
G-25 desalted extract	6,522	814	0.12	1	100
60°C, 5 min supernatant	397	ND[b]	ND	ND	ND
Hydroxylapatite	9	1,230	137	1,098	151
Phenyl Sepharose	2.5	587	235	1,883	72
Gel filtration	0.15	586	3,907	31,300	72
Anion exchange	~.01	202	ND	ND	25
Leaves (81 g fr wt)					
G-25 desalted extract	8,229	860	0.10	1	
PEG supernatant	3,558	3,133	0.88	8.4	100
PEG/ammonium sulfate lower phase	355	3,167	8.9	85	100
Phenyl Sepharose	79	1,485	18.8	180	47
Hydroxylapatite	29	1,269	43.8	419	40
Anion exchange	1.7	720	424	4,057	23

[a] 1 activity unit = 0.001 *A*/min at 520 nm. [b] Not determined.

RESULTS

Purification of DHPS from Cell Suspension Cultures

Representative data from one of three purification experiments are presented in Table I. DHPS activity was not detectable in the initial crude extract probably because of the presence of low mol wt inhibitors subsequently removed by passage through a desalting column. The stability of maize DHPS to a 5-min, 60°C treatment in the presence of 50 mM pyruvate provided a simple, effective means to remove >90% of the protein from the desalted extract. Although not assayed in this experiment (Table 1), recovery of DHPS activity was routinely 90 to 110% after the heat treatment (DE Myers, BG Gengenbach, unpublished data). Another reduction (>95%) in protein concentration and extract volume was obtained by elution from a hydroxylapatite column; after these two steps, DHPS activity exceeded the original activity detected in the desalted homogenate, and the apparent purification was at least 1000-fold. Subsequent chromatography on hydrophobic interaction, gel filtration, and anion exchange columns resulted in approximately 25% recovery of highly purified DHPS. It is likely that the DHPS activity recovered in desalted extracts underestimated the actual amount of DHPS protein present. Recovery percentages exceeding 100% in subsequent steps indicated that the extract may still have contained proteins or high mol wt compounds that interfered with the assay or inhibited activity. Thus, we expect that the calculated 30,000-fold purification value obtained here is an overestimate.

The protease inhibitor PMSF was found to inhibit DHPS activity and was not used in the isolation. The enzyme purified from the anion exchange step retained 80% of its activity when stored for 3 weeks at 4°C in 20 mM bis-tris propane (pH 8.0), 10 mM pyruvate, 300 mM NaCl (from the elution gradient), and 0.01% (w/v) sodium azide. The presence of 10 mM pyruvate throughout the isolation except during anion exchange chromatography may have contributed to the stability and yield of DHPS. Before using the purified DHPS for kinetic analysis, the enzyme was desalted on a G-25 column equilibrated with 50 mM Tris (pH 8.0) to remove salt and pyruvate.

Purification of DHPS from Maize Leaves

We were interested in developing a purification procedure (Table I) for DHPS from maize leaves so that a broad array of plants and genotypes could be compared. The desalted crude extract had approximately the same activity as that from the cell suspension cultures. In attempts to remove pigments from the extract, we found that 20% PEG 4000 precipitated most of the chlorophyll and other pigmented proteins but not DHPS. This step resulted in an apparent three- to fourfold increase in total activity, possibly because of the removal of additional inhibitors or pigments interfering with the colorimetric assay. The PEG was then removed by phase partitioning with 0.75 M ammonium sulfate which also removed 90% of the protein without loss of DHPS activity. The purification of DHPS from leaves was >4,000-fold compared with the desalted homogenate, and the yield was 23% compared to the PEG supernatant activity. Gel filtration was not used for the leaf preparations because of lower specific activities and larger volumes, but this step was effective in the purification of cell suspension culture DHPS and likely would be a good final step for the leaf DHPS procedure.

DHPS Molecular Weight and Subunit Composition

DHPS purified from cell suspension cultures through the anion exchange step (Table I) yielded one protein band as detected by silver staining after native gradient PAGE (Fig. 1, lane 1). This band corresponded with the position of DHPS activity determined by incubating and staining adjacent lanes for DHPS activity (Fig. 1, lane 2). The apparent mol wt of

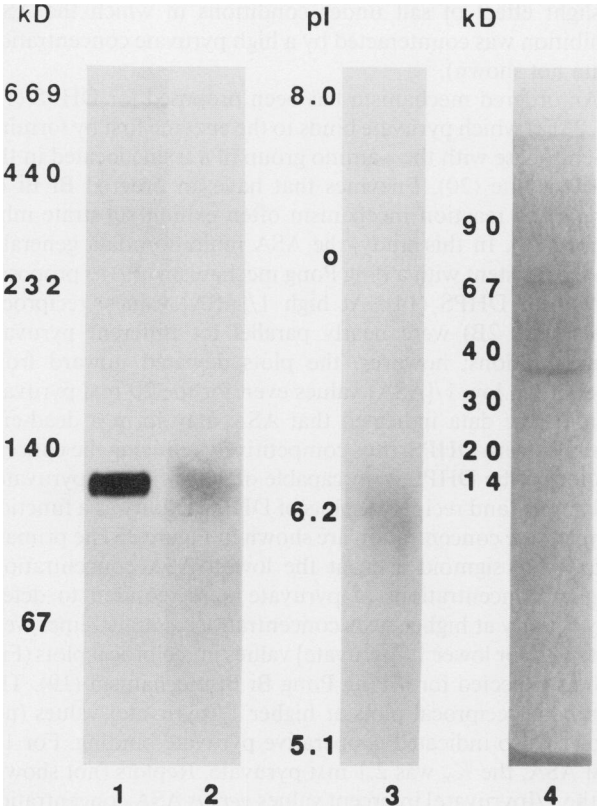

Figure 1. PAGE and isoelectric focusing analysis of purified DHPS from maize cell suspension cultures. DHPS from the anion exchange step was electrophoresed on a native 8 to 25% gradient PAGE Phast gel and stained for total protein (lane 1) or incubated in the DHPS reaction buffer and stained for activity (lane 2). The same DHPS preparation was run on an isoelectric focusing Phast gel (pH 5–8) (lane 3) followed by second dimension electrophoresis on an 8 to 25% gradient SDS-PAGE Phast gel (lane 4). Proteins in lanes 1, 3, and 4 were visualized by silver staining (11). Mol wt standard sizes and isoelectric point values are indicated; 0 = sample origin.

the native enzyme was estimated to be approximately 130,000 based on native PAGE. Isoelectric focusing gels also did not resolve more than one protein species (Fig. 1, lane 3); the protein band had an estimated isoelectric point of 6.2. When run in the second dimension on 8 to 25% SDS-PAGE, two different mol wt bands were visible (Fig. 1, lane 4). One polypeptide band had an apparent mol wt of 37,000 to 38,000 as expected for DHPS and the other band was approximately 72,000. Similar results were obtained when gel slices corresponding to the area of activity were cut out of the native PAGE gel and run in the second dimension (not shown). Two polypeptides could be attributed to co-purification of an unrelated protein that was not resolved from DHPS on native PAGE and isoelectric focusing gels. If so, the subunit size of approximately 72,000 mol wt suggests a dimeric structure for this unrelated native protein. The alternative explanation is that the 72,000 mol wt polypeptide is an authentic component of DHPS, which indicates that the native enzyme is a heteromer possibly with an $\alpha_1\beta_1$- or $\alpha_2\beta_1$-subunit composition in which α and β are the 38,000 and 72,000 mol wt polypeptides, respectively.

Because the 38,000 mol wt polypeptide was similar in size to DHPS monomers obtained from bacteria and other plants, it also was analyzed by N-terminal amino acid sequencing (8). Purified DHPS was run on preparative SDS-PAGE and electroblotted onto a Immobilon P (Millipore) membrane, and the 38,000 mol wt band was cut out and microsequenced by the University of Minnesota Microchemical Facility. The first eight N-terminal amino acids were determined to be Ala-Ile-Thr-Leu-Asp-Lys-Tyr-Leu (8). This amino acid sequence corresponded exactly with the predicted amino acid sequence derived from the nucleic acid sequence of a maize cDNA clone we had identified by complementation of an *E. coli* DHPS$^-$ auxotroph (8). The *E. coli* cells transformed with the maize cDNA expressed DHPS activity with lysine feedback inhibition similar to that of the enzyme purified from maize, indicating that both catalytic and regulatory properties were conferred by the 38,000 mol wt polypeptide. The mol wt of the mature DHPS polypeptide (minus a transit sequence for import into the plastid) was 35,854 based on the cDNA sequence. According to a homotetramer model for maize DHPS, these data would predict a native mol wt of 142,416.

Maize DHPS Kinetic Characteristics

Previous studies of the kinetics of plant DHPS have indicated that ASA concentrations exceeding 3 to 7 mM inhibit activity (3, 14, 16, 23). ASA inhibition has been attributed either to the increased ionic strength and high NaCl or KCl concentrations resulting from neutralization of ASA stocks (stored in concentrated HCl) before the assays (3, 14) or to substrate inhibition in studies in which the ASA stock was more concentrated, requiring less NaOH for neutralization and generating lower salt concentrations in the assay (16, 23).

For assays containing 2.5 to 20 mM pyruvate, the activity of highly purified maize DHPS was lower at 2.8 mM ASA than at 1.4 mM ASA (Fig. 2A). The relative inhibition was much less for 20 mM (9%) than for 2.5 mM pyruvate (79%), suggesting that increasing pyruvate concentration was competitive with respect to the decline in activity at high ASA concentrations. At 2.5 mM pyruvate, activity was maximal at 0.7 mM ASA and declined to 15% of the maximum at 2.8 mM ASA. To determine whether the reduced activity at 2.8 mM ASA was the result of substrate inhibition by ASA or a higher salt concentration, we added NaCl to give the same concentration (80 mM) in assays at 0.7 and 1.4 mM ASA as at 2.8 mM ASA (Table II). The added NaCl did not reduce DHPS activity at 2.5 and 5 mM pyruvate and could not account for the significant inhibition obtained as ASA concentrations increased. DHPS activity also was not affected by 1 mM L-aspartate which could have been present in the ASA as a result of excessive oxidation during ozonolysis of allylglycine. These results indicated that the reduction in DHPS activity observed at high ASA concentrations was due to inhibition by the substrate alone. Stahly (21) showed that only L-ASA was a substrate for *Bacillus licheniformis* DHPS but both L-ASA and D-ASA were equally inhibitory. Thus, the actual inhibitory concentrations of ASA should be considered to be twice those given here because we used a DL-ASA mixture. In assays containing 20 mM pyruvate and 1.4 mM ASA, 400 mM NaCl reduced DHPS activity 17%, indicating

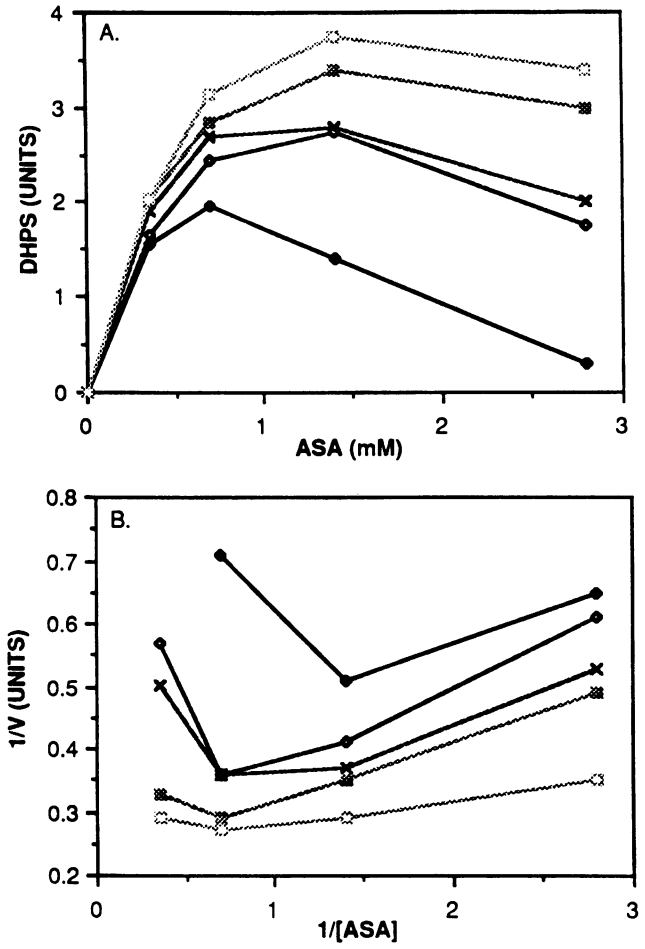

Figure 2. Maize DHPS activity *versus* ASA concentration. Primary (A) and reciprocal (B) plots for assays containing 0.35, 0.7, 1.4, or 2.8 mM ASA and fixed pyruvate concentrations of 2.5 (●), 4.0 (○), 5.0 (x), 10.0 (■), or 20.0 mM (□).

a slight effect of salt under conditions in which the ASA inhibition was counteracted by a high pyruvate concentration (data not shown).

An ordered mechanism has been proposed for DHPS (14, 21, 23) in which pyruvate binds to the enzyme first by forming a Schiff base with the ϵ-amino group of a lysine located in the catalytic site (20). Enzymes that have an ordered Bi Bi or Ping Pong reaction mechanism often exhibit substrate inhibition (19). In this study, the ASA inhibition data generally were consistent with a Ping Pong mechanism (19) as proposed for wheat DHPS (14). At high 1/[ASA] values, reciprocal plots (Fig. 2B) were nearly parallel for different pyruvate concentrations; however, the plots deviated upward from linearity at low 1/[ASA] values even for the 20 mM pyruvate plot. These data indicated that ASA may form a dead-end complex with DHPS, thus competitively reducing the concentration of the DHPS form capable of activation by pyruvate.

Primary and reciprocal plots of DHPS activity as a function of pyruvate concentration are shown in Figure 3. The primary plots were sigmoid even at the lowest ASA concentration. Higher concentrations of pyruvate were required to detect any activity at higher ASA concentrations. Parallel lines were obtained for lower 1/[pyruvate] values in reciprocal plots (Fig. 3B) as expected for a Ping Pong Bi Bi mechanism (19). The parabolic reciprocal plots at higher 1/[pyruvate] values (not shown) also indicated cooperative pyruvate binding. For 1.4 mM ASA, the K_m was 2.1 mM pyruvate. Replots (not shown) of the 1/[pyruvate] intercept values *versus* ASA concentration provided a K_m estimate of 0.6 mM ASA. The primary data for 0.7 mM ASA also were used to estimate the n_{app} of cooperative binding sites for pyruvate by testing $1/V$ *versus* 1/[pyruvate]^n plots for best fit to linearity, where $n = 1, 2, 3$, or 4 (19). The second order equation gave the best fit to linearity (not shown), indicating that $n_{app} \geq 2$ and that there are at least two pyruvate-binding sites on maize DHPS.

Maize DHPS Feedback Inhibition

The effect of increasing lysine concentrations at a fixed pyruvate concentration (10 mM) and three ASA concentrations is shown in Figure 4. The reciprocal plots intersect to the right of the $1/V$ axis and above the 1/[ASA] axis which is

Table II. *NaCl Effect on Activity of Purified Maize DHPS*

NaCl was added to a final concentration of 80 mM for comparison with standard assay conditions.

Pyruvate	ASA	NaCl in Control	DHPS	
			Control	80 mM NaCl
mM	*mM*	*mM*	*units*[a]	
2.5	0.7	20	2.4	2.6
	1.4	40	1.3	1.5
	2.8	80	0.07	0.07
5.0	0.7	20	3.6	3.6
	1.4	40	3.1	3.2
	2.8	80	2.2	2.2

[a] 1 unit = 0.001 *A*/min at 520 nm.

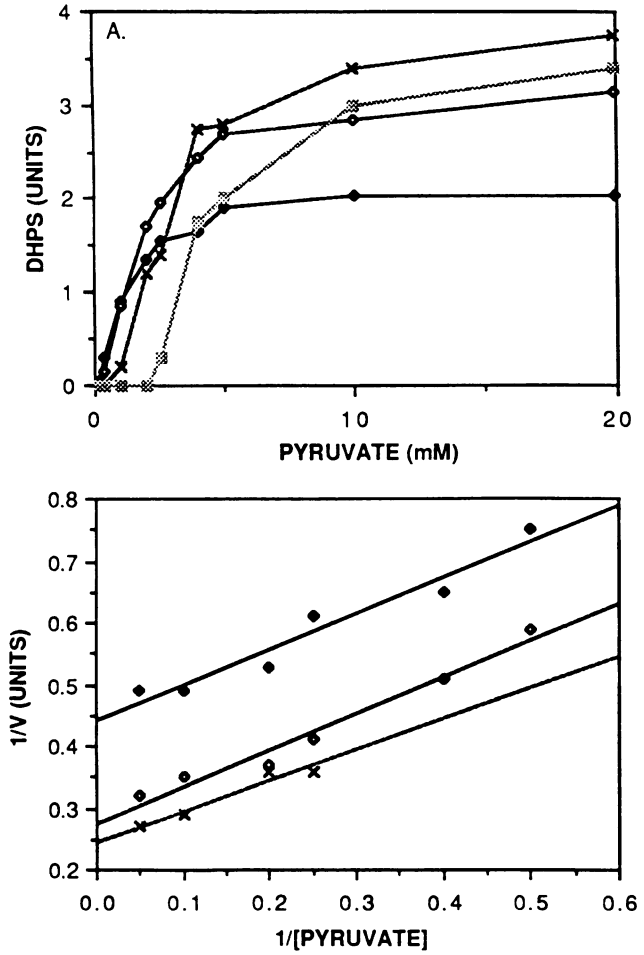

Figure 3. Maize DHPS activity *versus* pyruvate concentration. Primary (A) and reciprocal (B) plots for assays containing 0.2 to 20.0 mM pyruvate and fixed ASA concentrations of 0.35 (●), 0.7 (○), 1.4 (x), or 2.8 mM (■).

characteristic of a mixed-type inhibition (19), indicating that lysine binds to free DHPS and DHPS-ASA complexes with different affinities and affects both the K_m and V_{max} values.

The effect of increasing lysine concentrations at fixed ASA (0.35 mM) and varying pyruvate concentrations is shown in an Eadie-Scatchard plot in Figure 5. The curved lines characteristic of allosteric enzyme inhibition show that increased lysine concentrations shifted the peaks down on the V/S axis and to the left on the V axis. Decreased peak height is characteristic of an inhibitor that increases the $[S]_{0.5}$ value, which is analogous to increasing the K_m value, and indicates that lysine is a competitive inhibitor with respect to pyruvate. The shift of the peaks to the left on the V axis indicates that n_{app} decreases or that lysine decreases the cooperativity of pyruvate binding.

The effects of increasing lysine concentrations were determined for DHPS isolated from cell suspension cultures and leaves of A619, *Ask-LT19*, and *Ask2-LT20* plants. Plots of velocity *versus* [lysine] were sigmoidal and are shown as Hill plots (Fig. 6). The linear regions (10–80 μM lysine) of the plots provided estimates of n_{app} and $[I]_{0.5}$ values. The slope from the A619 plot (Fig. 6A) was 3.3, indicating that there were at least four cooperative lysine-binding sites. The slope of the lines from each of the other three sources was 2.6, again indicating three or more cooperative lysine-binding sites. The $[I]_{0.5}$ values for cell suspension and A619 leaf DHPS both were 23 μM lysine; *Ask-LT19* and *Ask2-LT20* values were 32 and 30 μM lysine, respectively.

Several analogs also inhibited DHPS activity but none as effectively as lysine (Table III). Lysine ethyl ester and *threo*-hydroxy lysine were approximately one-half as effective as lysine, and *S*-(2-aminoethyl)-L-cysteine, δ-hydroxy lysine, and arginine became inhibitory at 1 mM. Lysine analogs missing the α-amino group (ε-amino caproic acid) or the ε-amino group (norleucine) were not inhibitory, indicating the impor-

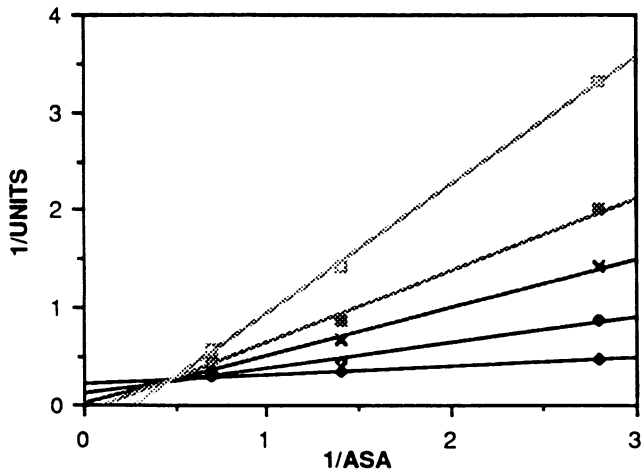

Figure 4. Reciprocal plot of DHPS activity *versus* ASA concentration in the presence of 0 (●), 15 (○), 20 (x), 25 (■), or 30 μM (□) L-lysine. Assays contained 10 mM pyruvate and 0.35, 0.7, or 1.4 mM ASA.

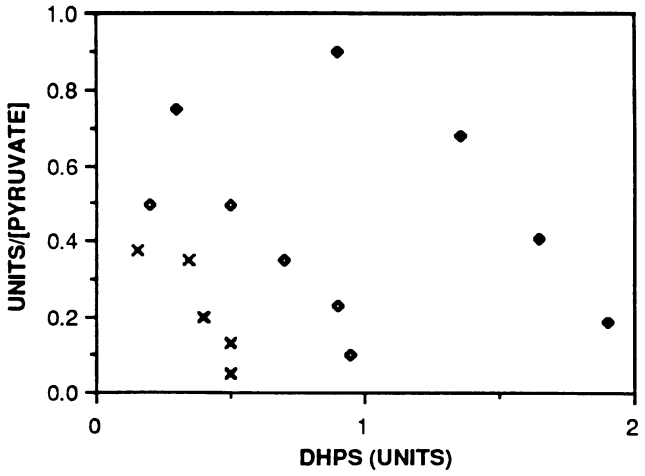

Figure 5. Eadie-Scatchard plots for DHPS activity *versus* pyruvate concentration in the presence of 0 (●), 15 (○), or 20 (x) μM L-lysine. Assays contained 0.35 mM ASA and 0.2, 0.4, 1, 2, 4, and 10 mM pyruvate.

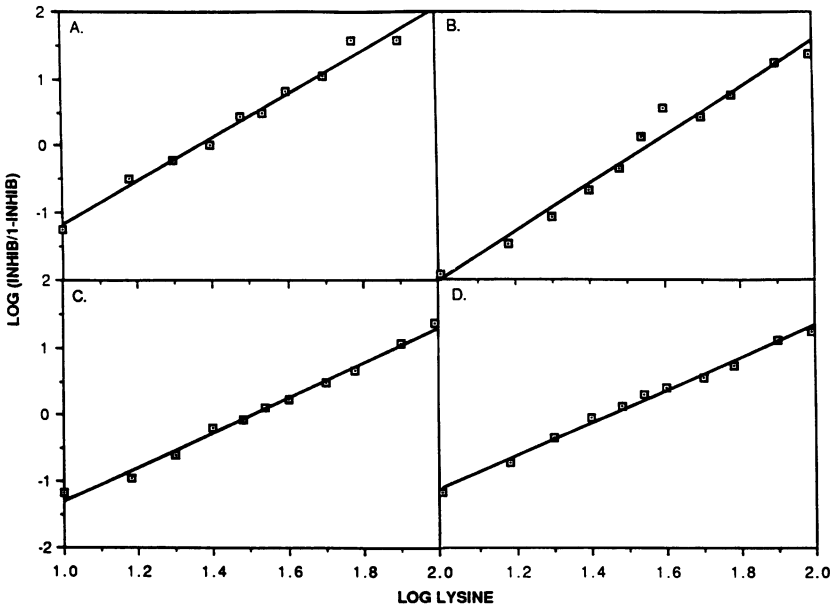

Figure 6. Hill plots for lysine inhibition of DHPS isolated from leaves of A619 (A), *Ask-LT19* (C), and *Ask2-LT20* (D) and from cell suspension cultures (B). Assays contained 10 mM pyruvate and 1.4 mM ASA.

tance of both amino groups. The lysine precursors aspartate and diaminopimelic acid were not inhibitory to maize DHPS.

DISCUSSION

Maize DHPS was reproducibly highly purified from cell suspension cultures and green leaves. The fold purification values (maximum estimates of 4,000 to approximately 30,000) compare favorably with the 5100-fold purification reported for wheat suspension cultures (14) and the 87-fold purification obtained for spinach leaves (23). DHPS recovery was approximately 25% for both maize sources. Although gel filtration was not used for leaves, we expect it could have resulted in an additional five- to 10-fold purification with little loss in yield.

When cell suspension culture DHPS purified through the anion exchange step was run on native PAGE or isoelectric focusing gels, only one protein band was detected by silver staining and it had DHPS activity on native gels (Fig. 1). When the same preparation was separated by SDS-PAGE, two polypeptides of approximately 38,000 and 72,000 mol wt were observed. The same pattern was obtained when the protein band from the native gel was excised and run in the second dimension. We confirmed that the 38,000 mol wt polypeptide was the DHPS monomer by comparing its N-terminal sequence to the predicted amino acid sequence of a maize DHPS cDNA (8). The eight N-terminal amino acids from the 38,000 mol wt polypeptide corresponded exactly to a region of a maize cell suspension culture cDNA clone selected for restoration of DHPS activity to an *E. coli* DHPS$^-$ auxotroph. We propose that the 72,000 mol wt polypeptide was a co-purifying unrelated protein with a native mol wt of approximately 140,000. The fact that a polypeptide with DHPS activity and lysine feedback inhibition was expressed in transformed DHPS$^-$ auxotrophic cells (8) supports this contention. We recently cloned the maize DHPS cDNA into the pGEX-2T (Pharmacia) expression vector and purified the

Table III. *Inhibition of DHPS Activity by Lysine, Lysine Analogs, and Related Amino Acids*
Control reaction contained 10 mM pyruvate and 1.4 mM ASA, and the activity was 2.5 units.

Compound	Concentration (μM)			
	25	50	100	1000
	% inhibition			
L-Lysine	51	87	97	100
Lysine ethyl ester	14	56	89	95
*threo*hydroxy lysine	4	38	83	100
S-(2-aminoethyl)-L-cysteine	0	4	18	93
Arginine	6	4	8	55
δ-Hydroxy lysine	0	0	2	45
D-Lysine	0	0	0	27
Diaminopimelic acid	0	0	0	0
ε-Amino caproic acid	0	0	0	0
Norleucine	0	0	0	0
Aspartic acid	0	0	0	0

glutathione *S*-transferase:DHPS fusion protein by affinity chromatography (AM Tommey, unpublished observation). We found that DHPS activity was retained after thrombin cleavage at the junction of the two polypeptides making up the fusion protein. Antibodies prepared against the purified DHPS polypeptide will be used to investigate the relationships of the 38,000 and 72,000 mol wt species. It is possible that the 72,000 mol wt polypeptide could modify the structure and kinetic properties of DHPS *in vivo*.

Based on estimations from native PAGE, purified maize DHPS appears to be a homotetramer of approximately 130,000 mol wt. This apparent size is less than that (143,416 mol wt) predicted from the cDNA sequence which indicates that, after cleavage of a transit peptide, the mature polypeptide would contain 326 amino acids and have a mol wt of 35,854 (8). Other mol wts reported for DHPS range from 115,000 for spinach (23), 123,000 and 124,000 for wheat and *B. subtilis*, respectively (14, 26), 134,000 for *E. coli* (20) to 164,000 for tobacco (9). DHPS from wheat, *E. coli*, and *B. subtilis* are reported to be homotetramers (14, 20, 26). The cDNA sequence recently obtained for wheat DHPS indicates mature polypeptide subunit sizes of 35,737 and 35,776 (13) which are nearly identical with that of maize. The maize cDNA sequence also indicates a 54 amino acid transit peptide which is consistent with a plastid location of DHPS as demonstrated for spinach DHPS (22). The pH optimum of 8.5 for maize DHPS (data not shown) is similar to that reported for other organisms (14, 16, 20, 23) and to the plastid stromal pH in the light (25).

Maize DHPS showed substrate inhibition by ASA that decreased as the pyruvate concentration was increased (Figs. 3 and 4), indicating that the reaction proceeds by an ordered binding mechanism (19). Kumpaisal *et al.* (14) proposed a Ping Pong mechanism for wheat DHPS in which pyruvate binds to DHPS first, followed by the release of water before ASA binds to the pyruvate-activated enzyme. The kinetic data obtained for maize DHPS are consistent with a Ping Pong mechanism, but the mechanism is not certain because of the confounding effects of ASA substrate inhibition and cooperative pyruvate binding. As has been reported for DHPS from spinach (23), wheat (14, 16), and *B. licheniformis* (10), maize DHPS exhibited cooperative binding for pyruvate with an $n_{app} \geq 2$. Unlike wheat germ and *B. licheniformis* DHPS, pyruvate cooperativity did not appear to increase with increasing ASA. If it is assumed that maize DHPS is composed of four identical subunits, there may be one pyruvate-binding site per subunit, each showing weak cooperativity. Most of the conformational changes could occur when the first and second pyruvates bind so that the third and fourth pyruvates bind with comparable kinetics. Conversely, there may be only two pyruvate-binding sites per molecule showing strong cooperativity, with each subunit providing part of the pyruvate-binding site.

Cooperative binding also was found for lysine with an $n_{app} \geq 3.3$ for DHPS from A619 leaves which implies a minimum of four lysine-binding sites per homotetrameric molecule, presumably one per subunit. The $[I]_{0.5}$ value of 23 μM lysine for DHPS from cell suspension cultures and A619 leaves was essentially the same as for homozygous *Ask-LT19* and *Ask-LT20* mutants (30–32 μM) that had been selected for altered regulation of amino acid biosynthesis (4, 7, 12). Lysine was a competitive inhibitor with respect to pyruvate and a mixed inhibitor with respect to ASA. Lysine ethyl ester was only slightly less inhibitory than lysine, indicating that the carboxyl group is not of major importance in lysine binding. *threo*hydroxy lysine and *S*-(2-aminoethyl)-L-cysteine, which involve substitution of a hydroxyl for a hydrogen and a sulfur for a carbon, respectively, were highly inhibitory at 100 and 1000 μM. Analogs lacking either the α-amino or ϵ-amino group were not inhibitory, indicating both amino groups are important in lysine binding. The common feature of two amino groups also might have accounted for the inhibition observed with 1 mM arginine.

This study has confirmed that lysine, one of the end products of the aspartate family amino acid biosynthesis pathway, exerts strong allosteric feedback inhibition on highly purified maize DHPS. Lysine also is highly inhibitory to purified maize aspartate kinase (5, 6), the first enzyme in this pathway. Two single-gene, dominant mutants, *Ask-LT19* and *Ask2-LT20* (4, 7), have aspartate kinase with reduced lysine feedback inhibition and have increased accumulation of aspartate family pathway products, especially free threonine in maize kernels. If increased capacity for lysine biosynthesis is to be obtained by modifications in the pathway, the strong control of lysine on DHPS, the first enzyme specific to the lysine biosynthesis branch, also must be circumvented. Mutations conferring reduced lysine feedback inhibition of DHPS, such as reported for tobacco (17), should be tested individually and in combination with the aspartate kinase mutations to determine their effect on the accumulation of lysine and other aspartate family pathway end products in maize. The information concerning the structural and kinetic properties of maize DHPS as well as availability of DHPS cDNA clones should contribute to a more complete description of the physiological and molecular regulation of lysine biosynthesis in maize and other cereals.

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