Purification and Characterization of a Novel Calcium-Dependent Protein Kinase from Soybean†

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ABSTRACT: A novel calcium-dependent protein kinase (CDPK) previously reported to be activated by the direct binding of Ca^{2+}, and requiring neither calmodulin nor phospholipids for activity [Harmon, A. C., Putnam-Evans, C. L., & Cormier, M. J. (1987) *Plant Physiol. 83*, 830–837], was purified to >95% homogeneity from suspension-cultured soybean cells (*Glycine max*, L. Wayne). Purification was achieved by chromatography on DEAE-cellulose, phenyl-Sepharose, Sephadex G-100, and Blue Sepharose. The purified enzyme (native molecular mass = 52 200 Da) resolved into two immunologically related protein bands of 52 and 55 kDa on 10% SDS gels. Enzyme activity was stimulated 40–100-fold by micromolar amounts of free calcium ($K_{0.5}$ = 1.5 μM free calcium) and was dependent upon millimolar Mg^{2+}. CDPK phosphorylated lysine-rich histone III-S and chicken gizzard myosin light chains but did not phosphorylate arginine-rich histone, phosvitin, casein, protamine, or Kemptide. Phosphorylation of histone III-S, but not autophosphorylation, was inhibited by KCl. CDPK displayed a broad pH optimum (pH 7–9), and kinetic studies revealed a K_m for Mg^{2+}-ATP of 8 μM and a V_{max} of 1.7 μmol min^{-1} mg^{-1} with histone III-S (K_m = 0.13 mg/mL) as substrate. Unlike many other protein kinases, CDPK was able to utilize Mg^{2+}-GTP, in addition to Mg^{2+}-ATP, as phosphate donor. The enzyme phosphorylated histone III-S exclusively on serine; however, CDPK autophosphorylated on both serine and threonine residues. These properties demonstrate that CDPK belongs to a new class of protein kinase.

Numerous processes from the contraction of muscle cells in animals to the streaming of cytoplasm in plants are affected by the concentrations of free calcium ions within the cell [reviewed in Potter and Johnson (1982), Helper and Wayne (1985), and Poovaiah and Reddy (1987)]. Calcium transients can affect cellular metabolism in part through the activation of Ca^{2+}-dependent enzymes, many of which are Ca^{2+}-dependent protein kinases.

In animal cells, two classes of Ca^{2+}-dependent protein kinase have been described: (1) Ca^{2+}/calmodulin-dependent and (2) Ca^{2+}/phospholipid-dependent protein kinases [reviewed in Cohen (1985), Nairn et al. (1985), and Nishizuka (1986)]. Members of these two classes of kinase are not active in the presence of calcium alone, but require effector molecules, either calmodulin or phospholipid, in addition to Ca^{2+} for activation (Nairn et al., 1985; Nishizuka, 1988). Protein kinases with these regulatory properties have not yet been purified from plants, even though Ca^{2+}-dependent protein phosphorylation has been observed in plant extracts.

We recently reported the presence of an unusual Ca^{2+}-dependent protein kinase (CDPK)[1] in suspension-cultured soybean cells whose activation is dependent upon the direct binding of Ca^{2+} and is independent of calmodulin or phospholipids (Putnam-Evans et al., 1986; Harmon et al., 1987). Soybean CDPK is one of only a few plant protein kinases described whose means of regulation is definitively known, and it is one of only seven plant protein kinases which have (now) been purified to homogeneity. Fluorescence immunocytochemical studies have shown that CDPK is co-localized with F-actin in plant cells (Putnam-Evans et al., 1989) and that a CDPK-like antigen is also co-localized with F-actin in animal cells (Harmon et al., 1989), suggesting a possible cellular role for this enzyme in both plant and animal cells. Indeed, CDPK is one of the first plant proteins identified that associates with actin fibers.

The elucidation of the molecular and kinetic properties of CDPK is of obvious importance to the understanding of the potential physiological role(s) of this enzyme in plants, and is also necessary for evaluating its relationship to other known protein kinases. Here we report the purification of soybean CDPK to >95% homogeneity and describe the physical and kinetic properties, substrate specificity, and other characteristics of this unique protein kinase.

Experimental Procedures

Materials. Leupeptin, aprotinin, PMSF, ATP, GTP, histone III-S (lysine-rich histone H-1), histone VIII-S (arginine-rich histone), Kemptide, BSA, phosphotyrosine, phosphothreonine, phosphoserine, phosphatidylserine, diolein, phosphorylase *b*, carbonic anhydrase, and cytochrome *c* were purchased from Sigma; [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham, and [γ-^{32}P]GTP (10–50 Ci/mmol) was from ICN; phenyl-Sepharose and Sephadex G-100 were purchased from Pharmacia; DEAE-cellulose and P81 filter paper were from Whatman; Chelex-100 was purchased from Bio-Rad; ninhydrin was obtained from Pierce; precoated cellulose MN3000 thin-layer plates (20 cm × 20 cm × 0.1 mm) were obtained from Brinkmann; $MgCl_2$ (Analar grade) was from BDH Chemical Co., and 100 mM stock $CaCl_2$ solution was from Orion. Cibacron blue–Sepharose was prepared by the

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[1] Abbreviations: CDPK, calcium-dependent protein kinase; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; mAb, monoclonal antibody.

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methods of Travis et al. (1976) except that 5 g of Cibacron F3GA and 500 mL of Sepharose 4B were used.

Cell Culture. Soybean (*Glycine max*, L. Wayne) suspension cultures were maintained as previously described (Harmon et al., 1987). Soybean cells in log-phase growth were harvested, washed in 0.4 M sorbitol, 20 mM Tris, pH 8.5, and 10 mM $MgCl_2$, and frozen at −80 °C until use.

Protein Kinase Assay. Protein kinase assays were performed in either Ca^{2+}/EGTA or Ca^{2+}/EDTA buffers to control the free calcium concentration. All assay components except histone were made in deionized H_2O which had been passed through a column containing Chelex-100 to remove contaminating Ca^{2+}. Histone III-S was exhaustively dialyzed before use in order to remove contaminating calcium. The amount of contaminating Ca^{2+} in the assay components was determined by plasma emission spectroscopy to be <1 μM. Free calcium concentrations were calculated by a computer program based on the method of Perrin and Sayce (1967), using the stability constants for metal–chelator and metal–ATP complexes reported in Sillén and Martel (1971). Assays were carried out in 1.2 cm × 7.5 cm polyethylene tubes.

Routine kinase assays were performed in a total volume of 0.15 mL containing 50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 0.2 mM EGTA, 0.21 mM $CaCl_2$ (free $[Ca^{2+}]$ = 12 μM), 1.2 mg/mL histone III-S, and 1.5 nM purified CDPK. Prior to addition to each assay tube, purified CDPK was diluted in 20 mM Tris, 10 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, and 0.02% NaN_3. Reactions were initiated by the addition of 50 μM [γ-^{32}P]ATP (240 cpm/pmol). Assay mixtures were incubated at 30 °C for 6 min. Reactions were terminated by the addition of 0.5 mL of 20% trichloroacetic acid containing 0.2% sodium pyrophosphate. Precipitates were collected on Whatman GF/A filters and washed with 10 mL of trichloroacetic acid/pyrophosphate quench solution followed by 2 mL of ethanol/ether (1:1). Filters were placed in scintillation vials with 2 mL of Scintiverse II (Fisher), and radioactivity was determined in a liquid scintillation counter. The concentrations of [γ-^{32}P]ATP, [γ-^{32}P]GTP, and histone III-S were varied in some kinetic experiments as indicated in the figure legends.

For assays in which Kemptide, synthetic myosin light chain peptide, or gizzard myosin light chains were compared to histone as substrates, the following changes were made in the assay procedure: (1) the total volume was 50 μL, and 1.5-mL conical centrifuge tubes were used; (2) 4.5 nM CDPK, 26 μM [γ-^{32}]ATP (1000–2000 cpm/pmol), and substrate concentrations of 200 and 100 μM for Kemptide and synthetic myosin light chain peptide, respectively, were used; for determination of the kinetic parameters of gizzard myosin light chains, the substrate concentration was varied; (3) after 12-min incubation at 30 °C, 15 μL of each reaction mixture was spotted on 2-cm-diameter P81 filter paper circles (Whatman). The filters were washed 30 min to overnight in five changes, 1 L each, of 150 mM H_3PO_4. They were then washed 1 min in 95% ethanol, air-dried, and counted in 2 mL of Scintiverse BD (Fisher).

During purification, assays were performed in a Ca^{2+}/EDTA buffer containing 50 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 5 mM EDTA, 2.8 mM $CaCl_2$ (free $[Ca^{2+}]$ = 86 μM, 0.5 mg/mL histone III-S, 30 μM [γ-^{32}P]ATP (240 cpm/pmol), and 5 μL of each column fraction, with the remainder of the assay conducted according to the routine kinase assay procedure.

CDPK autophosphorylation was assayed in 1.5-mL conical centrifuge tubes in a total volume of 25 μL containing Ca^{2+}/EGTA buffer (see above) and 2 μg of purified CDPK. Reactions were initiated by the addition of 12.5 μM [γ-^{32}P]ATP (240 cpm/pmol). Tubes were incubated at room temperature for 10 min, and then the reaction was stopped by the addition of 5× concentrated Laemmli SDS sample buffer. Samples were immediately boiled (2 min) and electrophoresed in 10% SDS gels.

Purification of CDPK. All steps were carried out at 4 °C. Soybean cells (1 kg fresh weight) were thawed, added to 3 L homogenization buffer (20 mM Tris, pH 7.2, 2.5 mM EDTA, 1 mM PMSF, 10 μg/mL leupeptin, and 20 μg/mL aprotinin), homogenized for 1 min with a Tekmar homogenizer, and then passed through a Manton-Gaulin homogenizer (Everett, MA). The resulting crude homogenate was then centrifuged at 11000*g* for 45 min to obtain a crude supernatant fraction.

The crude supernatant fraction was applied to a 10 cm × 20 cm column of DEAE-cellulose equilibrated in 20 mM Tris, pH 7.2, and 2.5 mM EDTA. The column was washed with 2 L of equilibration buffer containing 0.05 M NaCl, and the kinase was eluted with 2.5 L of equilibration buffer containing 0.4 M NaCl. The flow rate of the column ranged from 10 to 15 mL/min. Fractions containing kinase activity were pooled, PMSF and aprotinin (the same amounts as listed above) were added, and the DEAE pool was dialyzed against 10 mM Tris, pH 7.2, and 0.5 mM $CaCl_2$.

The DEAE pool was loaded on a 5 cm × 7.5 cm phenyl-Sepharose column equilibrated in 10 mM Tris, pH 7.2, and 0.5 mM $CaCl_2$. Flow rates ranged from 5 to 8 mL/min. The column was washed with 7 column volumes of equilibration buffer, followed by 3 column volumes of the same buffer containing 0.15 M NaCl. The kinase was eluted with 20 mM Tris, pH 8.0, and 10 mM EDTA. Aprotinin and PMSF were added to the phenyl-Sepharose pool of kinase activity, which was subsequently concentrated to a volume of 4 mL in an Amicon ultrafiltration device using a YM10 membrane.

After addition of NaCl and glycerol to final concentrations of 0.2 M and 3%, respectively, the concentrated enzyme was chromatographed on a Sephadex G-100 column (1.7 cm × 120 cm) equilibrated in 20 mM Tris, pH 8.0, 2.5 mM EDTA, 200 mM NaCl, and 2 mM NaN_3. The flow rate was approximately 15 mL/h. Leupeptin was added to the pool of fractions containing CDPK activity.

$MgCl_2$ was added (final concentration = 5.5 mM) to the G-100 pool, and this fraction was loaded onto a column of Cibacron blue–Sepharose (2.7 cm × 1.2 cm) equilibrated in 20 mM Tris, pH 8.0, 5.5 mM $MgCl_2$, and 2.5 mM EDTA. The flow rate was 1–2 mL/min. The column was washed with 5 column volumes of equilibration buffer containing 0.6 M NaCl, and CDPK was eluted in equilibration buffer containing 0.2% CHAPS. For storage, 10% glycerol was added to the purified CDPK pool which was divided into aliquots and frozen at −80 °C until use.

Stokes Radius Determination. The same Sephadex G-100 column and running conditions used in the purification procedure were used for the determination of Stokes radius. Blue dextran and $NaNO_2$ were used to determine the void volume and the included volume, respectively, of the column. CDPK was detected by its activity. The Stokes radius of the kinase was determined from a linear plot of $K_d^{1/3}$ vs Stokes radius for the following standard proteins: BSA, ovalbumin, carbonic anhydrase, and cytochrome *c*.

Velocity Sedimentation. Purified CDPK (2 μg) and the standard proteins BSA (0.64 mg), ovalbumin (0.6 mg), myoglobin (0.6 mg), fibrinogen (0.6 mg), and catalase (0.5 mg) were layered onto preformed linear 10–30% glycerol

Table I: Purification of Calcium-Dependent Protein Kinase from Soybean

fraction	volume (mL)	protein (mg)	specific activity (nmol min^{-1} mg^{-1})		Ca^{2+} sensitivity, (x-fold)	purification (x-fold)	yield (%)
			+Ca^{2+}	−Ca^{2+}			
(1) crude homogenate	3500	8720	1.5	0.6	2	1	100
(2) crude supernatant	3180	5620	1.8	0.6	3	1	77
(3) DEAE-cellulose	1060	1720	3.9	0.2	20	3	51
(4) phenyl-Sepharose	187	14.2	115	0.8	140	77	12
(5) Sephadex g-100	34	3.43	220	5.7	40	147	6
(6) Blue-Sepharose	21	0.33	1530	26.9	60	1020	4

gradients in 20 mM Tris, pH 8.0, 2.5 mM EDTA, and 0.2 M NaCl and centrifuged in a Beckman SW 40 rotor at 40 000 rpm for 40 h at 4 °C. Twenty-seven fractions (0.42 mL each) were collected from the bottom of the tubes. Standard proteins and CDPK were identified by measuring the absorbance at 280 nm and protein kinase activity, respectively. The sedimentation coefficient for CDPK was determined from a linear plot of $s_{20,w}$ vs fraction number for the standard proteins.

Phosphoamino Acid Analysis. Histone III-S was phosphorylated as described above. CDPK was autophosphorylated also as outlined above except that the specific activity of the [γ-^{32}P]ATP was 2400 cpm/pmol. Samples of phosphorylated histone III-S and autophosphorylated CDPK were dried in a Speed Vac (Savant Instruments, Hicksville, NY), dissolved in 6 M HCl, and subjected to partial acid hydrolysis under reduced pressure at 105 °C for either 1 or 4 h. Hydrolysates were mixed with standard P-Ser, P-Thr, and P-Tyr and subjected to thin-layer electrophoresis on cellulose plates as previously described (Glover et al., 1983). Radiolabeled amino acids were identified by autoradiography and the standards visualized by ninhydrin staining.

Monoclonal Antibodies. Monoclonal antibodies to CDPK were prepared in the monoclonal facility, University of Georgia, as previously described (Pratt et al., 1986).

Immunoblotting. Immunoblotting was carried out according to the method of Pratt et al. (1986) except that the nitrocellulose was blocked in 20 mM Tris, pH 7.5, 0.5 M NaCl, 0.02% NaN_3, and 5% non-fat dry milk. A mixture of five mAbs to CDPK was used to immunostain kinase which had been transferred to nitrocellulose.

SDS Gel Electrophoresis. Electrophoresis in 10% acrylamide gels was performed as described by Laemmli (1970). For quantitation, gels were scanned with a Quick Scan densitometer (Helena Laboratories). Radiolabeled gels were autoradiographed at −80 °C using Kodak X-OMAT film and a Lightening Plus (Du Pont) screen.

Protein Determination. Protein concentrations were measured by using a Bio-Rad protein assay kit based on the method of Bradford (1976), with BSA as standard. The concentration of histone III-S was determined by the method of Lowry et al. (1951).

Results

Purification. The purification of CDPK from suspension-cultured soybean cells is summarized in Table I. The standard purification procedure allowed recovery of 1–5% of the total available kinase activity, and yielded approximately 250–350 μg of purified CDPK from 1 kg of starting material (Table I). The final specific activity in the presence of micromolar concentrations of free Ca^{2+} was consistently 1–2 μmol min^{-1} mg^{-1}. However, the basal activity of purified CDPK varied between preparations, resulting in variations in the x-fold activation by Ca^{2+} from 40- to 100-fold. The large (140-fold) activation seen at the phenyl-Sepharose step in this preparation was unusually high, resulting from a lower than normal basal activity. We previously demonstrated that the activation of the enzyme by Ca^{2+} was not due to the presence of contaminating amounts of calmodulin and that the concentration of Ca^{2+} required for 50% activation of both autophosphorylation and histone phosphorylation (for 80% homogeneous CDPK) was 1.8 μM (Harmon et al., 1987). The $K_{0.5}$ for Ca^{2+} for CDPK which was >95% homogeneous was approximately the same at 1.5 μM (data not shown). Maximal activation occurred at 10 μM free Ca^{2+}.

Four column chromatography steps were used to purify CDPK over a period of 3–4 days. In order to perform the first ion-exchange chromatography step as quickly as possible, a large-diameter column was used, and the activity was eluted with a pulse of 0.4 M NaCl. Eluting the activity with a gradient of NaCl took longer and did not improve the results at this step. Only one peak of calcium-dependent histone III-S phosphorylating activity was observed when the DEAE-purified kinase was rechromatographed on a Mono-Q column on an FPLC system and eluted with a salt gradient (data not shown).

The elution profiles of the last three chromatographic steps are shown in Figure 1. Many Ca^{2+} binding proteins undergo a conformational change in the presence of Ca^{2+} such that a hydrophobic site is exposed (Moore & Dedman, 1982), allowing the protein to bind to hydrophobic resins such as phenyl-Sepharose. CDPK bound to phenyl-Sepharose in the presence of Ca^{2+} and was eluted in buffers containing EGTA or EDTA (Figure 1A). Similar results were obtained when the pH of the elution buffer was maintained at 7.2 or raised to 8.0. Observations indicating that this step separates CDPK from other kinases are (1) the flow-through fractions contained a large amount of histone phosphorylating activity which was only slightly activated by Ca^{2+} and (2) some Ca^{2+}-independent kinase activity remained associated with the column material after elution of CDPK. A large purification (77-fold) resulted from chromatography on phenyl-Sepharose. Gel filtration in Sephadex G-100 (Figure 1B) resulted in a further 2-fold purification. The final purification was achieved by chromatography on Blue-Sepharose, a resin to which CDPK binds very tightly. A 0.6 M NaCl wash removed a major low molecular weight contaminant and some CDPK activity (Figure 1C). The majority of the CDPK was eluted in buffer containing 0.2% CHAPS, which also served to stabilize the activity of the kinase. Calmodulin (M_r 16.7K) copurified with CDPK through the first three chromatographic steps and was the predominant Coomassie-staining band in the phenyl-Sepharose and G-100 elution pools (Figure 2, lanes 5 and 6). However, calmodulin does not bind to Blue-Sepharose and was absent from the purified CDPK pool (Figure 2, lane 7).

Purified CDPK, which eluted as a single peak of activity during column chromatography (Figure 1), resolved into two protein bands of molecular mass 52 and 55 kDa on 10% SDS gels (Figures 2 and 3A). Quantitative densitometry revealed

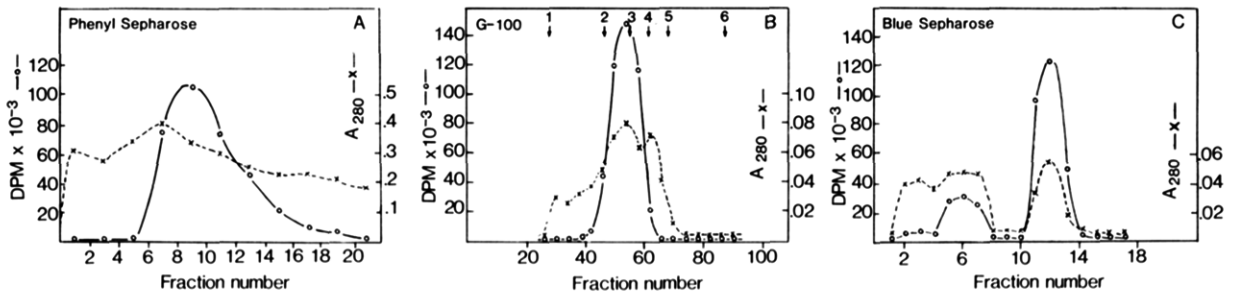

FIGURE 1: Column chromatography. Procedures for chromatography were described under Experimental Procedures. Panel A shows the elution of CDPK from phenyl-Sepharose. Fraction 1 marked the beginning of the EDTA-containing elution buffer. Fractions 7–12 were pooled. Panel B shows the elution of CDPK from a Sephadex G-100 column. Elution positions of markers used to calibrate the column are indicated by the numbered arrows as follows: (1) Blue dextran (used to determine the excluded volume, v_o); (2) BSA; (3) ovalbumin; (4) carbonic anhydrase; (5) cytochrome *c*; (6) $NaNO_2$ (used in the measurement of the included volume, v_i). Elution positions for the marker proteins and CDPK were converted to K_d values by the equation $(v_e - v_o)/v_i$, where $v_i = v_s - v_o$ and v_s was the elution volume of $NaNO_2$. Fractions 46–60 were pooled. Panel C shows the elution of CDPK from Blue-Sepharose. Fraction 1 began the column buffer wash and fraction 4 the column buffer plus 0.6 M NaCl wash. Fraction 10 marked the addition of the elution buffer (column buffer plus 0.2% CHAPS).

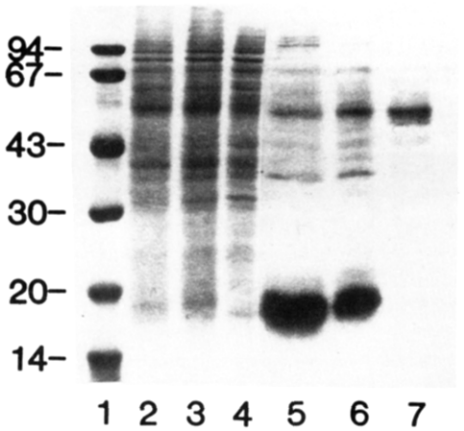

FIGURE 2: Analysis of purification pools by SDS–PAGE. Proteins from various stages of purification were resolved by electrophoresis in a 10% polyacrylamide gel in the presence of SDS and stained with Coomassie blue. Lane 1 contained the following molecular mass markers (in kilodaltons): phosphorylase *b*, 94; BSA, 67; ovalbumin, 43; carbonic anhydrase, 30; soybean trypsin inhibitor, 20; and α-lactalbumin, 14. Other lanes show the following fractions from each step of the purification procedure: lane 2, crude homogenate (10 μg); lane 3, crude supernatant (10 μg); lane 4, DEAE pool (10 μg); lane 5, phenyl-Sepharose pool (8 μg); lane 6, Sephadex G-100 pool (5 μg); and lane 7, Blue-Sepharose pool (2 μg).

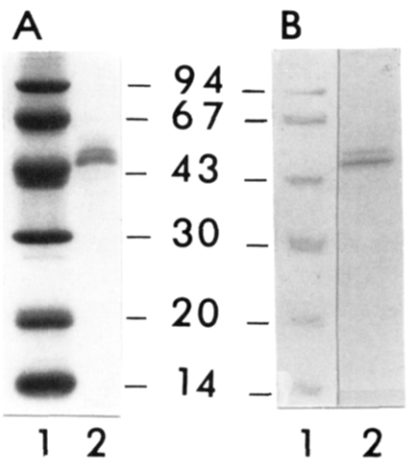

FIGURE 3: Monoclonal antibodies to CDPK recognize both CDPK bands. CDPK (2 μg) was electrophoresed in a 10% SDS gel, which was stained with Coomassie blue (panel A, lane 2). Lane 1 contained the molecular mass markers (the same as described in the legend to Figure 2). In panel B, CDPK (0.5 μg) was electrophoresed in a 10% SDS gel, transferred to nitrocellulose, and immunostained with a mixture of monoclonal antibodies specific for CDPK (lane 2). The molecular mass standards in lane 1 were stained with Ponceau S.

Table II: Structural and Kinetic Properties of CDPK

Structural Parameters	
Stokes radius[a] (Å)	29.5
sedimentation coefficient[b] (s)	4.06
molecular weight	52 200[c]
	52 000–55 000[d]
Kinetic Parameters[e]	
Mg^{2+}-ATP	
K_m (μM)	8
V_{max} (μmol min^{-1} mg^{-1})	1.7
Mg^{2+}-GTP	
$K_{m_{app}}$ (μM)	77
$V_{max_{app}}$ (μmol min^{-1} mg^{-1})	0.5
Mg^{2+}-ADP	
K_i (μM)	18
histone III-S	
K_m (mg/mL)	0.13
Comparison of Protein Substrates[f]	
histone III-S	
$K_{m_{app}}$ (mg/mL)	0.16
$V_{max_{app}}$ (μmol min^{-1} mg^{-1})	0.93
gizzard myosin light chains	
$K_{m_{app}}$ (mg/mL)	0.32
$V_{max_{app}}$ (μmol min^{-1} mg^{-1})	0.62

[a] Measured relative to internal standards by gel filtration chromatography on Sephadex G-100 in the presence of 200 mM NaCl. [b] Measured relative to internal standards by velocity sedimentation in linear 10–30% glycerol gradients in the presence of 150 mM NaCl. [c] Calculated from the Stokes radius and sedimentation coefficient assuming a partial specific volume of 0.74 cm^3/g. [d] Measured relative to molecular mass standards in SDS–polyacrylamide gels. [e] Values for nucleotides were determined with histone III-S as protein substrate. [f] Values were derived from reciprocal plots of data obtained under the assay conditions described under Experimental Procedures.

that the two bands represented >95% of the total protein present in the preparation. The two proteins appear to be related and may have resulted from proteolysis. Observations which support this conclusion are the following: (1) certain of our CDPK preparations have contained two bands whose molecular masses are smaller than 52 and 55 kDa (Harmon et al., 1987); (2) the relative proportions of the Coomassie-stained bands in SDS gels vary among preparations; (3) preliminary data from one-dimensional maps of ^{32}P-labeled peptides resulting from digestion with V-8 protease are consistent with the bands being generated by proteolysis (data not shown); and (4) monoclonal antibodies to CDPK recognize both bands (Figure 3B). Additionally, the two bands resolve into two sets of bands when CDPK preparations are electrophoresed in 10–15% gradient gels, and all of these bands are recognized by each of four monoclonal antibodies to CDPK

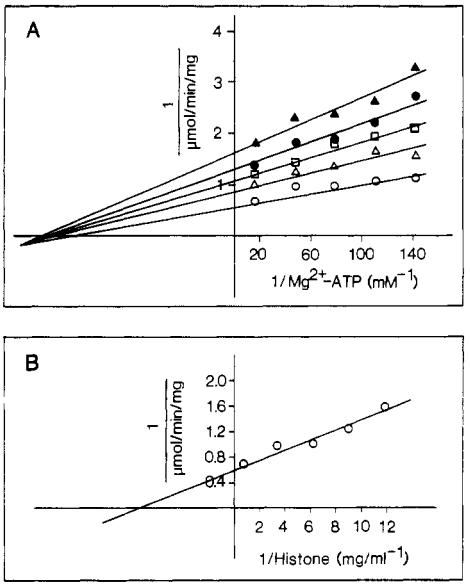

FIGURE 4: Initial velocity studies. Assays were performed in Ca^{2+}/EGTA buffer as described under Experimental Procedures, with 1.5 nM CDPK. (Panel A) $1/v$ vs $1/[Mg^{2}$-ATP] at different fixed concentrations of histone III-S. Histone concentrations were 1.46 (○), 0.30 (△), 0.16 (□), 0.11 (●), and 0.085 (▲) mg/mL. Panel B is a replot of the $1/v$ axis intercepts from panel A vs 1/[histone III-S].

(A. C. Harmon and M. J. Cormier, unpublished results). We are continuing to investigate the origin of the multiple CDPK bands.

Structural and Kinetic Properties. The physical parameters of purified CDPK are summarized in Table II. The molecular mass calculated from the Stokes radius (29.5 Å) and sedimentation coefficient (4.06 *s*) was 52 200 Da, which agrees well with the size of the two bands determined in 10% SDS gels (52 and 55 kDa; Figures 2 and 3A). This indicates that the active form of the kinase is a monomer.

The plot of $1/v$ vs $1/[Mg^{2+}$-ATP] at different fixed concentrations of histone III-S is shown in Figure 4A. The K_m for histone III-S was estimated from a secondary plot of the $1/v$ axis intercepts vs 1/[histone III-S] at saturating [Mg^{2+}-ATP] (Figure 4B) to be 0.13 mg/mL (Table II). The same data were plotted as $1/v$ vs 1/[histone III-S], and the K_m for Mg^{2+}-ATP at saturating [histone III-S] estimated from the secondary replot (data not shown) was 8 μM (Table II). The V_{max} from both replots was 1.7 μmol min^{-1} mg^{-1}. The families of converging lines in Figure 4A and in the plot of $1/v$ vs 1/[histone III-S] are consistent with a sequential reaction mechanism and inconsistent with a ping-pong mechanism. Product inhibition studies with Mg^{2+}-ADP showed it to be a competitive inhibitor with respect to Mg^{2+}-ATP (data not shown). The K_i estimated from a replot of slope vs [Mg^{2+}-ADP] was 18 μM.

Substrate Specificity. CDPK was able to utilize Mg^{2+}-GTP in addition to Mg^{2+}-ATP as phosphate donor, though Mg^{2+}-GTP proved to be a poorer substrate (Table II). The $K_{m_{app}}$ for Mg^{2+}-GTP estimated from kinetic plots of $1/v$ vs $1/[Mg^{2+}$-GTP] at saturating [histone III-S] was 77 μM, approximately 10 times the K_m for Mg^{2+}-ATP. $V_{max_{app}}$ was 0.5 μmol min^{-1} mg^{-1}, almost one-fourth that seen with Mg^{2+}-ATP.

CDPK displayed a somewhat narrow protein substrate specificity. CDPK did not phosphorylate (0% incorporation of ^{32}P compared to 100% incorporation into 1 mg/mL histone III-S) any of the following proteins assayed at 1 mg/mL: arginine-rich histone, α-casein, phosvitin, bovine serum albumin, protamine, or actin. Nor did CDPK phosphorylate the synthetic peptide Kemptide (LRRASLG) used at a concentration of 200 μM. Both β-casein (1 mg/mL) and 100 μM myosin light chain peptide (KKRAARATSNVFA) were phosphorylated 7% and 16%, respectively, compared to histone III-S. In addition to histone III-S, gizzard myosin light chains proved to be a good substrate for CDPK (Table II). The $V_{max_{app}}$ to $K_{m_{app}}$ ratios for histone III-S and gizzard myosin light chain were 5.8 and 1.9, respectively, in the assays used for comparison. These values are similar in magnitude and indicate that both proteins are good substrates for CDPK.

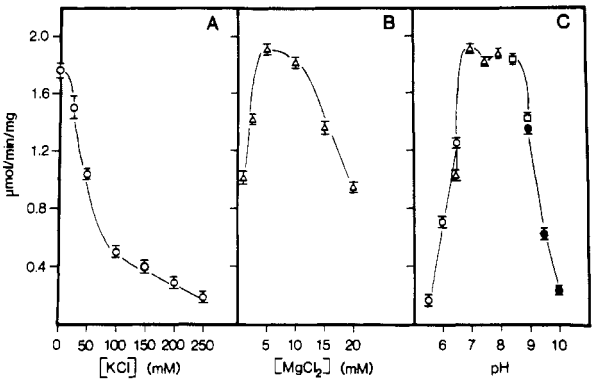

FIGURE 5: Effect of KCl, Mg^{2+}, and pH on CDPK activity. Assays were performed as outlined under Experimental Procedures in Ca^{2+}/EGTA buffer with 1.5 nM CDPK and histone III-S as substrate. (Panel A) Effect of KCl concentration. (Panel B) Effect of Mg^{2+} concentration. (Panel C) Effect of pH. The following buffers were used at 50 mM: MES (○), HEPES (△), Tris (□), and CHES (●).

Effects of Salt, Mg^{2+}, and pH on CDPK Activity. Enzyme activity was inhibited in the presence of increasing concentrations of KCl (Figure 5A), with histone III-S as substrate. This could be a result of the salt disrupting ionic interactions between the positively charged histone and a negatively charged site(s) on the enzyme. The same concentrations of KCl had no effect on CDPK autophosphorylation (data not shown).

CDPK has a requirement for Mg^{2+} independent of the Mg^{2+} needed to form a stable Mg^{2+}–ATP complex, indicating additional function(s) for Mg^{2+}. The Mg^{2+} optimum of the kinase was 5–10 mM, with inhibition of enzyme activity observed above 10 mM Mg^{2+} (Figure 5B).

The enzyme displayed a broad pH optimum, with maximal activity occurring between pH 7 and 9 (Figure 5C).

Effect of Calmodulin, Phospholipids, and Polycations on CDPK Activity. We observed that CDPK activity can be stimulated less than 2-fold, in the presence of calcium, by high concentrations (micromolar) of a number of proteins including murine IgG (Table III, experiment 2), bovine brain calmodulin, bovine serum albumin, ovine IgG, and rabbit skeletal muscle actin (data not shown). This apparent stimulation of activity was not due to phosphorylation of these proteins by CDPK (data not shown). Calmodulin at 20 nM had little to no effect on CDPK activity. These results were similar to those obtained with less pure CDPK (Harmon et al., 1987) and demonstrate that stimulation by protein at high concentration is nonspecific.

It was inferred from previous data that CDPK activity was independent of phospholipids, since Ca^{2+} alone was required to activate the enzyme (Harmon et al., 1987). To confirm this conclusion, we examined the effects of phospholipid and diacylglycerol on CDPK activity. The purified CDPK used in this experiment was activated 100-fold by Ca^{2+} alone (Table III). Addition of phosphatidylserine alone caused a slight increase in activity (13%), as did diolein alone (15%). CDPK activity was increased 29% by the addition of phosphatidylserine plus diolein. We interpret these small increases in

Table III: Effects of Phospholipids and Polycations on CDPK Activity[a]

free calcium concn (μM)	addition	% activity
	Experiment 1	
13	none	100 ± 5
0	none	1.4 ± 0.2
13	10 μg of phosphatidylserine	113 ± 1
0	10 μg of phosphatidylserine	0.5 ± 0.0
13	0.2 μg of diolein	115 ± 4
0	0.2 μg of diolein	1.5 ± 0.2
13	10 μg of phosphatidylserine + 0.2 μg of diolein	129 ± 11
0	10 μg of phosphatidylserine + 0.2 μg of diolein	0.6 ± 0.2
	Experiment 2	
12	none	100 ± 4
12	100 μg/mL polylysine	5.5 ● 1.9
12	100 μg/mL protamine	19 ± 2
12	1.7 mM spermine	82 ± 6
12	1.7 mM spermidine	88 ± 10
12	1.0 μM nonimmune IgG (mouse)	118 ± 4

[a] CDPK assays were performed in Ca^{2+}/EGTA buffer with lysine-rich histone as substrate (see Experimental Procedures). Each value is the average and standard deviation of triplicate determinations. 100% activity in both experiments was 1.6 μmol min^{-1} mg^{-1}.

activity as nonspecific effects, since we have shown that CDPK activity can be stimulated 1.2–2-fold by high concentrations (micromolar) of the variety of compounds discussed above. Additionally, exogenous calmodulin stimulated the Ca^{2+}-independent activity of CDPK (Harmon et al., 1987), whereas phosphatidylserine inhibited the basal CDPK activity (Table III), further demonstrating the nonspecificity of these compounds.

Polycations have been documented to affect the activity of a number of protein kinases (Ahmed et al., 1985). Spermine and spermidine were found to slightly inhibit CDPK activity (18% and 12% inhibition, respectively), while protamine and polylysine have more pronounced effects (Table III). Protamine caused an 80% inhibition of enzyme activity while polylysine caused almost complete inhibition (95%). Since CDPK utilizes the positively charged histone III-S as substrate, it is possible that a negatively charged site exists on the enzyme which is necessary for interaction with the histone. Polycations might inhibit CDPK activity by interacting with such a site.

Phosphoamino Acid Analysis. The amino acid residue(s) phosphorylated by CDPK were identified by thin-layer chromatography of hydrolyzed samples of both histone III-S and the enzyme which were labeled with [γ-^{32}P]ATP. CDPK phosphorylated histone III-S on serine (Figure 6A). No phosphorylation of either threonine or tyrosine was observed.

Like all known protein kinases, CDPK undergoes autophosphorylation. We have previously shown that both CDPK polypeptides undergo Ca^{2+}-dependent autophosphorylation (Harmon et al., 1987). CDPK autophosphorylated on both serine and threonine residues (Figure 6B), indicating that there are at least two phosphorylation sites on the enzyme.

Discussion

Signal transduction mechanisms involving free calcium as a second messenger are understood in much detail in animal systems. In plants, however, little is known regarding the means by which free calcium can control intracellular events. Several laboratories have recently begun to study potential Ca^{2+}-regulated protein kinases from plants, with the hopes of gaining information on how Ca^{2+} may function as a second messenger in plants. To that end, we discovered that soybean

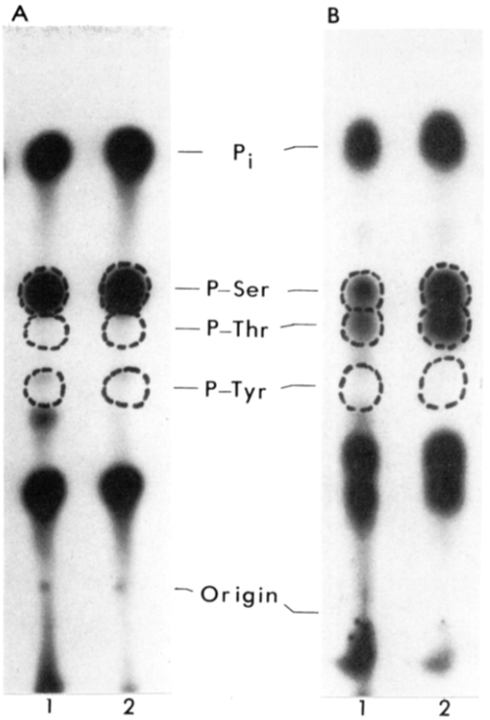

FIGURE 6: Identification of phosphorylated amino acids. Histone III-S (1 mg/mL) was phosphorylated as described under Experimental Procedures except that the specific activity of the ATP used was 2400 cpm/pmol. CDPK (2 μg) was autophosphorylated also as described except that the concentration of ATP used was 1 μM [γ-^{32}P]ATP (4800 cpm/pmol). The labeled proteins were hydrolyzed in 6 M HCl for 1 or 4 h, mixed with standard P-serine, P-threonine, and P-tyrosine, and subjected to thin-layer electrophoresis at pH 3.5. The data are an autoradiogram of the thin-layer plate. (Panel A) Analysis of histone III-S phosphorylation. (Panel B) Analysis of CDPK autophosphorylation. Lane 1 in each panel represents a 1-h hydrolysis and lane 4 in each a 4-h hydrolysis. Positions of the standards, visualized by ninhydrin staining, are indicated by the dashed circles. The mobility of inorganic phosphate is indicated as P_i. Other spots are small, incompletely hydrolyzed phosphopeptides.

cells contain a calcium binding protein which possesses protein kinase activity (Harmon et al., 1987). We have used the designation "CDPK" to distinguish this kinase, which is activated by the direct binding of Ca^{2+}, from other calcium-regulated kinases. Other protein kinases isolated from plant tissues have been reported to be activated by Ca^{2+} or by fatty acids, with no effect of exogenous calmodulin (Battey & Venis, 1988; Polya et al., 1987). Whether these kinases directly bind Ca^{2+} and are related to soybean CDPK is not yet known.

Soybean CDPK was purified to >95% homogeneity and its biochemical properties assessed. The purified enzyme consisted of two related polypeptides of molecular mass 52 and 55 kDa in 10% SDS–polyacrylamide gels (Figures 2 and 3A). These did not represent two different subunits since CDPK eluted from a gel filtration column with a molecular mass of 52 kDa (Table II), and several lines of evidence indicate these bands were generated by proteolysis (see Results). Preliminary sequencing data (Harry Charbonneau, Biochemistry Department, University of Washington, Seattle, personal communication) have indicated the presence of one major protein species. The amino acid sequence of one of the longest and most abundant tryptic peptides shows homology with the catalytic domain of other protein kinases.

The in vitro protein substrates identified for CDPK thus far are histone III-S, myosin light chain peptide, gizzard myosin light chains (Table II), the enzyme ADPglucose pyrophosphorylase (Preiss et al., 1987), and soybean ribosomal proteins (data not shown). Histone is probably not an in vivo

substrate, since its phosphorylation by CDPK is inhibited in the presence of physiological concentrations of KCl (Figure 5A). CDPK catalyzes the incorporation of 0.5–1.0 mol of PO_4/mol of subunit into either spinach or maize ADPglucose pyrophosphorylase (Preiss et al., 1987). Since there is as yet no observed effect of phosphorylation on the kinetic properties of this enzyme from either source (Jack Preiss, Biochemistry Department, Michigan State University, personal communication), the role of phosphorylation of this enzyme is unclear. We observed the phosphorylation of a number of proteins in a mixture of purified soybean ribosomal proteins (data not shown); however, we do not as yet know the identity of the individual proteins. Determination of the amino acid substrates of the enzyme showed that CDPK is a serine/threonine protein kinase (Figure 6).

CDPK is unusual, though not unique, in that it utilized Mg^{2+}-GTP (K_m = 77 μM) in addition to Mg^{2+}-ATP (K_m = 8 μM) as phosphate donor (Table II). Similarly, K_ms of 16 and 66 μM have been observed for ATP and GTP, respectively, for *Drosophila* casein kinase II (Glover et al., 1983). In contrast, the K_ms reported for ATP (45 μM) and GTP (940 μM) for calmodulin-dependent protein kinase II are much higher (Woodgett et al., 1983) and suggest that GTP is an extremely poor substrate for this calcium-regulated enzyme. Other calcium-regulated protein kinases such as myosin light chain kinase, phosphorylase kinase, and protein kinase C do not utilize GTP as phosphate donor [reviewed in Edelman et al. (1987)].

Soybean CDPK is one of two members of a new class of Ca^{2+}-dependent protein kinase. Another protein kinase which directly binds Ca^{2+} has been purified from the protozoan *Paramecium tetraurelia* (Gundersen & Nelson, 1987). This kinase is remarkably similar to the soybean enzyme in that (1) it is a monomer of molecular mass 51 kDa, (2) electrophoretic analysis shows the presence of two polypeptides of 50 and 55 kDa, (3) both kinase bands directly bind $^{45}Ca^{2+}$ on nitrocellulose blots, (4) the $K_{0.5}$ for Ca^{2+} for catalytic activity is 1–1.5 μM, (5) one of the two kinase bands exhibits a Ca^{2+}-dependent mobility shift in SDS gels, (6) both kinase polypeptides undergo Ca^{2+}-dependent autophosphorylation in situ after electrophoresis in SDS gels, and (7) neither calmodulin nor phospholipids are required for activity. Despite the similarity in the two kinases, there are some important differences between them. The *Paramecium* CDPK preferentially phosphorylates casein, whereas soybean CDPK preferred histone III-S and did not phosphorylate casein under the standard assay conditions. Unlike soybean CDPK, the protozoan enzyme does not use Mg^{2+}-GTP as phosphate donor. *Paramecium* CDPK is unstable in the absence of DTT and is sensitive to inhibition by sulfhydryl reagents, indicating a requirement for an –SH group for activity. The soybean enzyme displayed no such requirement. Thus, these are nonidentical but highly related enzymes which comprise a novel class of Ca^{2+}-regulated protein kinase.

While the biological role of soybean CDPK remains unclear, recent data suggest that this enzyme may function in the regulation of the plant cytoskeleton. In immunocytochemical localization studies using a monoclonal antibody specific for CDPK, the enzyme was shown to co-localize with F-actin in three different types of plant cells (Putnam-Evans et al., 1989). Though the enzyme did not bind to F-actin in vitro, the data indicate that it may associate with an as yet unidentified actin binding protein.

Using immunoblotting techniques with monoclonal antibodies to CDPK, we observe that proteins immunologically related to CDPK are present in rabbit tissues and HeLa cells (A. C. Harmon and M. J. Cormier, unpublished results). Additionally, the same antibodies stain stress fibers in HeLa cells, and the data show that a CDPK-related antigen is co-localized with F-actin in these cells (Harmon et al., 1989), just as it is in plant cells (Putnam-Evans et al., 1989). It is interesting to note that gizzard myosin light chain is a good substrate for CDPK, since the interaction of myosin and actin in smooth muscle and nonmuscle cells is regulated by phosphorylation. Experiments designed to investigate whether CDPK may play such a role are in progress.

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Cysteinyl Peptides of Rabbit Muscle Pyruvate Kinase Labeled by the Affinity Label 8-[(4-Bromo-2,3-dioxobutyl)thio]adenosine 5′-Triphosphate†

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ABSTRACT: The affinity label 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate (8-BDB-TA-5′-TP) reacts covalently with rabbit muscle pyruvate kinase, incorporating 2 mol of reagent/mol of enzyme subunit upon complete inactivation. Protection against inactivation is provided by phosphoenolpyruvate, K^+, and Mn^{2+} and only 1 mol of reagent/mol of subunit is incorporated [DeCamp, D. L., Lim, S., & Colman, R. F. (1988) *Biochemistry 27*, 7651–7658]. We have now identified the resultant modified residues. After reaction with 8-BDB-TA-5′-TP at pH 7.0, modified enzyme was incubated with [^{3}H]$NaBH_4$ to reduce the carbonyl groups of enzyme-bound 8-BDB-TA-5′-TP and to introduce a radioactive tracer into the modified residues. Following carboxymethylation and digestion with trypsin, the radioactive peptides were separated on a phenylboronate agarose column followed by reverse-phase high-performance liquid chromatography in 0.1% trifluoroacetic acid with an acetonitrile gradient. Gas-phase sequencing gave the cysteine-modified peptides Asn^{162}-Ile-Cys-Lys^{165} and Cys^{151}-Asp-Glu-Asn-Ile-Leu-Trp-Leu-Asp-Tyr-Lys^{161}, with a smaller amount of Asn^{43}-Thr-Gly-Ile-Ile-Cys-Thr-Ile-Gly-Pro-Ala-Ser-Arg^{55}. Reaction in the presence of the protectants phosphoenolpyruvate, K^+, and Mn^{2+} yielded Asn-Ile-Cys-Lys as the only labeled peptide, indicating that inactivation is caused by modification of Cys^{151} and Cys^{48}.

Pyruvate kinase catalyzes the transfer of phosphate from phosphoenolpyruvate (PEP)[1] to ADP, the last step in the glycolytic conversion of glucose to 2 molecules of pyruvate/molecule of glucose. The active site of the enzyme has been investigated through NMR techniques, which has led to predictions regarding the spatial arrangement of substrates and the structural changes that occur upon substrate binding and catalysis (Mildvan & Cohn, 1966; Gupta et al., 1976; Mildvan et al., 1976; Nageswara Rao et al., 1979; Dunaway-Mariano et al., 1979; Rosevear et al., 1987). X-ray crystallographic analysis at 2.6-Å resolution has suggested the location of the active site and of amino acid residues that participate in substrate binding (Muirhead et al., 1986). Affinity-labeling studies, which correlate loss of activity with reaction at certain residues, can yield information concerning the role and importance of regions of the active site. Such studies have identified tyrosine^{147} at the entrance to the active site as a target residue for the reactive nucleotide analogue 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 5′-diphosphate (2-BDB-TεA-5′-DP) (DeCamp & Colman, 1989). Reaction of this residue with the affinity label was shown to inactivate the enzyme. Protection against inactivation of the enzyme and modification of Tyr^{147} was provided by phosphoenolpyruvate, KCl, and $MnSO_4$, indicating that reaction occurred in the region of the PEP binding site.

The related nucleotide analogues 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate and 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate, which feature the reactive group at a position of the purine ring different from that of 2-BDB-TεA-5′-DP, were also shown to function as affinity labels in activating rabbit muscle pyruvate kinase (DeCamp et al., 1988). As in the case of 2-BDB-TεA-5′-DP, the inactivation rate constants for 8-BDB-TA-5′-DP and 8-BDB-TA-5′-TP were markedly decreased by PEP in the presence of KCl and $MnSO_4$, whereas the coenzymes ADP and ATP were less effective in preventing inactivation. The two 8-BDB nucleotides exhibited similar kinetic characteristics in their reaction with pyruvate kinase, except that k_{max} for 8-BDB-TA-5′-TP was about 3 times greater than for 8-BDB-TA-5′-DP. After an 80-min incubation with 175 μM 8-BDB-TA-5′-TP, the enzyme was 97% inactive and 2 mol of reagent/mol of subunit were incorporated. Reaction of pyruvate kinase with 8-BDB-TA-5′-TP in the presence of the

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[1] Abbreviations: 2-BDB-TεA-5′-DP, 2-[(4-bromo-2,3-dioxobutyl)-thio]-1,N^6-ethenoadenosine 5′-diphosphate; 2-BOP-TεA-5′-DP, 2-[(3-bromo-2-oxopropyl)thio]-1,N^6-ethenoadenosine 5′-diphosphate; 8-BDB-TA-5′-TP, 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate; 8-BOP-TA-5′-TP, 8-[(3-bromo-2-oxopropyl)thio]adenosine 5′-triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

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