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Purification and Characterization of Lysine-Sensitive Aspartate Kinase from Maize Cell Cultures[1]

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ABSTRACT

Aspartate kinase is a feedback-regulated enzyme that controls the first step common to the biosynthesis of lysine, threonine, isoleucine, and methionine in plants. Aspartate kinase was purified from Black Mexican Sweet maize (*Zea mays* L.) cell suspension cultures for physical and kinetic characterization studies. Partial purification and elution from an anion exchange column resolved two lysine-sensitive aspartate kinase isoforms. Both isoforms were purified >1,200-fold to a minimum specific activity of 18 units/milligram of protein. Both isoforms were sensitive to the lysine analogues S-2-aminoethyl-L-cysteine, L-lysine ethyl ester, and δ-hydroxylysine. No threonine-sensitive form of aspartate kinase was detected at any stage during the purification. Additional purification steps were combined with preparative gel electrophoresis to obtain apparently homogeneous lysine-sensitive aspartate kinase. Aspartate kinase appeared to be a tetramer with a holoenzyme molecular weight of 254,000 and to be composed of 49,000 and 60,000 subunits. The tetramer appeared to disassociate during native gel electrophoresis to 113,000 dalton species that retained aspartate kinase activity.

The aspartate family amino acid biosynthesis pathway results in the synthesis of lysine, threonine, and methionine in plants and bacteria, and threonine and methionine in fungi (3). These amino acids are important to plants beyond their use in protein synthesis. Threonine is a precursor of isoleucine via the pyrvuate family amino acid biosynthesis pathway. Methionine is a precursor to S-adenosyl methionine, which is the primary donor for one carbon methylation reactions and a precursor for polyamine and ethylene biosynthesis. Lysine, threonine, methionine, and isoleucine are also essential amino acids in the diets of humans and nonruminant animals. Their nutritional importance is further enhanced in most cereal grains such as maize (*Zea mays* L.) because lysine is limiting and threonine, methionine, and isoleucine are in near limiting amounts. An understanding of the regulation of the aspartate family pathway is of interest in basic plant biochemistry as well as for modification of nutritional quality in cereals.

Aspartate kinase (EC 2.7.2.4) is the first enzyme in the aspartate family amino acid biosynthesis pathway and regulates the entry of aspartate into the pathway (3). In plants, AK[3] exists as one or more isozymes which have been resolved by anion exchange chromatography and may be individually feedback-regulated by either threonine or lysine (7, 14, 15, 22, 24). Maize lysine-sensitive AK has been partially characterized (4, 6, 8, 13), and there is one report of a minor threonine-sensitive maize AK (4). Lysine-sensitive AK also may be inhibited synergistically by lysine plus S-adenosyl methionine. The lysine concentration required for 50% inhibition of partially purified maize leaf AK was reduced by half when 100 μM S-adenosyl methionine was included in the assay mixture (23).

Plant AK has not been extensively characterized in purified preparations except for the partial purification and characterization of a lysine-sensitive AK from carrot (*Daucas carota* L.) suspension cultures (21). The objectives of this study were to develop a purification procedure for maize AK and to characterize the enzyme with respect to isozyme composition, feedback regulators, native mol wt, and subunit composition.

MATERIALS AND METHODS

Plant Material

Black Mexican Sweet (BMS) maize (*Zea mays* L.) suspension cultures previously initiated from stem sections were grown on Murashige and Skoog medium as modified by Green (9). Cells (18 mL) were subcultured into 320 mL medium in 1 L flasks and grown for a 7 d culture cycle at 28°C in the dark on an orbital shaker at 150 rpm. Cells were harvested for enzyme extraction during mid-log phase 5 d after subculture.

Aspartate Kinase Activity Hydroxamate Assay

During the purification procedure, AK was assayed by the aspartyl hydroxamate method (19) modified to reduce the reaction volume to 1 mL by proportionately adjusting reagent concentrations. Assays were run at 6 mM ATP, 10 mM $MgCl_2$, and 70 mM aspartate for 80 min at 30°C. Just prior to the assay, two volumes of assay buffer (25 mM Mops [pH 7.1], 75 mM KCl, 20% [v/v] glycerol, 3 mM DTT) were added to

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[3] Abbreviations: AK, aspartate kinase; FPLC, Pharmacia Fast Protein Liquid Chromatography system; BMS, Black Mexican Sweet maize; Bistris propane, bistris(hydroxymethyl)aminomethane propane.

1 volume of a 4.8 M NH_2OH-HCl stock and 1 volume of NaOH (the NaOH stock had previously been titrated so that 1 volume of NaOH neutralized 1 volume of hydroxylamine to pH 7.1). Each assay was conducted in a 5 mL conical centrifuge tube containing 0.5 mL neutralized hydroxylamine, 0.07 mL 1 M aspartate, 0.07 mL MgATP (2 volumes assay buffer were added to 1 volume 0.36 M ATP and 1 volume 0.6 M $MgCl_2$), and 0 to 0.36 mL enzyme preparation and buffer so that final volume was 1 mL. Assays were stopped and color developed by adding 0.25 mL 14% (w/v) TCA immediately followed by 0.5 mL 0.26 M $FeCl_3$ in 0.58 N HCl, vortexing for 15 s, and centrifuging for 5 min at 2000*g*. A corresponding blank was determined for each assay where aspartate was omitted during the enzyme incubation and added to the assay mix immediately prior to stopping the reaction with TCA. Absorbance was measured at 505 nm within 20 min after the assay was stopped. An extinction coefficient of 540 M^{-1} cm^{-1} was used (19). Enzyme activity units were calculated in nktal (nmol/s).

Aspartate Kinase Activity Coupled Assay

For inhibition studies, AK activity was determined spectrophotometrically as the aspartate-dependent formation of ADP by coupling the reaction to the pyruvate kinase-lactate dehydrogenase assay (27). The required units of coupling enzymes were determined based on a 6-s lag period before the reaction approached 99% of the true initial rate (16). Under the AK assay conditions, pyruvate kinase typically had a K_m for ADP of 0.35 mM and lactate dehydrogenase a K_m for pyruvate of 1.0 mM. Prior to each experiment, linearity of the coupled assay velocity *versus* volume of added AK preparation was confirmed.

The coupled assay mixture (500 μL) consisted of 250 μL 2X coupling buffer (25 mM Tris [pH 8.0], 15 mM $MgCl_2$, 300 mM KCL, 25% [v/v] glycerol, 6 mM DTT, 0.426 mM NADH, 1.2 mM phospho*enol*pyruvate, 11.5 IU pyruvate kinase, and 32.5 IU lactate dehydrogenase); 100 μL of AK preparation; 50 μL of an inhibitor (0–10 mM); 50 μL of aspartate (0–10 mM); and 50 μL of ATP (0–5 mM). Coupling enzymes were desalted into 25 mM Tris (pH 8.0), 300 mM KCl, and 25% (v/v) glycerol using PD10 columns (Pharmacia/LKB) just prior to inclusion in the coupling buffer. The coupling buffer container was wrapped in foil and the buffer used within 2 h after adding NADH. Aspartate, ATP, and inhibitors were dissolved in 25 mM Tris (pH 8.0). Reaction progress was measured by following the change in absorbance at 340 nm using temperature controlled cuvettes in a Beckman DU-60 spectrophotometer. Reactions were initiated with ATP and run at 25°C for 5 min. Aspartate-independent background activities were determined for each different ATP concentration and subtracted from the reaction rate for each assay.

Enzyme Purification

All buffers were adjusted to their stated pH at room temperature. All procedures were carried out at 4°C unless stated otherwise.

Cells were vacuum-filtered to remove medium, and fresh weight was recorded. Cells were generally extracted immediately for enzyme purification, although, in some cases, they were frozen in liquid nitrogen and stored at −70°C prior to use without loss of enzyme activity. Up to 150 g BMS cells were suspended in liquid nitrogen and pulverized in a stainless steel Waring blender. After the nitrogen had evaporated, cells were extracted by grinding in a mortar and pestle with sand and 1 mL buffer/gm fresh weight cells (200 mM Mops [pH 7.1), 200 mM KCl, 20% [v/v] glycerol, 1 mM EDTA, and 3 mM DTT). The extract was centrifuged at 10,000*g* for 20 min, filtered through glass wool, and desalted on a 4 × 60 cm Sephadex G-50 (Pharmacia/LKB) column equilibrated with desalting buffer (25 mM Mops [pH 7.1], 25 mM KCl, 20% [v/v] glycerol, 1 mM EDTA, and 3 mM DTT).

The desalted extract was brought to 10% saturation with $(NH_4)_2SO_4$, centrifuged at 10,000*g* for 20 min to remove precipitates, and loaded onto a 2.5 × 20 cm phenyl sepharose (Pharmacia/LKB) column, which was washed overnight with desalting buffer containing 7.5% saturation $(NH_4)_2SO_4$. AK was eluted with desalting buffer in which 50% (v/v) ethylene glycol replaced the glycerol. After elution from phenyl sepharose, AK was precipitated by adding 1.5 volumes of a saturated solution of $(NH_4)_2SO_4$ and centrifuging at 10,000*g* for 30 min. The protein pellet was resuspended in a final volume of about 8 mL in 25 mM Bistris propane (pH 7.1), containing 75 mM KCl, 0.1 mM EDTA, 15% (v/v) ethylene glycol, and 3 mM DTT, and loaded onto a 2.5 × 120 cm Sephacryl S300HR (Pharmacia/LKB) gel filtration column equilibrated in the same buffer. The gel filtration column was eluted at 20 mL/h overnight. Fractions containing AK activity were pooled and diluted with one volume of AE1 buffer (25 mM Bistris propane [pH 7.1], 20% [v/v] glycerol, and 3 mM DTT) for anion exchange chromatography. The pooled AK fractions were loaded onto a Pharmacia FPLC Mono Q HR5/5 anion exchange column. The column was washed with a KCl gradient (0–75 mM) in 5 mL AE1 buffer and then AK activity was eluted with a 75 to 275 mM KCl gradient in 30 mL AE1 buffer.

To further purify AK, fractions containing AK activity from the first anion exchange step were pooled and diluted with two volumes AE2 buffer (25 mM Tris [pH 8.0], 20% [v/v] glycerol, 3 mM DTT). The enzyme was loaded at this higher pH onto the Mono Q HR5/5 column and eluted with a 30-mL KCl gradient (0–500 mM) into 0.5 mL fractions. Fractions containing the highest activity were pooled and diluted with two volumes AE2 buffer containing 100 μM lysine and rechromatographed on the Mono Q column using the same conditions except that 100 μM lysine was added to all buffers. Fractions with the highest AK activity were then desalted on a PD10 column (Pharmacia/LKB) into 5 mM K_2HPO_4, (pH 7.1) 15% (v/v) glycerol, 3 mM DTT, and loaded onto a 0.8 × 5 cm FPLC column (Upchurch Scientific) containing hydroxylapatite (Bio-Rad) equilibrated in the same buffer. AK was eluted with a 20-mL, 5 to 405 mM phosphate gradient in 0.5 mL fractions collected at 6.0 mL/h. Active fractions were desalted into 25 mM Tris (pH 8.0), 15 mM $MgCl_2$, 20% (v/v) glycerol, and 3 mM DTT, and then loaded onto a Red A agarose (Amicon Co.) FPLC column. AK was eluted with a linear 30-mL, 0 to 500 mM KCl gradient in 0.5 mL fractions. Active fractions were desalted with a PD10 column equili-

brated with AE2 buffer and chromatographed again on a Mono Q column to concentrate the enzyme. Protein was determined according to Bradford (1) using BSA as a reference.

Polyacrylamide Gel Electrophoresis

Native and SDS-PAGE gels were run on a Pharmacia Phastgel electrophoresis system. AK was applied (1 μL per lane) on 10 to 15% (w/v) acrylamide gradient Phastgels and run using the appropriate buffer strips according to the supplier's instructions (Pharmacia Phastsystem Owner's Manual, 1987).

Phastgels to be stained for activity were equilibrated overnight in 0.112 M Tris, 0.112 M acetate (pH 6.4), 0.1% (v/v) Triton X-100, and 15% (v/v) glycerol at 4°C prior to electrophoresis. After electrophoresis, AK activity was assayed using the calcium phosphate precipitate method (21) by incubating the gels in 25 mM Bistris propane, 20% (v/v) glycerol, 75 mM KCl, 50 mM $MgCl_2$, 100 mM $CaCl_2$, 10 mM ATP, and 70 mM aspartate. Activity was visualized as a white precipitate within 4 h. Control reactions in which aspartate was omitted or 10 mM lysine was included were performed by cutting the gel into thirds and incubating each third in the appropriate assay mix. As little as 2 pkta/μL could be visualized on the Phastgel. This assay was only useful after completing the first anion exchange step which removed an active ATPase that migrated to the same position as AK.

Subunit composition of AK was determined by excising a 2 mm strip from the center of an AK activity band on a native Phastgel. Aspartate kinase was denatured by boiling the gel strip for 2.5 min in 0.112 M Tris, 0.112 M acetate (pH 6.4), 2.5% (w/v) SDS, and 3 mM DTT followed by soaking the strip for 2.5 min in Tris-acetate buffer, 2.5% (w/v) SDS, 3 mM DTT, 0.1% (v/v) bromophenyl blue, and 0.26 M iodoacetamide. The strips were then placed gel side down on 10 to 15% (w/v) acrylamide gradient Phastgels and run according to the instructions for the SDS dimension of the Pharmacia two-dimensional electrophoresis procedure (Pharmacia Phastsystem Technical Note No. 2, 1988). Phastgels were silver stained by a modified method (12). Mol wt were determined by using high mol wt native or SDS gel electrophoresis calibration kits.

Standard 0.75 mm slab native PAGE was run using a discontinuous buffer system stacking at pH 7.3 and resolving at pH 8.5 (10). The gels were prepared from a 30% acrylamide plus 0.8% bisacrylamide stock; the stacking gel was 4% (w/v) acrylamide and the resolving gel was a linear 5 to 10% (w/v) acrylamide gradient. Both the stacking and resolving gel were modified to include 0.1% (v/v) Triton X-100 and 15% (v/v) glycerol to maintain AK activity and the 10% resolving gel also included 15% (w/v) sucrose to stabilize the gradient.

Chemicals

Buffers, salts, EDTA, Triton X-100, and DTT were from Research Organics. Ultrapure glycerol and ammonium sulfate were from Bethesda Research Laboratories. Hydroxylamine and ethylene glycol (fresh 99+% pure) were from Aldrich Chemical Co. Gel filtration standards were from the high mol wt kit from Sigma Diagnostics. Electrophoresis standards were from the high mol wt native and SDS kits from Pharmacia/LKB. All other chemicals were the highest grade available from Sigma Chemical Co.

RESULTS

Purification of Aspartate Kinase Isoforms

Partially purified AK was resolved reproducibly into two peaks of activity eluting from an FPLC MonoQ anion exchange column as shown in Figure 1. The early and late eluting AK activity peaks were individually rechromatographed under identical conditions. Each peak eluted as a single activity peak indicating little interconversion between the two isoforms (Fig. 1). Based on their anion exchange elution patterns, the two isoforms were designated AK Early and AK Late. The two AK isoforms were purified more than 1200-fold to a minimum specific activity of 18 nktal/mg by the procedures given in Table I. Total AK activity recoveries were near 90% based on the desalted enzyme activity and were greater than 30% of the highest total activity observed at any step of the procedure. Aspartate kinase activity in crude extracts was not reliably determined presumably due to inhibitors that were removed by desalting and phenylsepharose column chromatography, which might explain the gain in total AK activity following these steps. Therefore, the purification determinations were minimum estimates. Purified AK Early and AK Late were stable for more than 1 year when stored at −70°C in the AE1 Buffer containing 200 mM KCl and 2 mg/mL BSA and were found to be >90% stable at 23°C for up to 1 week. These preparations also were essentially free of ATPase activity as evidenced by the absence of aspartate-independent activity bands on native gels and as verified with the coupled assay.

AK Activity pH Dependence

AK Early and AK Late isoforms exhibited a pH maximum activity just above pH 8.0 (data not shown) which is consistent with their chloroplast localization (17).

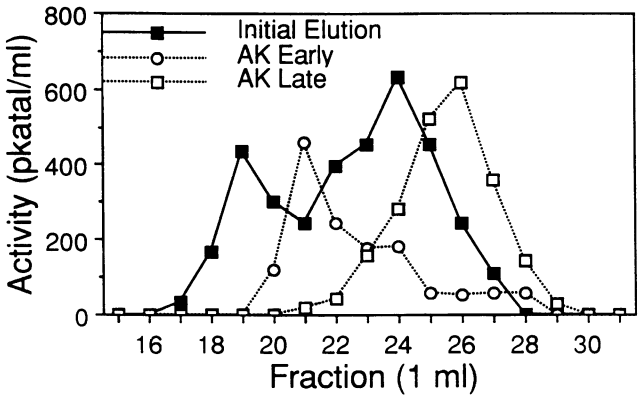

Figure 1. Elution of BMS maize AK activity from an FPLC MonoQ anion exchange column (75–275 mM KCl gradient in 30 mL). Two peaks of activity were initially resolved. Fractions 17 to 21 and 22 to 27, respectively, were combined and individually rechromatographed under identical conditions yielding AK Early and AK Late.

Table I. *Partial Purification of AK from BMS Maize Cell Cultures*

BMS suspension culture cells (80 g) were harvested 5 d after subculture (mid-log growth stage). Two isoforms were resolved by anion exchange chromatography and were individually rechromatographed to further separate them from each other before final specific activity was determined.

Step	Total Protein	Total Activity	Specific Activity	Purification	Percent Recovery
	mg	*units*	*units/mg*	*-fold*	
Crude	1300				
G-50	1500	21	0.014	1	100
Phenyl sepharose	223	54	0.240	17	257
60% $(NH_4)_2SO_4$ ppt	116	61	0.528	37	290
Gel filtration	15	45	3.06	216	214
Anion exchange					
AK Early	0.36	6	18.0	1273	29
AK Late	0.62	12	18.9	1339	57

Survey of Potential Regulators

AK Early and AK Late isoforms were sensitive to lysine and lysine analogs as presented in Table II. No threonine-sensitive isoform was detected in extracts from BMS suspension cell cultures at any stage of purification. Furthermore, no significant activation by valine or isoleucine was observed for either isoform in contrast to previously published reports for maize seedling AK (4, 6).

Native Mol Wt

The AK isoforms coeluted from a Sephacryl S300 gel filtration column as a single symmetrical peak (data not shown) with a relative native mol wt of 255,000 as shown in Figure 2. Anion exchange purified isoforms were recombined and run on a Superose 6 FPLC column. Both isoforms again eluted as a single peak but in this case with a mol wt of 233,000.

Table II. *Effects of Potential Regulators on Maize AK Activity*

AK isoforms were assayed by coupling to pyruvate kinase and lactate dehydrogenase. Substrate concentrations were constant at 5 mM ATP and 10 mM aspartate ™about5 K_m). Data are averages of duplicate assays.

Potential Regulators (10 mM)	Activity	
	AK Early	AK Late
	%	
L-Lysine	20	20
δ-Hydroxylysine	17	19
L-Lysine ethyl ester	12	13
S-2-aminoethyl-L-cysteine	17	15
L-threonine	109	98
L-methionine	100	95
L-isoleucine	93	100
L-homoserine	94	93
DL-α,ε-diaminopimelic acid	102	98
L-valine	98	100
L-arginine	94	90
L-asparagine	100	101
Aspartate hydroxamate	94	102

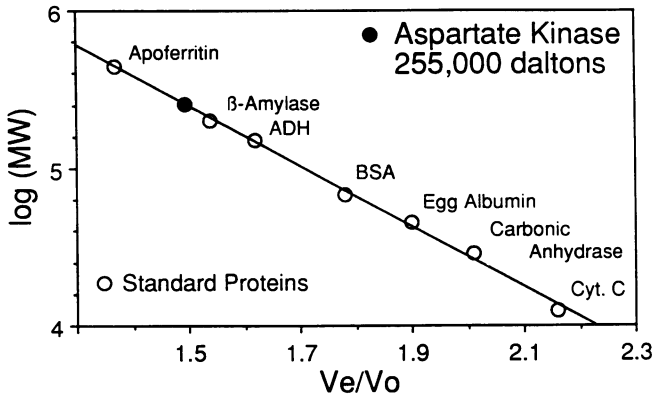

Figure 2. Gel filtration of maize AK. A 3-mL sample of AK was applied to a 2.5 × 120 cm Sephacryl S300 column and eluted at 20 mL/h in 2-mL fractions. Mol wt standards were run before and after the AK sample.

Characterization of AK Late

The AK Late isoform was further purified by the four step sequence of (a) anion exchange chromatography at pH 8.0 instead of 7.1, (b) anion exchange chromatography in the presence of lysine, (c) hydroxylapatite chromatography, and (d) by RedA agarose dye ligand affinity chromatography. Based on estimations of protein from UV absorbance of column elution profiles, each step gave 2- to 5-fold increases in purification that together consistently yielded a final sample containing seven proteins as shown in Figure 3 following native PAGE and silver staining. Recovery of AK following these four final steps was typically 5% and the enzyme was stable at 4°C for >1 week. The protein concentration of the final preparation was too low to be determined without using the whole sample; therefore, specific activity estimates were not made.

The calcium phosphate precipitate gel activity assay (21) was used to identify AK activity after native gradient PAGE of the highly purified AK Late isoform (Fig. 3). The calcium phosphate precipitate was associated with the most rapidly migrating protein in the preparation and was resolved as a single broad band with a mean mol wt less than 140,000. The

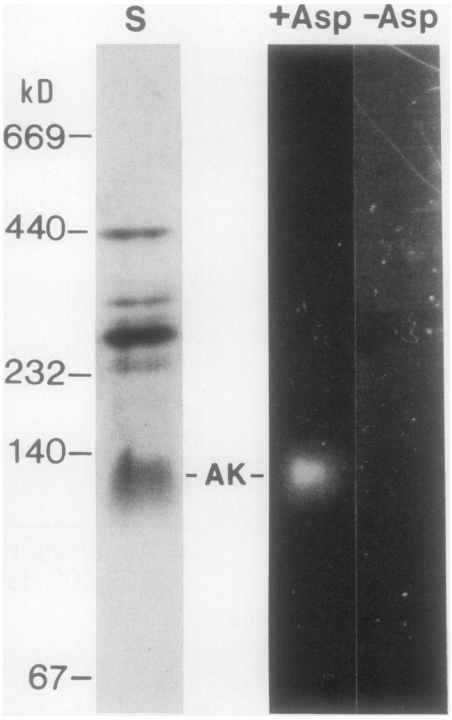

Figure 3. Highly purified AK Late preparation separated by native 10 to 15% acrylamide gradient PAGE. S, Gel silver stained for total protein; +Asp, Gel stained for AK activity visualized by a white calcium phosphate precipitate. The activity was aspartate dependent (+Asp *versus* −Asp) and was lysine-sensitive (data not shown). Mol wt standards were run on each side of the AK Late lanes.

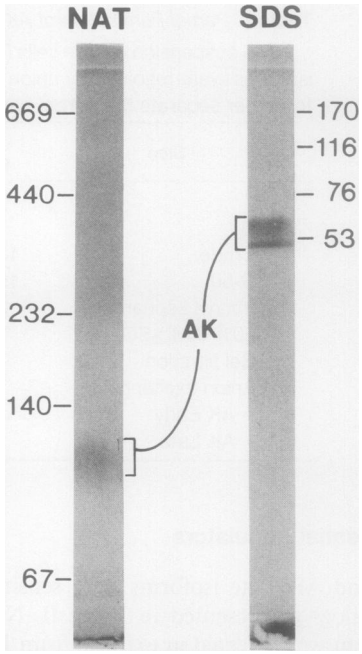

Figure 4. Characterization of homogeneous maize AK Late purified by excising a 2 mm band of AK activity from a native gradient PAGE identical to that shown in Figure 3. Native gradient PAGE (NAT) and SDS-PAGE (SDS) gels were silver stained. Mol wt markers were run on each side of the AK Late lanes as indicated.

calcium phosphate activity stain was aspartate dependent (Fig. 3) and lysine sensitive (data not shown). No aspartate-independent ADP production was observed on these gels even after incubation for 48 h. Partially purified AK also was separated on preparative native gradient slab gels and assayed. The white calcium phosphate activity band correlated perfectly with activity determined in gel slices by the hydroxamate method further indicating that the calcium phosphate gel stain reflected AK. In more than 10 gel analyses of three different highly purified AK Late isoform preparations, the AK activity stain always was resolved as the broad band that corresponded to a broad silver-stained band. A broad band of activity on PAGE also was consistent with the broad elution of AK activity from the Mono Q anion exchange column either for total AK activity or individual isoforms (Fig. 1).

Because glycerol and Triton X-100 retarded migration of the mol wt standards in Figure 3, a more accurate mol wt estimation of AK was obtained by slicing the activity band from the gel and repeating the native gradient PAGE electrophoresis procedure in the absence of glycerol and Triton X-100. In four tests at these conditions, AK Late had an apparent mean mol wt of 113,000 and migrated as a broad band ranging in size from 104,000 to 124,000 as shown in Figure 4. In separate native PAGE experiments, migration of AK Early was similar to that of AK Late (data not shown). This nondenaturing PAGE system separates proteins primarily by size; therefore, the AK isoforms were not separated from each other even though they exhibited different ion exchange elution profiles.

Aspartate kinase subunit composition was determined using homogeneous AK sliced from native gradient Phastgels stained for AK activity. Two putative AK subunits with mol wt of 49,000 and 60,000 were resolved by SDS gradient PAGE (Fig. 4).

DISCUSSION

Two maize isoforms were purified to a minimum specific activity of 18 units/mg, which was comparable with the highest published report for a plant AK (21). The AK isoforms were separated by anion exchange column chromatography. Both maize AK isoforms were lysine sensitive and had similar apparent native mol wt and activity pH maxima. Substrate kinetic constants and lysine inhibition properties were also similar for both isoforms (SB Dotson, unpublished data). The two isoforms may result from some type of microheterogeneity rather than from different genes. Microheterogeneity might include posttranslational modification or *in vitro* modifications such as degradation by proteases or phenolic derivitization during the purification procedure. Alternatively, the AK isoforms may be isozymes but we prefer to consider AK Early and AK Late isoforms rather than isozymes until their gene-enzyme relationships are established.

Aspartate kinase activity in BMS maize suspension cultures was present as two lysine-sensitive AK isoforms. Only lysine-sensitive AK activity was observed in wheat (*Triticum aestivum* L.) seedlings and embryos (2) and suspension cultures (28). The absence of a detectable threonine-sensitive AK in

maize is different from the situation reported for many plant species and tissues where distinct threonine-sensitive isozymes have been identified (3). Relton *et al.* (21) suggested the lysine-sensitive form predominates in rapidly dividing cells to explain the low threonine-sensitive AK activity observed in their carrot cell suspension cultures. Although this is certainly a possibility, significant threonine-sensitive AK activities have been detected in soybean (*Glycine max*) (15), carrot (7, 14), and *Vinca rosea* (24) cell suspension cultures. BMS cell cultures may not have expressed the threonine-sensitive activity which has been detected at low levels in maize seedlings (4), or perhaps the threonine-sensitive form was unstable during extraction and purification. However, our data and those previously reported (6, 8, 11) favor only lysine-sensitive AK isoforms in maize.

Maize AK exhibited a holoenzyme mol wt of 255,000 based on gel filtration chromatography which agreed with the mol wt of 253,000 reported for the lysine-sensitive carrot AK (21). BMS maize AK isoforms migrated during native gradient PAGE as active species with a mol wt ranging from 104,000 to 124,000, suggesting that 255,000 holoenzyme dissociated during native PAGE. Carrot AK was also shown to dissociate into independently migrating active species of 100,000 and 150,000 during sucrose density gradient centrifugation (21). *Escherichia coli* aspartokinase I/homoserine dehydrogenase I also exists in different active aggregation states, a 320,000 tetramer and a 122,000 dimer (25, 26). The 113,000 gel-purified maize AK Late isoform dissociated into subunits of 49,000 and 60,000 on SDS gradient PAGE. Therefore, the native maize AK Late isoform likely was a tetramer which dissociated into active dimers during native gradient PAGE. The genetic relationship of the different size subunits has not yet been determined for maize AK, and there are no other reports of AK subunit composition in plants for comparison. AKII in *Bacillus subtilis* (18) was composed of two subunits of 43,000 and 17,000 and the mol wt deduced from the DNA sequences for AK monomers from *Saccharomyces cerevisiae* (20) and for AKIII in *E. coli* (5) are 45,700 and 48,500, respectively.

The different aggregation states and multiple subunits observed for maize AK suggest a complex quaternary structure for this regulatory enzyme. The different mol wt AK subunits may perform separate catalytic and regulatory functions. Maize AK late might be an $(\alpha\beta)_2$ tetramer wherein each heterodimer consists of a 49,000 and 60,000 subunit. *B. subtilis* AKII is an $\alpha_2\beta_2$ tetramer composed of functionally different monomers (18). Alternatively, the maize AK Late isoform may be composed of functionally equivalent subunits that both have catalytic and regulatory sites such as demonstrated for yeast AK (20) and *E. coli* AKIII (5). Heterogeneity of AK detected in native PAGE separations as indicated by the broad band of AK activity and protein stain (Figs. 3 and 4) may indicate that different monomers can interact to form an array of $\alpha_n\beta_n$ tetrameric conformations. Although the subunit composition of AK early was not determined, two specific, structurally preferred $\alpha_n\beta_n$ complexes of the same subunits detected for AK late may account for the prevalence of the AK early and AK late isoforms that differ in ion exchange elution but have similar holoenzyme mol wt.

The two subunits resolved from AK Late may be products of two genes or result from *in vitro* or *in vivo* modification of a single gene product. At least two genes encode different barley AK isozymes (22); however, barley AK isozymes have not been characterized at the structural level to determine subunit composition. We are pursuing further kinetic and genetic analyses to determine the origin and conformation of these maize AK isoforms.

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