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Maize Leaf Adenylate Kinase[1]

PURIFICATION AND PARTIAL CHARACTERIZATION

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ABSTRACT

Adenylate kinase (EC 2.7.4.3) from leaves of maize (*Zea mays*) was purified to homogeneity using $(NH_4)_2SO_4$ fractionation, followed by chromatography on DEAE-cellulose, hydroxyapatite, Sephadex G-75SF, and Green A dye-ligand columns. The purified enzyme had specific activity of about 1,550 micromoles ADP produced per minute per milligram protein, and the ratio of velocities of the reverse (utilization of ATP) to forward (formation of ATP) reaction was about 1.5. The M_r value of adenylate kinase, determined by electrophoresis in dissociating conditions and by gel filtration, was 29,000 and 31,000 respectively, suggesting monomeric nature of the enzyme. Purified preparations were stable for at least 1 month at 0 to 4°C. Magnesium ions were essential for activity of adenylate kinase in both directions of the reaction. Optimal rates in the forward direction were observed at the magnesium to ADP ratio of about 0.6 to 0.8. For the reverse reaction, ATP served as a substrate only when complexed with magnesium, while AMP reacted as a free species. The enzyme preferentially utilized adenine ribonucleotides in both directions of the reaction. The nucleoside triphosphate-binding site of adenylate kinase was fairly nonspecific with regard to nucleotide species. On the other hand, the primary amino group of either adenine and cytosine moieties was essential for effective binding to the nucleoside monophosphate site of the enzyme.

Adenylate kinase (AK)[3] is a key enzyme involved in the interconversion of adenine nucleotides in living tissues. AK catalyzes a freely reversible reaction: 2 ADP ↔ AMP + ATP. The enzyme is an important component of the so called 'energy charge' concept and maintains an equilibrium or near-equilibrium of adenine nucleotides in the cell (2, 23). AK was extensively purified and characterized from a number of animal tissues (see Noda [21] for review), as well as some bacteria (*e.g.* [28]) and yeast (10).

In leaves, AK is involved in regulation of adenine nucleotide levels in the stroma of chloroplasts as well as in the cytosol (3, 7, 8, 15, 20, 23, 27). In C_4 plants activity of AK is at least one order of magnitude higher than in C_3 species (9), and the enzyme is closely coupled to the metabolic pathway of C_4-photosynthesis, removing AMP formed by pyruvate, Pi dikinase in the mesophyll chloroplasts (8, 9). Although the role of AK in regulation of plant metabolism is widely acknowledged (see Pradet and Raymond [23] for review), to our knowledge no report has been published describing an effective purification procedure for leaf AK. Partial purification has been achieved for the enzyme from wheat (3), pea (26), and maize (8) leaves, and a typical purification procedure usually consisted of $(NH_4)_2SO_4$ precipitation and chromatography on the gel filtration and/or ion exchange columns. Our task was to modify these procedures and to purify AK to the extent which would allow general characterization of its physical and enzymic properties (in this paper) as well as more detailed kinetic analysis (LA Kleczkowsik, DD Randall, unpublished data). A preliminary report describing some of the data presented in this paper has been published (13).

MATERIALS AND METHODS

Plant Material. Maize (*Zea mays*) seeds were obtained from local seed supplier. Seedlings were grown in greenhouse as previously described (14).

Reagents. ATP, ADP, CDP, UDP, AMP, GMP, and UMP were from PL-Biochemicals. All other nucleotides were from Sigma. Pyruvate kinase, lactate dehydrogenase (both from rabbit muscle), hexokinase (yeast), and glucose-6-P dehydrogenase (*Leuconostoc mesenteroides*) were from Sigma.

Purification of Adenylate Kinase. All purification procedures were performed at 0 to 4°C. Extraction medium contained 40 mM Tricine (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 1 mM ε-aminocaproic acid, 5 μM leupeptin, and 100 mM 2-ME. The DEAE-cellulose and Green A dye-ligand (Amicon) columns were equilibrated and eluted with buffer A containing 20 mM Tricine (pH 7.8), 2 mM $MgCl_2$, and 28 mM 2-ME. The hydroxyapatite column was equilibrated with 20 mM Tricine (pH 7.8) and 28 mM 2-ME (buffer B). The Sephadex G-75SF column was equilibrated and eluted with buffer C containing 20 mM Mops (pH 7.0), 2 mM $MgCl_2$, and 28 mM 2-ME. All buffers were degassed prior to use.

Adenylate Kinase Assay. AK was assayed both in the direction of ATP and AMP formation as well as in the direction of ADP production. The 1 ml assay mixture for the former reaction contained, unless otherwise indicated, 100 mM Tricine (pH 7.8), 2 mM ADP, 2 mM $MgCl_2$ 0.5 mM NAD^+, 60 mM KCl, 5 mM D-glucose, varying amounts of AK which was previously dialyzed against buffer A, and 5 units each of hexokinase and glucose-6-P dehydrogenase. Reactions were initiated by addition of AK and run at 25°C. Rates in the direction of ADP formation were assayed using a 1 ml reaction mixture containing, unless otherwise indicated, 100 mM Tricine (pH 7.8), 2 mM ATP, 0.5 mM AMP, 2 mM $MgCl_2$, 1 mM PEP, 60 mM KCl, 0.33 mM NADH, varying amounts of AK, and 5 units each of pyruvate kinase and lactate dehydrogenase. Reactions were initiated by addition of AMP and run at 25°C. One unit of AK activity was defined as amount of the enzyme required to reduce 1 μmol NAD/min (forward reaction) or to oxidize 1 μmol NADH/min (reverse

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[3] Abbreviations: AK, adenylate kinase; 2-ME, 2-mercaptoethanol; Mops, 4-morpholine propane sulfonic acid; PEP, phosphoenolpyruvate.

reaction) under conditions of the assay.

Prior to assays, the coupling enzymes were desalted on a small Sephadex G-25 column. Assay concentrations of free AMP and ATP, as well as Mg-AMP and Mg-ATP were calculated using the following stability constants (22): $K_{Mg\text{-}AMP} = 69.7\ M^{-1}$, $K_{Mg\text{-}ATP} = 69{,}700\ M^{-1}$, $K_{Mg\text{-}PEP} = 182\ M^{-1}$.

Analytical Methods. Determination of protein and Chl was done according to Bradford (4) and Arnon (1) procedures, respectively. Slab gel electrophoresis was done as described by Laemmli (16).

RESULTS

Purification of Adenylate Kinase. Leaves of 16-d-old maize seedlings (250 g) were homogenized in a Waring Blendor for 20 s in the presence of 1,000 ml extraction medium and the homogenate squeezed through 4 layers of cheesecloth and 1 layer of Miracloth, and centrifuged at 15,000*g* for 15 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 35% saturation, the suspension equilibrated by stirring for 30 min, and centrifuged at 15,000*g* for 10 min. Resulting supernatant was further adjusted to 70% saturation with solid $(NH_4)_2SO_4$ and then equilibrated for 30 min and centrifuged at 15,000*g* for 10 min. The pellet was dissolved in the extraction medium and concentrated/desalted with buffer A using a Diaflo PM10 filter (Amicon). AK was chromatographed on a DEAE-cellulose column (2.5 × 15 cm), using a 480 ml linear gradient of 0 to 0.5 M NaCl in buffer A. The peak of AK activity eluted at about 0.17 M NaCl. The most active fractions were applied onto a hydroxyapatite column (1.5 × 10 cm) and eluted with a 300 ml linear gradient of 0 to 0.5 M sodium phosphate (pH 7.8) in buffer B. AK eluted at about 0.1 M sodium phosphate. The enzyme was concentrated/desalted with buffer C using a Diaflo PM10 filter and applied (3 ml) onto a Sephadex G-75SF column (2.5 × 68 cm) operating at a flow rate of 15 ml/h. AK eluted after the major protein peak. Following gel filtration, AK was chromatographed on a Green A column (1.0 × 2.5 cm) and eluted with a step gradient of 1 mM ATP in buffer A. Results of the purification procedure are summarized in Table I.

Purity and Molecular Weight. The final enzyme preparation exhibited a single band following SDS-PAGE when silver-stained (Fig. 1). The enzyme was purified almost 300-fold (Table I) at a high yield of about 60%. Since most of the maize AK is believed to occur in mesophyll chloroplasts (8, 19) it seems probable that the purified protein is in fact the chloroplastic isozyme of AK. Specific activity of the homogeneous AK (1550 U/mg protein) is at least 7-fold higher than values previously reported for the partially purified maize enzyme (215 units/mg protein [8]) and similar to the activity found for AK from yeast (1900 units/mg protein [10]). The position of AK on the SDS-polyacrylamide gel (Fig. 1) corresponded to M_r value of 29,000, while chromatography of native AK on a calibrated Sephadex G-75SF column gave the M_r of 31,500 (data not shown). The similar M_r values observed for both SDS-treated and native AK indicate that the maize protein is a monomer of about 30,000.

Stability. Purified AK was stable when kept at 0 to 4°C, losing only 10 to 15% of its activity in 30 d. Dilution of the final preparation with buffer A, resulting in protein concentration of 1 to 2 μg/ml, did not affect the stability of AK. These results are contrary to what was previously observed for partially purified maize AK (8), which was very labile—especially at diluted conditions. However, the enzyme in (8) was isolated in the absence of protease inhibitors and thus it seems possible that the partially purified preparations contained some proteolytic activities responsible for poor stability of AK. The use of degassed buffers as well as high thiol content utilized in the present studies (see "Materials and Methods") might also be crucial for stability of the enzyme, since AK from several tissues was reportedly very sensitive to inhibition by oxidizing agents (21, 28, 29).

Magnesium Requirement. Activities of both forward and reverse reactions of AK were strictly dependent upon concentration of magnesium ions (Fig. 2, A and B). In the direction of ATP and AMP formation, the enzyme showed some activity without Mg (Fig. 2A); however, this low activity was fully inhibited when the assay was supplemented with 0.1 mM EDTA. This probably indicates that the enzyme contains some bound Mg, which can be removed by the chelator. Low rates in absence of Mg were previously observed for crude AK from leaves of several species (18, 20). In the forward direction, the optimal ratio of [Mg]/[ADP] was about 0.6 to 0.8 (Fig. 2A) for two concentrations of ADP, while in the reverse direction the optimal activity was observed at 3 to 5 mM $MgCl_2$ when total ATP and total AMP were kept at 2.0 and 0.5 mM, respectively (Fig. 2B). For the latter reaction, it was calculated (see "Materials and Methods") that at 5 mM $MgCl_2$ more than 99% of ATP was in the form of Mg-ATP, strongly suggesting that ATP reacts only as a Mg-chelated species. On the other hand, similar calculations done for the assay containing 1 mM $MgCl_2$ revealed that, at these conditions, free AMP constituted more than 99.9% of total AMP, and thus practically eliminating possibility of Mg-AMP serving as a substrate. This is contrary to the conclusion expressed by Hatch (8) that partially purified maize AK utilizes Mg-AMP rather than free AMP as a substrate in the reverse direction. He observed that at 1 mM $MgCl_2$, 2 mM total ATP and up to 2 mM total AMP (pH 7.8) the activity of AK was reduced and the apparent Michaelis constant for AMP increased several-fold over that found at higher concentration of $MgCl_2$ "suggesting that AMP may be active only in a Mg-complexed form." However, we feel that these results could be readily related to inhibition of AK activity by free ATP rather than to limiting concentration of Mg-AMP. Free ATP has been known as a potent inhibitor of the 'kinase'-type phosphotransferases (24), and under the assay conditions used in Hatch (8) it would constitute almost 50% of the total ATP. Our own data on the effect of Mg on AK activity (Fig. 2B) are, in fact, analogous to those described by Hatch (lower rates at limiting Mg^{2+} and inhibition by an excess of the divalent cation). A decrease in rates of AK at high concentration

Table I. *Purification Summary of Maize Leaf Adenylate Kinase*
Assays were monitored in the direction of ADP formation.

Procedure	Volume	Total Activity	Specific Activity	Yield	Enrichment
	ml	*units*	*units/mg protein*	*%*	*-fold*
Crude extract	982	4837	4.3	100	1
Ammonium sulfate	47.5	5587	8.5	115	2
DEAE-cellulose	122	5021	35	104	8
Hydroxyapatite	25.5	4233	48	88	11
Sephadex G-75SF	23.8	3252	260	67	60
Green A	14	2863	1270 (1550[a])	59	295

[a] Assay contained 3.0 mM $MgCl_2$.

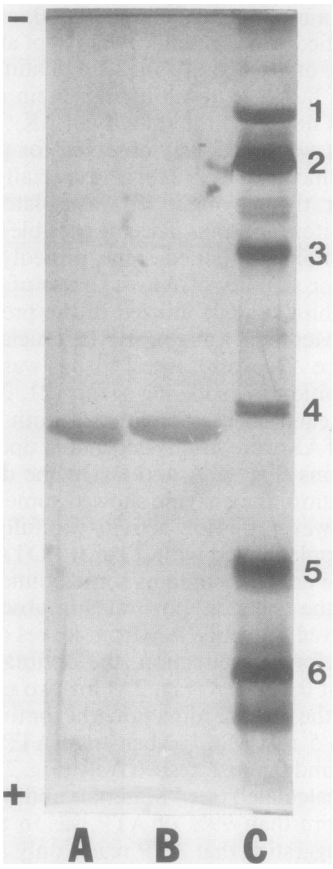

FIG. 1. SDS-PAGE of purified adenylate kinase. AK was mixed with an equal volume of the solution of 6 M urea, 4% 2-ME, and 4% SDS, heated at 80°C for 20 min, and applied to a 12.5% polyacrylamide gel. A and B, 3 and 6 μg of AK, respectively; C, 20 μg of standard proteins. The gel was stained with silver stain (Bio-Rad). Standard proteins kit (Bio-Rad) contained: phosphorylase b (1), M_r 92,500; BSA (2), M_r 66,200; ovalbumin (3), M_r 45,000; carbonic anhydrase (4), M_r 31,000; soybean trypsin inhibitor (5), M_r 21,500; and lysozyme (6), M_r 14,400.

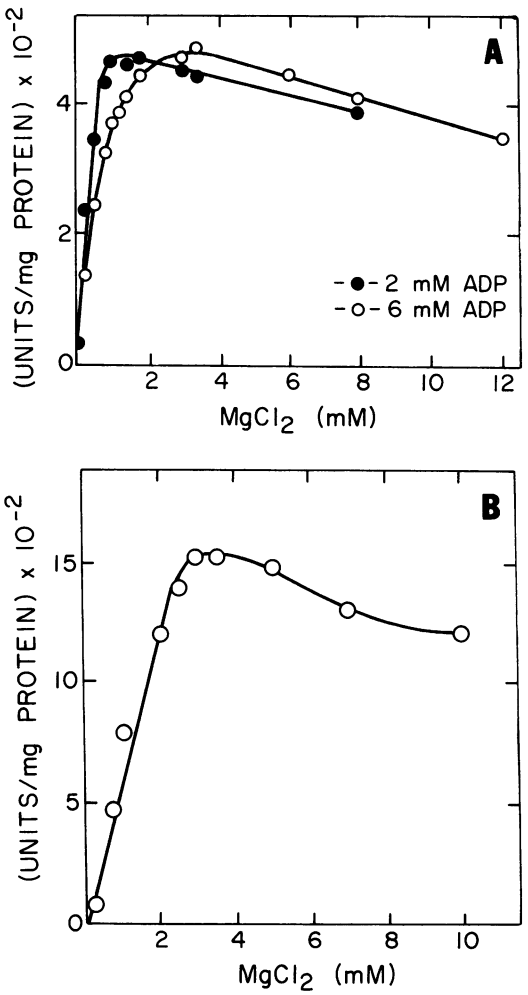

FIG. 2. Effect of Mg on adenylate kinase activity assayed in the forward (A) and reverse (B) direction, respectively. Conditions of the assays were as described in "Materials and Methods."

of Mg (Fig. 2B) might be explained in terms of either inhibition by increased levels of Mg-AMP (*e.g.* at 10 mM $MgCl_2$, Mg-AMP constituted about 33% of total AMP) or depletion in the amount of free AMP. Both Mg-ATP and free AMP were previously postulated as substrates for AK from several nongreen tissues (12, 21, 25).

Concerning the forward reaction of AK (from ADP), there are three possible sets of substrates to be considered: (a) two molecules of free ADP, (b) two molecules of Mg-ADP, and (c) one molecule each of free ADP and Mg-ADP. Data presented in Figure 2A seem to eliminate the first possibility; however, they are not sufficient to distinguish between two other combinations. The best approach to this problem might be a computer-assisted substrate kinetics analysis, with carefully defined concentrations of free ADP, Mg-ADP, and free Mg. Such studies applied to myokinase (25) and AK from yeast (12) allowed for unequivocal demonstration of one molecule each of ADP and Mg-ADP as substrates of the forward reaction.

Activity of AK in the reverse reaction, monitored by ADP production, was about 3-fold higher than the rate in the forward direction, determined by ATP production (Fig. 2, A, B). However, since two ADP are formed from one ATP molecule, the adjusted ratio of the reverse to forward reactions was about 1.5. Similar data were obtained by Hatch (8) (value of 3.7 for unadjusted ratio) who suggested that higher rates of AK in the reverse reaction may be related to a special role of this enzyme in C_4 photosynthesis (coupling to AMP formation by pyruvate, Pi dikinase).

Nucleotide Specificity. Table II summarizes the nucleotide specificity of AK in both directions of the reaction. For the reverse reaction, when ATP was replaced by either dATP, CTP, GTP, ITP, or UTP, activity with AMP was greatly reduced. There was at least 10-fold decrease in activity with ATP when AMP was replaced by dAMP or CMP, while substitution of either GMP, IMP, or UMP resulted in no activity at all. No ATPase activity was associated with purified maize AK. For the forward reaction, ADP was by far the most reactive species. Besides ADP, only CDP and dADP could serve as substrates, and no activity was detected using either GDP, IDP, or UDP.

It should be noted that pyruvate kinase used as a coupling enzyme in the reverse direction has been known to have wide substrate specificity with respect to nucleoside bisphosphates (6), and therefore could not be a limiting step in the assays employed. Similar comment can be made with regard to the nucleoside triphosphate specificity of hexokinase (6) which served as a coupling enzyme for the forward reaction of AK.

The 4-fold reduction of AK activity observed with dATP when AMP served as a second substrate indicated that the 2′-OH group of sugar moiety was preferred over 2′-deoxy group in either binding to the nucleoside triphosphate site on the enzyme or catalysis or both. An even more pronounced decrease in activity when either CTP, GTP, ITP, or UTP were substituted

Table II. *Nucleotide Specificity of Maize Leaf Adenylate Kinase*

Assays contained 2.0 mM nucleoside diphosphate and 1 mM $MgCl_2$ (forward reaction), or 0.5 mM nucleoside monophosphate, 2.0 mM nucleoside triphosphate and 2 mM $MgCl_2$ (reverse reaction). An excess of coupling enzymes (more than 15 units each) was used for assays in both directions. For other details see "Materials and Methods."

Substrates	Activity
	%
ADP	100[a]
dADP	1
CDP	7
GDP	0
IDP	0
UDP	0
ATP, AMP	100[b]
dATP, AMP	25
CTP, AMP	13
GTP, AMP	3
ITP, AMP	8
UTP, AMP	12
ATP, dAMP	7
ATP, CMP	10
ATP, GMP	0
ATP, IMP	0
ATP, UMP	0
ATP alone	0

[a] Corresponds to 480 μmol nucleoside triphosphate produced/min·mg protein. [b] Corresponds to 1300 μmol nucleoside diphosphate produced/min·mg protein.

for ATP suggested preference for the adenine moiety as well. Concerning specificity of the nucleoside monophosphate-binding site of AK, the lack of activity with either GMP, IMP, or UMP and low rates with CMP and dAMP suggested that the primary amino group of either adenine and cytosine moieties was essential for catalysis. This presumption was supported by studies on specificity of the forward reaction (Table II) which exhibited low activities in presence of CDP and dADP, and no rates with GDP, IDP, nor UDP.

DISCUSSION

The complete purification scheme presented for AK from maize is the first reported for this enzyme from leaf tissues. Purified preparations contained essentially homogeneous enzyme, as determined by silver-staining of the SDS-polyacrylamide gel (Fig. 1). Homogeneity was confirmed by Western blotting of crude maize leaf extracts using rabbit anti-maize AK IgG as a probe (data not shown). Properties of the purified maize enzyme are generally similar to those described for the corresponding protein from bacteria, yeast, and animal tissues. AK from almost any source considered is a low mol wt monomer (11, 21, 28), and uses free AMP as a substrate in the reverse direction (12, 25). AK is actually the only kinase known to require a free nucleotide for activity (5).

An interesting property of maize AK is that the enzyme has the ability to carry out an exchange of phosphate group not only between two adenine ribonucleotides but also between cytosine ribonucleotides and adenine deoxyribonucleotides as well (Fig. 3). The analogous nucleotide specificity has previously been shown for AK from human erythrocytes (29). AK from other tissues is usually very specific for AMP and shows no activity with other nucleoside monophosphates (6, 10, 17). On the other hand, the fact that guanosine, inosine, and uridine triphosphates could be readily interconverted with corresponding nucleoside diphosphates but not with nucleoside monophosphates (Fig. 3)

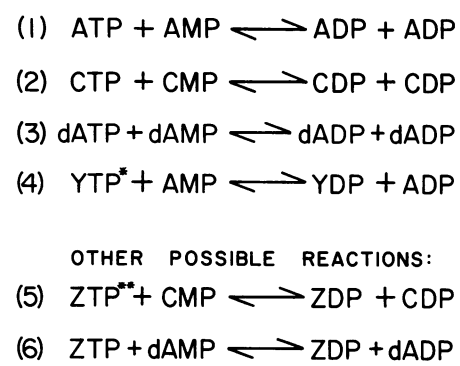

FIG. 3. Reactions catalyzed by maize leaf adenylate kinase. * Letter 'Y' denotes either guanosine, inosine, or uridine. ** Letter 'Z' denotes either adenine ribonucleoside, adenine deoxyribonucleoside, cytidine, guanosine, inosine, or uridine.

is rather typical for AK from variety of sources (10, 21) (myokinase is an important exception being specific only for adenine ribonucleotides [6]). Based on the specificity of each of the binding sites of maize AK, such interexchange of phosphate can occur with AMP (or ADP) as a second substrate and possibly with CMP (or CDP) or dAMP (or dADP) (reactions [4], [5], and [6] in Fig. 3). Whether the AK-catalyzed exchange of phosphate group(s) of nonadenosine nucleotides actually occurs *in vivo* is uncertain since adenylates are the most abundant nucleotide species in leaves (30) and would probably effectively compete for the binding sites on the enzyme. One may argue, however, that pending very high activities of AK in maize leaves (in excess of 2200 μmol/h·mg Chl), the nonspecific rates of the order of well below 1% of those found with adenylates could still be sufficient for considerable flux of phosphate through pools of nonadenosine nucleotides in the cell. Whatever is the magnitude, if any, of such reactions *in vivo*, it seems worth noting that cells do contain specific enzymes involved in transfer of phosphate between nonadenylate species (30).

Our data are in support of the contention (8, 9) that in C_4 plants the primary role of AK is coupling to AMP production by pyruvate, Pi dikinase in mesophyll chloroplasts. In our hands, the enzyme was highly specific for AMP as a substrate, had more than adequate activity for an effective removal of AMP *in vivo*, and showed higher rates in the reverse (utilization of AMP) direction of the reaction. The latter property could be quite unique for the maize enzyme, since AKs from spinach chloroplast stroma (11) and spinach chloroplast membranes (20) have been reported to have higher activity in the forward direction, measured as ATP formation (in case of membrane-bound AK, the value published for activity of the reverse reaction, monitored as ADP formation, should be reduced by half to account for utilization of one ATP per two ADP produced). Additional comparative studies on properties of AK from various plants (*e.g.* C_3 *versus* C_4 plants) are required to ascertain the proposed function and importance of AK in a C_4-type of photosynthesis.

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