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Kinetic Studies of Lysine-Sensitive Aspartate Kinase Purified from Maize Suspension Cultures[1]

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ABSTRACT

Steady state substrate kinetics and feedback regulation properties were determined for lysine-sensitive aspartate kinase (AK) purified from Black Mexican Sweet maize (*Zea mays* L.) cell suspension cultures. Two AK isoforms (AK Early and AK Late) were separated by two passages through an anion exchange column as the final steps in a procedure giving 1200-fold purification. Kinetic properties were determined for the major AK Late eluting isoform. Assays were conducted at the pH activity maximum (8.0) and with excess Mg^{2+} to favor a two-substrate reaction involving aspartate and complexed MgATP. AK catalyzed a sequential reaction in which MgATP and aspartate both bind to the enzyme complex before the ADP and aspartyl-phosphate products are released. The K_m value calculated for MgATP was 0.43 millimolar and for aspartate was 1.04 millimolar. Cooperativity in substrate binding was not observed and was not induced by lysine. The lysine concentration required for 50% inhibition of AK activity was 7 micromolar. An apparent Hill coefficient of 1.4 indicated a minimum of two lysine-binding sites on the active AK complex. At nonsaturating substrate concentrations, lysine inhibition was characteristic of an S-parabolic, I-parabolic noncompetitive allosteric inhibitor. The parabolic inhibitor replot, Hill coefficients >1, and the lack of substrate cooperativity were consistent with a model for multiple lysine-binding sites per active AK subunit. Similar kinetic properties were observed for the AK Early isoform.

Lysine, threonine, and methionine are synthesized from aspartate via the aspartate family amino acid biosynthesis pathway. AK[3] fits the classical criteria for a regulatory enzyme (4) that could control the flux of carbon through this pathway. AK is the first enzyme in the pathway and catalyzes an ATP-utilizing step. Plant AK isozymes are subject to regulation by feedback inhibition by either lysine or threonine (5). S-Adenosyl methionine acts synergistically with lysine to increase inhibition of some lysine-sensitive AK isozymes including AK from maize (*Zea mays* L.) (26). When compared to the specific activity of other enzymes later in the pathway, AK may be rate limiting (16, 23, 28). Recent information about the specific activity of *Lemna paucicostata* AK indicates that it should not be considered to be rate limiting *in vivo* even when subjected to feedback inhibition by lysine or threonine (15). In contrast, recovery of single gene mutations causing significant accumulation of threonine (12, 18) and reduced feedback inhibition of maize AK by lysine (SB Dotson, DA Somers, DA Frisch, BG Gengenbach, unpublished data) suggests that AK is an important regulatory enzyme of the pathway in maize and emphasizes the need for additional characterization of higher plant AK.

Previously, we purified and characterized AK from BMS maize cell cultures (13). AK activity was resolved during purification by anion exchange chromatography into two distinct isoforms that were designated AK Early and AK Late corresponding to their relative elution from the anion exchange column. These isoforms were purified >1200-fold with 85% recovery and both were lysine sensitive. The yield of AK Late activity was about 2-fold greater than AK Early. Additional purification and gel electrophoresis of homogeneous AK Late indicated a tetrameric holoenzyme of 254,000 D and subunits of 49,000 and 60,000 D (13). During native PAGE both isoforms dissociated into active dimers of 113,000 D. The objectives of this study were to determine the kinetic properties of the major AK Late isoform with respect to the substrates, ATP and aspartate, and the allosteric feedback regulator, lysine. Detailed kinetic analyses of AK were conducted to determine the relationship between enzyme structure and function and to understand the regulation of the aspartate family amino acid biosynthetic pathway in maize.

MATERIALS AND METHODS

Plant Material

Black Mexican Sweet maize (*Zea mays* L.) suspension cultures previously initiated from stem sections (17) were maintained on modified MS medium by a 1:20 dilution into fresh medium every 7 d. Cells were grown in 1 L flasks with 320 mL medium at 28°C in the dark on an orbital shaker at 150 rpm and harvested during midlog phase 5 d after subculture.

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[3] Abbreviations: AK, aspartate kinase (ATP: 4-L-aspartate-4-phosphotransferase [EC 2.7.2.4]); AK I/HDH I, aspartate kinase-homoserine dehydrogenase bifunctional enzyme; BMS, Black Mexican Sweet maize suspension culture; FPLC, Pharmacia fast protein liquid chromatography system; R_s = ratio of substrate or inhibitor concentration giving 0.9 V_1 to concentration giving 0.1 V_1.

Enzyme Purification

Aspartate kinase was extracted and purified according to Dotson *et al.* (13). Crude extracts were desalted to remove inhibitors and phenolic compounds followed by purification using phenyl sepharose hydrophobic interaction chromatography and Sephacryl S300HR gel filtration. Two peaks of AK activity then were resolved during FPLC Mono Q anion exchange column chromatography and designated AK Early and AK Late based on elution order from the column. Fractions containing peak activity of AK Early and AK Late were rechromatographed separately on the Mono Q column to further purify them from each other. Specific activity of AK Early and AK Late were both greater than 18 nktal/mg. At this stage of purification (>1200-fold, 85% recovery), 2 mg/mL BSA was added to the AK preparation, which was stored at −70°C. Preparations of both isoforms were substantially free of ATPase and retained high activity under storage conditions. Total enzyme activity of purified AK Late was on average 2-fold greater than that of AK Early providing more accurate kinetic analyses of AK Late. Accordingly, only data from AK Late are presented.

Aspartate Kinase Assay

For kinetic analysis, AK was assayed spectrophotometrically by the pyruvate kinase-lactate dehydrogenase coupled assay (13). Prior to each set of kinetic determinations, linearity for velocity *versus* volume of added AK preparation was experimentally confirmed. The AK reaction mix was as described previously (13). Dilutions of aspartate (0–10 mM), MgATP (0–5 mM), and L-lysine (0–10 mM) were made in 25 mM Tris (pH 8.0) from 100 mM (pH 8.0) stocks. AK catalyzes the transfer of the gamma phosphate from ATP to the side chain carboxyl of aspartate. To reduce the reaction to a two substrate system, $MgCl_2$ was added to the ATP stock before dilution so that Mg^{2+} was always in 1.67-fold excess of total ATP and the concentration of MgATP was approximately equal to the concentration of ATP (27). Reactions were run at pH 8.0 keeping most of the ATP in the ionized form. This pH also is the pH activity maxima for maize AK (13) indicative of the plastid subcellular localization of AK (5). AK activity was measured in a coupled assay as the decrease in absorbance at 340 nm using a Beckman DU-60 spectrophotometer. Reactions were run for 5 min at 25°C. Activity units were expressed as nktal (nmol s^{-1}).

Data Analyses

Absorbance readings were plotted by a printer-plotter and reaction rates were determined by regression over the linear portion of the decreasing absorbance curve. Rates due to aspartate-independent ATPases were determined for each ATP concentration and subtracted. Because the amount of AK activity purified was limiting even in large scale isolations, single assays were conducted over a broad range of substrate and inhibitor concentrations. When specific data needed to be confirmed, *e.g.* linearity of double reciprocal plots, the analyses were repeated in separate experiments as indicated in the "Results" section. In other experiments, the standard deviation of replicated assays determined under optimal substrate concentration in the absence of inhibitor was ≤2% of the mean activity value. The graphical methods and nomenclature of Cleland (8, 9) were used to analyze and report the data. After graphical analysis (8, 27), models were tested and kinetic constants determined using statistical treatments assisted by computer programs (10). Reciprocal plots of 1/[V] *versus* 1/[S] or 1/[I] were fitted according to a weighted least-squares analysis with the Hypero program and the derived slope and intercept values were used in replots. The program Sequeno was used to obtain kinetic constants and standard errors and to determine the effect of lysine at infinite substrate concentrations. Sigmoil and Eqordo programs were used to test for sigmoidal velocity curves and an equilibrium ordered mechanism, respectively.

Chemicals

Buffers and salts were obtained from Research Organics. Glycerol (99+% pure) was from Bethesda Research Laboratories. ATP, NADH, ADP, phosphoenol pyruvate, and L-lysine were from Sigma Chemical Co. Pyruvate kinase and lactate dehydrogenase were from Sigma or Boehringer-Mannheim Biochemicals.

RESULTS

Substrate Kinetics

The reaction mechanism and steady state substrate kinetics are reported for the AK Late isoform of maize resolved by anion exchange chromatography because the yield of this isoform was twofold greater than the early eluting AK isoform. The double reciprocal plots are shown in Figure 1. The family of lines for velocity *versus* variable aspartate concentrations with the ATP concentration fixed-changing intersected above the ordinate and to the left of the abscissa. Similar plots for variable ATP concentrations also intersected above the ordinate and to the left of the abscissa. These points of intersection indicated a sequential mechanism in which both substrates bind to the enzyme to form a central complex before products are released (7, 27). The reciprocal plots appeared to be linear indicating a lack of cooperativity in binding successive substrate molecules.

Because the initial tests of only five and four substrate concentrations (Fig. 1) might not have been adequate to detect certian sigmoidal velocity curves, we repeated the analyses over 12 different substrate concentrations with the other substrate concentration constant at about fivefold higher than the K_m. Aspartate was varied from 0.1 to 10 mM with nonsaturating 5 mM MgATP, and MgATP was varied from 0.2 to 5 mM with nonsaturating 10 mM aspartate. Results of this experiment (data not shown) also indicated absence of cooperativity because the primary data plots conformed to a right rectangular hyperbolic curve (R_s for substrate concentration = 81), reciprocal plots were linear, and Hill coefficients for substrate binding were not significantly different from 1. Additionally, no evidence of substrate cooperativity was observed for the AK Early isoform. Therefore, the data from Figure 1 were fitted to a sequential model using the statistical

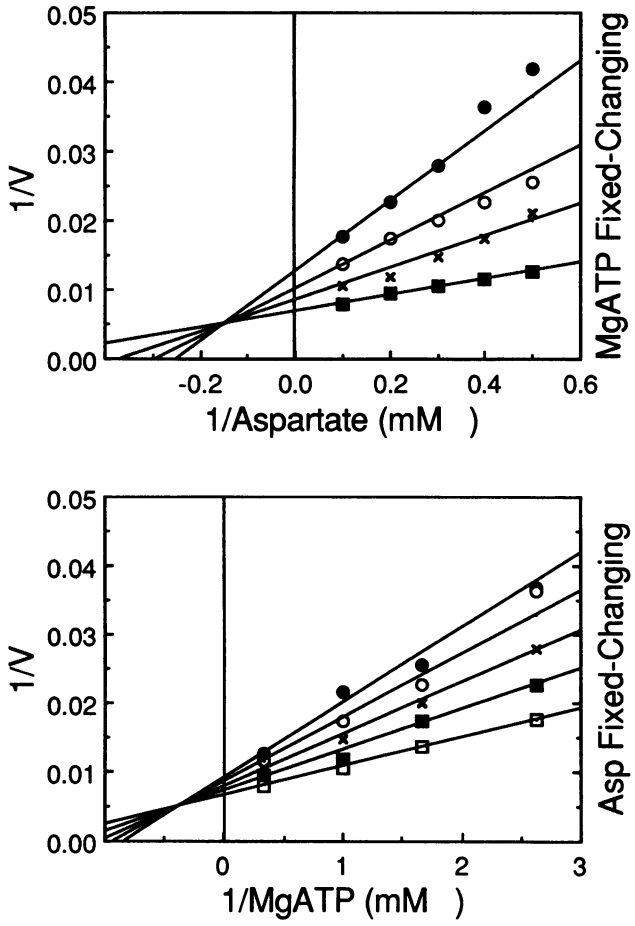

Figure 1. Reciprocal plots of velocity *versus* substrate concentration for maize AK. Values are from single AK assays conducted over a matrix of 0.38 (●), 0.6 (○), 1.0 (×), 3.0 (■) mM MgATP, and 2.0 (●), 2.5 (○), 3.33 (×), 5.0 ■), 10.0 (□) mM aspartate. The lines through each family of data points were calculated using the Hypero statistical computer program (10).

programs of Cleland (10). The fitted kinetic constants as defined by Cleland (7, 9) are presented in Table I.

Table I. *Kinetic Constants Determined from Double Reciprocal Plots shown in Figure 1 for Lysine-Sensitive Maize AK*

After correction for background, the data were fitted to a sequential mechanism model using the Sequeno statistical computer program (10) and the constants with standard errors were derived as described by Cleland (7).

Constant[a]	Calculated Value	SE
	mM	
K_{Asp}	1.04	0.21
K_{ATP}	0.43	0.06
Ki_{Asp}	6.34	1.25
Ki_{ATP}	2.49	0.57
	nktal	
V_1	167.1	6.7

[a] K_{Asp}, Michaelis constant for aspartate with saturating ATP. K_{ATP}, Michaelis constant for ATP with saturating aspartate. Ki_{Asp}, Dissociation constant for AK-aspartate complex. Ki_{ATP}, Dissociation constant for AK-ATP complex. V_1, Maximum velocity with saturating aspartate and ATP.

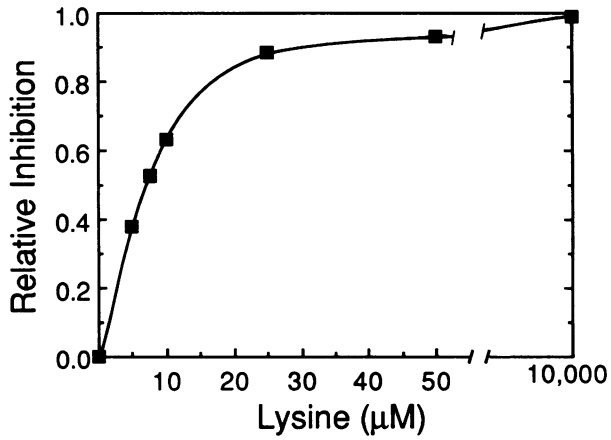

Figure 2. Effect of lysine on activity of maize AK. AK activity was determined from duplicate assays at 10 mM aspartate and 5 mM MgATP. Relative inhibition was determined by calculating $1\text{-}V_i/V_o$ at each lysine concentration, where V_i = rate with lysine and V_o = rate without lysine.

Lysine Inhibition Kinetics

The effect of lysine on velocity was examined over a wide range of lysine concentrations with both substrates at fixed, nonsaturating concentrations (Fig. 2). Lysine completely inhibited AK activity; however, the inhibition curve deviated from a right rectangular hyperbola. The calculated R_s value for lysine concentration was about 20 instead of 81 as expected for Michaelis-Menten inhibition kinetics (27). The data, therefore, were fitted to the Hill equation to determine whether inhibition exhibited cooperativity characteristics. The lysine concentration that inhibited 50% of the AK activity was 7 μM and the n_{app} coefficient for lysine binding was 1.4. The constant n_{app} denotes the slope of the Hill plot over its linear range rather than strictly at $V = 0.5\ V_1$ and indicates at least two lysine-binding sites are present on the active enzyme complex.

To better understand lysine inhibition, the effects of lysine on the velocity-substrate concentration relationships were studied. Double reciprocal plots were constructed for velocity *versus* variable concentrations of one substrate with lysine fixed-changing as shown in Figure 3 for one nonvaried concentration of the second substrate. The variable slopes and abscissa intercepts among the family of lines indicated that lysine was a noncompetitive inhibitor with respect to aspartate and MgATP when the constant substrate concentration was nonsaturating. Each of the primary reciprocal plots in Figure 3 represents one of a set of plots obtained at different constant second substrate concentrations. The same patten of variable slopes and intercepts as shown in Figure 3 was observed in all nine plots (data not shown).

The Hypero program (10) was used to calculate slope and intercept values from the primary reciprocal plots. These slope

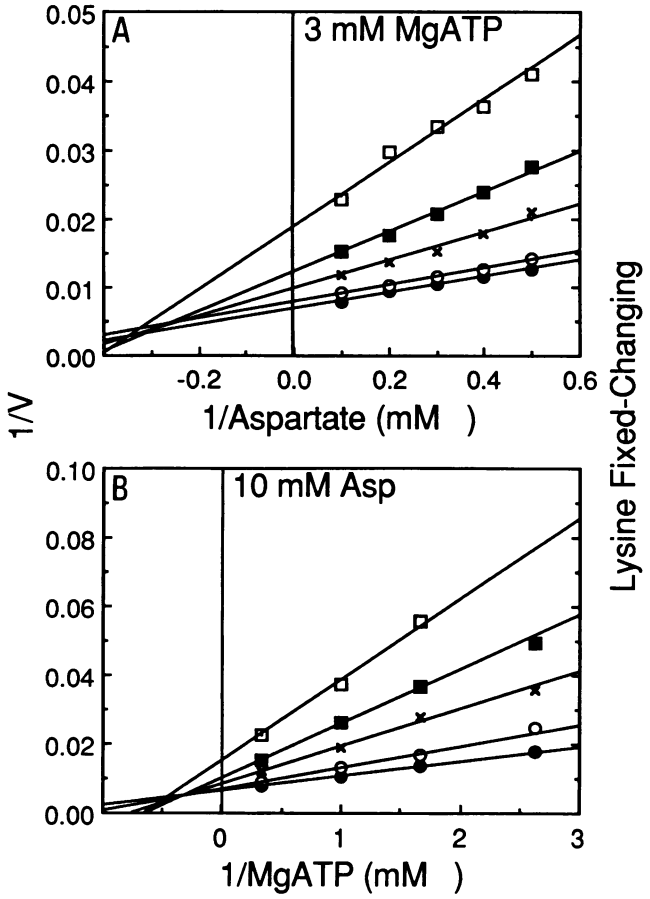

Figure 3. Primary reciprocal plots for velocity *versus* variable substrate concentrations at fixed-changing lysine concentrations for maize AK. A, Aspartate was varied at 2.0, 2.5, 3.33, 5.0, and 10.0 mM with 3 mM ATP; B, ATP was varied at 0.38, 0.60, 1.0, and 3.0 mM with 10 mM aspartate. For each plot lysine was fixed-changing at 0 (●), 2.5 (○), 5.5 (×), 8.5 (■), and 11.5 (□) μM. These plots are representative of the nine plots derived for AK from velocity measurements at five aspartate by four MgATP by five lysine concentrations.

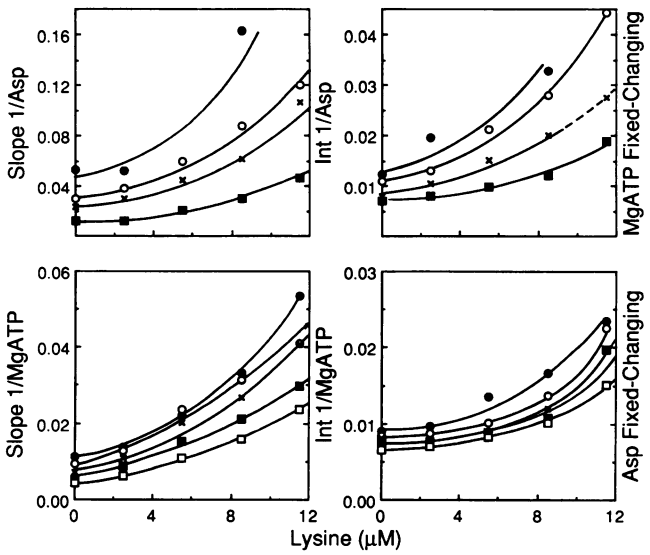

Figure 4. Replots constructed from the slopes (slope 1/Asp and slope 1/MgATP) and intercepts (Int 1/Asp and Int 1/MgATP) of primary reciprocal plots as described in Figure 3. The slope and intercept values were determined using the Hypero statistical computer program (10) and replotted against lysine concentrations of 0, 2.5, 5.5, 8.5, and 11.5 μM. For 1/Asp plots, MgATP was fixed-changing at 0.38 (●), 0.6 (○), 1.0 (×) and 3 mM (■). For 1/MgATP plots, aspartate was fixed-changing at 2 (●), 2.5 (○), 3.33 (×), 5 (■), and 10 mM (□).

Table II. *Effect of Lysine Inhibition on Kinetic Constants Determined from Figure 3 for Lysine-Sensitive Maize AK*

After correction for background, the data were fitted to a sequential mechanism model using the Sequeno statistical computer program (10). Values not followed by the same letter were significantly different according to LSD (0.05).

Lysine	V_1	K_{Asp}	K_{ATP}
μM	nktal	mM	
0.0	167a	1.08a	0.43a
2.5	156ab	0.94a	0.65b
5.5	138b	1.17a	1.06b
8.5	116c	1.51a	1.28b
11.5	75d	1.75a	1.22b

and intercept values were then replotted *versus* lysine concentration for the different nonvaried substrate levels (Fig. 4). The sets of nonlinear replots in Figure 4 indicated that lysine was a parabolic noncompetitive inhibitor (27) with respect to both MgATP and aspartate at each fixed level of the second substrate. Furthermore, Dixon plots (27) of 1/V *versus* lysine concentration were also parabolic concave up as expected for noncompetitive inhibition (data not shown). In the case of parabolic noncompetitive inhibition with multisubstrate enzymes, the primary reciprocal plots (Fig. 3) need not intersect at a common point (27). Inhibition constants (K_i) for a noncompetitive inhibitor of a two substrate enzyme can be determined independently for each substrate from the replots (27). The constants are apparent values ($K_{i\,app}$) because they are a function of the second fixed substrate. However, the parabolic nature of lysine inhibition of maize AK precluded simple extrapolation to the x-axis to determine the $K_{i\,app}$ values. The slope and intercept values of double reciprocal plots are expected to be an exponential function of lysine concentration (27). The exponent of the function is dependent on the number of lysine-binding sites and can be influenced by interaction between the sites. The number of sites and the nature of the interactions between sites have not been established for maize AK.

Assays performed using a matrix of five aspartate and four MgATP concentrations as shown in Figure 1 were repeated for four lysine concentrations in a separate experiment (data not shown). This allowed the data for each lysine concentration to be fitted to a sequential equation using the Sequeno program (10). The extrapolated constants in Table II represent the true V_1 for saturation conditions of both substrates, and the true K_m values under saturating concentrations of the nonvaried substrate. At saturating substrate levels, lysine ap-

peared to act as a noncompetitive inhibitor. Increasing lysine concentration resulted in decreases in V_1 whereas relatively smaller and mostly insignificant changes were observed for the K_m values.

Lack of Lysine-Induced Cooperativity in Substrate Binding

Sigmoidal velocity curves and cooperativity in substrate and ligand binding are common characteristics of allosterically regulated multimeric enzymes. Maize AK was allosterically regulated by lysine (Table II: Figs. 2–4) and physical characterization of highly purified maize AK suggested that it was most likely a tetramer (13). Furthermore, lysine inhibition curves deviated from a right rectangular hyperbola (Fig. 2). Even though there was no evidence suggesting substrate cooperativity in the absence of inhibitor (Fig. 1), we investigated whether AK exhibited lysine-induced substrate cooperativity because some allosteric enzymes show substrate cooperativity only in the presence of a ligand. The linearity of reciprocal plots in Figure 3 are consistent with hyperbolic substrate kinetics over the different lysine concentrations. To test this possibility further, we inspected the relationships of AK velocities over 12 substrate concentrations in the presence of 0, 4, 8, and 12 μM lysine (data not shown). Based on the observations that $R_s = 81$, reciprocal plots were linear and n_{app} estimates for substrate binding = 1, there again was no evidence of cooperative substrate binding induced by lysine. The same kinetic analyses were conducted on the early eluting AK isoform. No substantial differences in kinetic constants or properties were observed for the two isoforms.

DISCUSSION

Aspartate kinase from BMS maize cells was found to catalyze a sequential reaction in which both substrates were bound to a central complex before the release of products. Aspartate and MgATP were bound noncooperatively under the assay conditions used. In the absence of inhibition analyses using dead-end enzyme-ligand complexes (other than with allosteric ligands), random sequential *versus* ordered sequential mechanisms could not be rigorously distinguished from each other. One assay for AK activity utilizes hydroxylamine to convert the product, aspartyl phosphate, to aspartate hydroxamate which then can be assayed colorimetrically (21). Because AK is the active in the presence of hydroxylamine, an enzyme-acyl phosphate intermediate is unlikely. This observation is consistent with a sequential kinetic model in which the γ-phosphate from ATP is transferred directly to aspartate in the enzyme-substrate complex. The aspartate and MgATP kinetic constants determined for maize AK (Table I) were comparable to those reported previously (1, 2, 5, 6, 11, 24) for other plant AK.

Maize AK was previously shown to be sensitive to lysine and lysine analogues (13, 14). In this study, lysine was shown to be an S-parabolic (slope replots), I-parabolic (intercept replots) noncompetitive allosteric feedback regulator of AK at nonsaturating substrate concentrations (Fig. 4). Noncompetitive inhibition with respect to aspartate and sigmoidal inhibitor binding kinetics have been reported for lysine inhibition of barley (*Hordeum vulgare* L.) AK (1) and for threonine inhibition of pea (*Pisum sativum* L.) AK (2). Additional kinetic studies of AK from these species might also reveal a general phenomenon of parabolic noncompetitive lysine inhibition of AK with respect to aspartate and MgATP. For tobacco (*Nicotiana tabacum* L. cv Xanthi) AK (16), lysine was an S-parabolic, I-linear noncompetitive inhibitor with respect to aspartate at saturating ATP (40 mM ATP + 20 mM $MgSO_4$) which is similar to results reported here. Perhaps at lower nonvaried ATP concentrations, the tobacco AK intercept replots might also have been parabolic. In previous studies of maize seedling AK (6, 11), however, lysine was found to be a competitive inhibitor with respect to aspartate, to give nonsigmoidal kinetics and to have K_i values of 13 to 29 μM. In those studies, the observed nonlinear double reciprocal plots for velocity versus aspartate may have reflected a mixture of two isoforms in the enzyme preparation. The two isoforms that we detected were isolated from suspension culture cells; therefore, it is not known if other isoforms are expressed or have greater kinetic differences in other plant tissues. Furthermore, lysine inhibition kinetics were evaluated at nearly saturating (8 and 50 mM) aspartate concentrations in one of the previous studies (6). The non-saturating substrate conditions used in the present study provide a second possible explanation for the different kinetic data and our alternative interpretation relative to cooperativity and competitive lysine inhibition of maize AK.

Lysine is also a noncompetitive inhibitor of *Escherichia coli* AK with respect to aspartate and MgATP (29). Furthermore, plots of reciprocal velocity *versus* lysine (Dixon plots) for *E. coli* AK also were concave up as for maize AK isoforms (data not shown). In the *E. coli* study (29), the nonlinear reciprocal plots were attributed to cooperative binding of lysine. Because maize AK is a tetramer (13), each subunit may have lysine-binding sites which might exhibit cooperativity if binding on one subunit increases the lysine affinity of an interacting subunit. In applying cooperative models to maize AK, lysine would be assumed to bind to a catalytically inactive state according to the Monod, Wyman and Changeux model (22). Under the Koshland, Nemethy, and Filmer model (20), the binding of lysine would cause a transition of AK from an active to an inactive state. However, maize AK appeared unique when the general mathematical models for cooperative enzymes (19) were considered. No simple model for a ligand accounts for decreases in the V_1, small changes in K_m, and a substrate cooperativity index of 81 in the absence and presence of lysine such as was observed for both AK isoforms in this study. If lysine inhibition exhibits cooperativity and affects the affinity of AK for the substrates, then cooperativity in substrate kinetics also would be expected as the higher substrate concentrations begin to overcome the cooperativity for inhibition. According to this argument, the binding of the first substrate to the enzyme complex would disrupt the cooperativity between inhibitors and promote the binding of substrate to another subunit. However, the fact that cooperativity was not observed for substrate kinetics even in the presence of lysine provides evidence against this model.

The data for maize AK best fit a model that assumes multiple lysine-binding sites per active subunit. Parabolic

inhibitor replots are usually diagnostic of multiple inhibitor-binding sites (8). The deviation from hyperbolic lysine inhibition curves (Fig. 2) and a n_{app} value of 1.4 for lysine also are consistent with this model. As clearly demonstrated by Segal (27), an inhibitor with more than one binding site can result in Hill coefficients greater than 1 in the absence of binding cooperativity. Without cooperativity in lysine binding, substrate kinetics are expected to remain hyperbolic in the presence of inhibitor as was observed. The slight concave up curvature of the inhibitor replots (Fig. 4) might suggest that binding of the first lysine has less effect than binding of the next lysine to AK.

Characterization of AK from prokaryotes provides evidence that the enzyme can be regulated by multiple allosteric inhibitor binding sites. *Bacillus polymyxa* AK is subject to multivalent regulation by lysine and threonine (23). Presumably there are distinct allosteric sites for each of these structurally dissimilar regulators. In the case of *E. coli* AK (3), threonine is cooperatively bound by interacting subunits of the AK I/HDH I tetramer, but binding of a second threonine per subunit provides additional kinetic cooperativity.

The two maize AK isoforms initially separated by anion exchange chromatography exhibited similar kinetic properties. The isoform K_m values were not so different as to indicate distinct physiological roles. Both isoforms also were inhibited 50% by about 10 μM lysine, had apparent Hill coefficients >1 and parabolic inhibitor replots. Neither isoform appeared to bind the substrates with strong cooperativity. Lysine-sensitive plant AKs, including maize AK, are synergistically inhibited by S-adenosyl methionine and lysine (26). Given the similarity of the maize AK isoforms based on enzyme structure (13) and kinetic properties, it seems unlikely that the two isoforms would be distinguishable based on interaction with S-adenosyl methionine. However, the synergistic inhibition by S-adenosyl methionine and lysine can now be defined more thoroughly based on our characterization of purified maize AK. Differences in anion exchange elution of the two isoforms may have been due to post-translational modification or even degradation during the purification procedure of a single AK product rather than two distinct isozymes. However, three isozymes have been reported in barley including two that are lysine sensitive (25). The barley AK isozymes, as in maize, were first separated by anion exchange chromatography. Threonine sensitivity provided clear distinction for one isozyme. Furthermore, two genes that encode the two lysine-sensitive AK isozymes were identified in barley (25). Two maize mutants that accumulate high concentrations of threonine in the free amino acid pool are controlled by separate genes (12, 18) and have AK with reduced sensitivity to lysine inhibition (SB Dotson, DA Somers, DA Frisch, BG Gengenbach, unpublished data). The origin of the maize AK isoforms and the structural subunit relationships may be clarified by further analysis of these mutants.

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LITERATURE CITED

1. **Aarnes H** (1977) A lysine-sensitive aspartate kinase and two molecular forms of homoserine dehydrogenase from barley seedlings. Plant Sci Lett **9:** 137–145
2. **Aarnes H, Rognes SE** (1974) Threonine-sensitive aspartate kinase and homoserine dehydrogenase from *Pisum sativum*. Phytochemistry **13:** 2717–2724
3. **Bearer CF, Neet KE** (1978) Threonine inhibition of the aspartokinase-homoserine dehydrogenase I of *Escherichia coli*. A slow transient and cooperativity of inhibition of the aspartokinase activity. Biochemistry **17:** 3523–3530
4. **Beevers H** (1974) Conceptual developments in metabolic control, 1924–1974. Plant Physiol **54:** 437–442
5. **Bryan JK** (1980) Synthesis of the aspartate family and branched-chain amino acids. *In* BJ Miflin, ed, The Biochemistry of Plants, Amino Acids and Derivatives, Vol 5. Academic Press, New York, pp 403–452
6. **Bryan PA, Cawley RD, Brunner CR, Bryan JK** (1970) Isolation and characterization of a lysine-sensitive aspartokinase from a multicellular plant. Biochem Biophys Res Commun **41:** 1211–1217
7. **Cleland WW** (1963) The kinetics of enzyme-catalyzed reactions with two or more substrates or products I. Nomenclature and rate equations. Biochim Biophys Acta **67:** 104–137
8. **Cleland WW** (1963) The kinetics of enzyme-catalyzed reactions with two or more substrates or products III. Prediction of initial velocity and inhibition patterns by inspection. Biochim Biophys Acta **67:** 173–187
9. **Cleland WW** (1970) Steady state kinetics. *In* PD Boyer, ed, The Enzymes, Ed 3, Vol 2. Academic Press, New York, pp 1–65
10. **Cleland WW** (1979) Statistical analysis of enzyme kinetic data. Methods Enzymol **63:** 103–138
11. **Cheshire RM, Miflin BJ** (1975) The control of lysine biosynthesis in maize. Phytochemistry **14:** 695–698
12. **Diedrick TH, Frisch DA, Gengenbach BG** (1990) Tissue culture isolation of a second mutant locus for increased threonine accumulation in maize. Theor Appl Genet **79:** 209–215
13. **Dotson SB, Somers DA, Gengenbach BG** (1989) Purification and characterization of lysine-sensitive aspartate kinase from maize cell cultures. Plant Physiol **91:** 1602–1608
14. **Gengenbach BG, Walter TJ, Green CE, Hibberd KA** (1978) Feedback regulation of lysine, threonine, and methionine biosynthetic enzymes in corn. Crop Sci **18:** 472–476
15. **Giovanelli J, Mudd S, Datko AH** (1989) Aspartate kinase of *Lemna paucicostata* Hegelm. 6746. Plant Physiol **90:** 1577–1583
16. **Gonzales RA, Das PK, Widholm JM** (1984) Characterization of cultured tobacco cell lines resistant to ethionine, a methionine analog. Plant Physiol **74:** 640–644
17. **Green CE** (1977) Prospects for crop improvement in the field of cell culture. Hort Sci **12:** 131–134
18. **Hibberd KA, Green CE** (1982) Inheritance and expression of lysine plus threonine resistance selected in maize tissue culture. Proc Natl Acad Sci USA **79:** 559–563
19. **Kirtley ME, Koshland DE Jr** (1967) Models for cooperative effects in proteins containing subunits. J Biol Chem **242:** 4192–4205
20. **Koshland DE Jr, Nemethy G, Filmer D** (1966) Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry **5:** 365–385
21. **Lipman F, Tuttle LC** (1945) A specific micromethod for the determination of acylphosphates. J Biol Chem **159:** 21–28
22. **Monod J, Wyman J, Changeux JP** (1965) On the nature of allosteric transitions: a plausible model. J Mol Biol **12:** 88–118
23. **Paulus H, Gray E** (1964) Multivalent feedback inhibition of aspartokinase in *Bacillus polymyxa*. J Biol Chem **239:** 4008–4009
24. **Relton JM, Bonner PLR, Wallsgrove RM, Lea PJ** (1988) Phys-

ical and kinetic properties of lysine-sensitive aspartate kinase purified from carrot cell suspension culture. Biochim Biophys Acta **953**: 48–60

25. **Rognes SE, Bright SWJ, Miflin BJ** (1983) Feedback-insensitive aspartate kinase isoenzymes in barley mutants resistant to lysine plus threonine. Planta **157**: 32–38
26. **Rognes SE, Lea PJ, Miflin BJ** (1980) S-adenosylmethionine—a novel regulator of aspartate kinase. Nature **287**: 357–359
27. **Segal I** (1975) Enzyme kinetics. Wiley-Interscience, New York
28. **Shewry PR, Miflin BJ** (1977) Properties and regulation of aspartate kinase from barley seedlings (Hordeum vulgare L.) Plant Physiol **59**: 69–73
29. **Wampler DE, Westhead EW** (1968) Two aspartokinases from *Escherichia coli*. Nature of the inhibition and molecular changes accompanying reversible inactivation. Biochemistry **7**: 1661–1671