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Protection of Pyruvate,Pi Dikinase from Maize against Cold Lability by Compatible Solutes[1]

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ABSTRACT

Most C_4 species are chilling sensitive and certain enzymes like pyruvate,Pi dikinase of the C_4 pathway are also cold labile. The ability of cations and compatible solutes to protect maize (*Zea mays*) dikinase against cold lability was examined. The enzyme in desalted extracts at pH 8 from preilluminated leaves could be protected against cold lability (at 0°C) by the divalent cations Mn^{2+}, Mg^{2+}, and Ca^{2+}. There was substantial protection by sulfate based salts but little protection by chloride based salts of potassium or ammonium (concentration 250 millimolar). The degree of protection against cold lability under limiting $MgCl_2$ (5 millimolar) was pH sensitive (maximum protection at pH 8), but independent of ionic strength (up to 250 millimolar by addition of KCl). In catalysis Mg^{2+} is required and Mn^{2+} could not substitute as a cofactor. Several compatible solutes reduced or prevented the cold inactivation of dikinase (in desalted extracts and the partially purified enzyme), including glycerol, proline, glycinebetaine and trimethylamine-*N*-oxide (TMAO). TMAO and Mg^{2+} had an additive effect in protecting dikinase against cold inactivation. TMAO could largely substitute for the divalent cation and addition of TMAO during cold treatment prevented further inactivation. Cold inactivation was partially reversed by incubation at room temperature; with addition of TMAO reversal was complete. The temperature dependence of inactivation at pH 8 and 3 millimolar $MgCl_2$ was evaluated by incubation at 2 to 17°C for 45 minutes, followed by assay at room temperature. At preincubation temperatures below 11°C there was a progressive inactivation which could be prevented by TMAO (450 millimolar). The results are discussed relative to possible effects of the solutes on the quaternary structure of this enzyme, which is known to dissociate at low temperatures.

Pyruvate,Pi dikinase, an enzyme in the C_4 pathway of photosynthesis, is located in the chloroplasts of mesophyll cells. The enzyme is highly regulated and undergoes light/dark activation/inactivation through a complex, protein mediated mechanism (5, 8). The enzyme is also cold labile, dissociating from a tetramer to dimers and monomers (12, 22, 23). In these studies factors previously shown to give some protection of the enzyme against cold inactivation are Mg^{2+}, glycerol, sorbitol, the substrates phosphoenolpyruvate and pyruvate, and high protein concentration. There is evidence that cold treatment of leaves of the C_4 plants sorghum, maize (12, 25) and *Digitaria sanguinalis* (24) causes a loss of dikinase[2] activity which may be linked to dissociation. There is some variation among C_4 plants in their tolerance to chilling (16, 25) although it is not known if differences in cold lability of enzymes is a factor in vivo. From research on mammalian proteins it is known that a number of highly regulated enzymes with quaternary structure are cold labile (3) but there has been less research and fewer examples from studies with plants.

A number of studies demonstrate that specific organic solutes, namely polyols, certain free amino acids, and their derivatives, can protect proteins against a range of perturbations. For example, glycinebetaine protects against low temperature inactivation of rabbit muscle phosphofructiose kinase (11), against high temperature inactivation of several enzymes (including plant malate dehydrogenase) (17), and it partially protects against inactivation of barley NAD malate dehydrogenase by high salt (18). Thus, in addition to evidence that such solutes may serve as compatible osmolytes which are nontoxic to proteins and membranes under salt or water stress (1, 13, 14, 28), in some cases they may serve to stabilize proteins and prevent their denaturation or dissociation (11, 17, 21, 27). Certain types of compatible solutes have been found in a range of organisms under salinity or water stress, suggesting evolutionary convergence in the physiological role of these compounds (29).

Whether naturally occurring compatible solutes can protect cold labile plant enzymes is uncertain. Thus, we have examined the ability of cations and compatible solutes to protect against cold inactivation of dikinase from maize. Representative compatible solutes, glycerol, proline, glycinebetaine, and TMAO, were found to protect the enzyme against cold inactivation.

MATERIALS AND METHODS

Plant Material

Seed of *Zea mays* (hybrid 130 Garland Flint) was from Johnny's Selected Seed, Albion, Maine. Plants were grown in soil in a growth chamber and irrigated daily with Fe-EDTA-modified ½ strength Hoagland nutrient solution. The light intensity was 600 $\mu E\ m^{-2}\ s^{-1}$, the photoperiod was 16 h day/8 h night and the temperature was 25°C day/18°C night. Plants were sampled 10 to 13 d after emergence using the youngest fully emerged leaves.

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[2] Abbreviations: dikinase, pyruvate,Pi dikinase; TMAO, trimethylamine-*N*-oxide.

Extraction and Desalting of Extracts

Plants were preilluminated at 600 μE m^{-2} s^{-1} for at least 1 h prior to extraction and then ground in a chilled mortar under the same light intensity. Four ml of grinding medium were used per g tissue, resulting in a dikinase activity in the extract of about 0.5 μmol min^{-1} mL^{-1}. The grinding medium, which was at room temperature, contained 100 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM Na-EDTA, 0.5% (w/v) sodium ascorbate, 4 mM DTT, and 2% (w/v) PVPP (insoluble). To desalt the extract and remove low mol wt substances, a column of Sephadex G-25 was equilibrated at room temperature with 100 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5% ascorbate, and 4 mM DTT. When evaluating the effect of various cations on the cold lability of the enzyme, the cation was included in the equilibration medium at the same concentration. Following desalting, part of the extract was incubated at room temperature and part at 0°C (on ice) for the time indicated. Cold stability is defined as the activity after incubation at 0°C divided by the activity after incubation at room temperature times 100. In one experiment (Fig. 5) the temperature was varied from 2 to 17°C using an aluminum block 15 cm wide × 41.5 cm long × 10 cm deep. Temperature controlled water was circulated through holes in each end of the block using two separate water baths to generate the temperature gradient. Test tubes were placed in holes in the top of the block along the temperature gradient for incubation of the enzyme.

Dikinase Purification

In some cases, partially purified dikinase prepared according to Burnell and Hatch (4), but with increased concentrations of glycerol (20% v/v) and β-mercaptoethanol (20 mM) in the extraction and column media, was used to make comparisons with results obtained with crude extracts.

Assay Medium

All assays of the enzyme were performed spectrophotometrically at 25°C by coupling phosphoenolpyruvate formation with phosphoenolpyruvate carboxylase and NAD malate dehydrogenase. The assay medium contained 100 mM Tris-HCl (pH 8), 10 mM $MgSO_4$, 1 mM EDTA (Sodium salt), 10 mM sodium bicarbonate, 1.25 mM sodium pyruvate, 2 mM DTT, 0.2 mM NADH, 2.5 mM K_2HPO_4, 2 units of NAD-malate dehydrogenase, 2 units of phosphoenolpyruvate carboxylase, and 1.25 mM ATP (potassium). In all cases 25 μL of leaf extract (preincubated under the conditions indicated) was added to the assay medium to make a final volume of 1 mL. The reaction was initiated by addition of ATP.

RESULTS

At room temperature dikinase was stable for 30 min with or without addition of cations, whereas it was very unstable at 0°C without divalent cations. Among the cations tested Mn^{2+}, Mg^{2+}, and Ca^{2+} were effective in protecting the dikinase against cold inactivation (Table I). Mn^{2+} at a concentration of 1 mM gave a high degree of protection, whereas the protection with Mg^{2+} progressively decreased below 10 mM (Fig. 1). In additional experiments not shown the cold stability with 10 mM $MgCl_2$ ranged from 85 to 95% (several experiments), and 10 mM Ca^{2+} was more effective than 1 mM Ca^{2+}. There was little protection by 250 mM KCl or NH_4Cl, but substitution of sulfate for chloride gave substantial protection. The effect of cations on cold lability is different from that on catalysis in that Mg^{2+} (10 mM) was required as a cofactor in catalysis and Mn^{2+} (1 mM) could not substitute for this requirement (data not shown).

Table I. *Effect on Activity of Dikinase by Incubation of Leaf Extracts of Maize with Various Salts for 30 min at Room Temperature versus 0 °C*

Leaf extracts were desalted before addition of the salts indicated. Each value in parenthesis is the percentage activity at 0°C relative to that at room temperature.

Additions	Dikinase Activity	
	30 min at room temperature	30 min at 0 °C
	μmol mg⁻¹ Chl h⁻¹	
None	122	9.3 (8)
NH_4Cl (250 mM)	131	24.8 (19)
$(NH_4)_2SO_4$ (250 mM)	136	100 (74)
KCl (250 mM)	134	32.0 (24)
K_2SO_4 (250 mM)	138	74.3 (54)
$MgCl_2$ (10 mM)	143	134 (94)
$MnCl_2$ (1 mM)	133	133 (100)
$MnCl_2$ (2 mM)	128	129 (101)
$CaCl_2$ (10 mM)	134	121 (91)

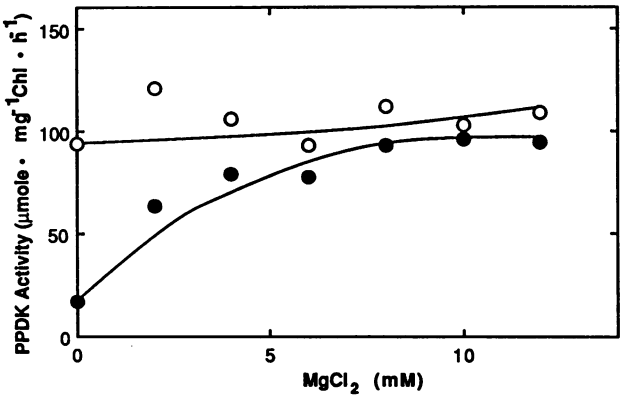

Figure 1. Influence of $MgCl_2$ on the cold lability of pyruvate,Pi dikinase. Desalted leaf extracts of maize were incubated at room temperature (O——O) or at 0°C (●——●) for 30 min prior to assay.

In subsequent experiments the influence of various factors on the cold lability of dikinase was examined with limiting Mg^{2+}, or in some cases without Mg^{2+}. In examining the effect of pH, in the presence of 5 mM $MgCl_2$ (adopted as a standard from previous studies) (22, 24) the cold stability was found to be pH dependent, with maximum stability occurring around pH 8 (Fig. 2A). In contrast, varying ionic strength (addition of KCl) at pH 8 up to 250 mM had no effect on the cold lability of dikinase (Fig. 2B).

The effects of a representative polyol, amino acid, and two methylamines on the cold stability of dikinase in leaf extracts were examined. The cold stability of the enzyme was much greater in the presence of compatible solutes (Table II). At a concentration of 250 mM, glycinebetaine and TMAO gave

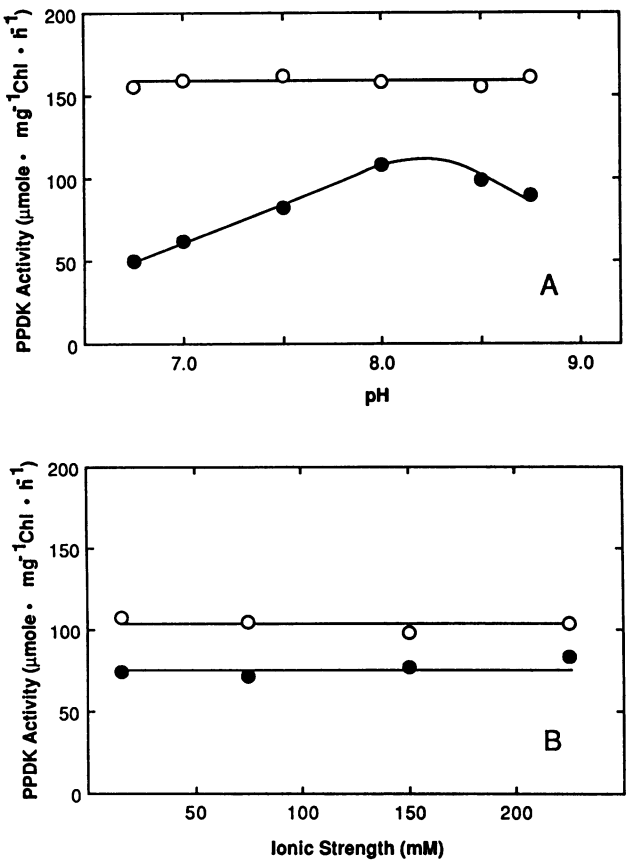

Figure 2. Influence of pH and changing ionic strength by increasing KCl concentration on the cold lability of pyruvate,Pi dikinase in the presence of 5 mM $MgCl_2$. A. The pH was varied using 3-[*N*-morpholino]propanesulfonic acid buffer (pH 6.75–pH 7.5), Hepes (pH 7.5–8.0), and Bicine (pH 8.0–8.75). At pH 7.5 and 8.0 there is only one data point as the results were the same with both buffers. The concentration of buffer in each case was 100 mM and solutions were titrated with KCl to maintain constant ionic strength. B. Ionic strength was varied with KCl at pH 8, 100 mM Tris buffer. Desalted leaf extracts of maize were incubated at room temperature (○——○) or at 0°C (●——●) for 30 min prior to assay.

similar protection, whereas proline gave less protection. Essentially the same results were obtained with the partially purified enzyme.

The concentration of TMAO required to give maximum cold stability of the enzyme, with 5 mM $MgCl_2$, was approximately 400 mM, and the degree of stability approached that of the enzyme stored at room temperature (Fig. 3A). At 0°C a time dependent loss of activity occurred over 1 h without TMAO, while in the presence of 280 mM TMAO the enzyme was stable (Fig. 3B). Similar results were obtained with increasing concentrations of glycinebetaine (data not shown).

The effect of TMAO on the cold inactivation was examined after 1 h at 0°C in the presence of 5 mM $MgCl_2$ (Fig. 4). Addition of TMAO (310 mM) after 1 h of cold treatment protected the enzyme against any further cold inactivation, but the original activity was not recovered. When a sample was transferred to room temperature after 1 h at 0°C, there was reactivation of the enzyme in the absence of TMAO, and full recovery in the presence of TMAO.

Table II. *Effect on the Activity of Dikinase by Incubating Leaf Extracts or Partially Purified Enzyme from Maize with Various Compatible Solutes for 30 min at Room Temperature versus 0°C*

Leaf extracts (A) were desalted before addition of the compatible solutes indicated. The incubation medium include 5 mM $MgCl_2$. Each value in parenthesis is the percentage activity at 0 °C relative to that at room temperature.

Additions	30 min at Room Temperature	30 min at 0°C
A. Leaf extracts	*μmol mg⁻¹ Chl h⁻¹*	
None	154	64.7 (42)
Glycerol (20%, v/v)	164	142 (86)
Proline (250 mM)	143	87.6 (61)
TMAO (250 mM)	141	117 (83)
Glycinebetaine (250 mM)	154	118 (76)
B. Partially purified enzyme	*units mg⁻¹ protein*	
None	0.432	0.235 (54)
Glycerol (20%, v/v)	0.418	0.478 (114)
Proline (250 mM)	0.391	0.252 (64)
TMAO (250 mM)	0.387	0.345 (89)
Glycinebetaine (250 mM)	0.415	0.327 (79)

Since both divalent cations and compatible solutes can protect dikinase against cold inactivation, the effect of a combination of Mg^{2+} and TMAO on cold stability of the enzyme was determined (Table III). The cold stability progressively increased from 4% with no Mg^{2+}, to 16% with 1 mM Mg^{2+}, to 70% with 5 mM Mg^{2+}. With addition of 300 mM TMAO the cold stability increased to 50% in the absence of Mg^{2+}, and TMAO had an additive effect with 1 and 5 mM Mg^{2+}. In the absence of Mg^{2+}, 600 mM TMAO gave a high level of cold stability, indicating that TMAO can largely substitute for Mg^{2+}.

In order to evaluate the temperature dependence of inactivation of dikinase with and without compatible solutes, the enzyme was incubated for 30 min between 2°C and 17°C in the presence of 3 mM $MgCl_2$ (Fig. 5). Activity was then measured at 25°C. In control incubations, below 11°C, cold inactivation became apparent with a progressive loss of activity down to 2°C. TMAO (450 mM) protected against cold inactivation over the entire temperature range. Proline (300 mM) protected against cold inactivation down to about 5°C; however, substantial inactivation occurred at lower temperature. Similar results were obtained with partially purified dikinase from maize, with TMAO giving complete protection, while proline gave limited protection at the lowest temperatures (data not shown).

DISCUSSION

Dikinase of C_4 plants is cold labile, since it dissociates and loses activity when stored at low temperatures (12, 22, 23). In the present study, the effect of various salts and compatible solutes on the cold stability of the enzyme was evaluated. Among the salts tested the divalent cations Mn^{2+}, Mg^{2+}, and Ca^{2+} protected against cold inactivation with Mn^{2+} being more effective than Mg^{2+} or Ca^{2+}. This effect of divalent cations is probably at a different site than that involved in catalysis, since Mn^{2+} could not substitute for Mg^{2+} as a cofactor in catalysis. The monovalent cations, ammonium and potassium, offered little or no protection against cold

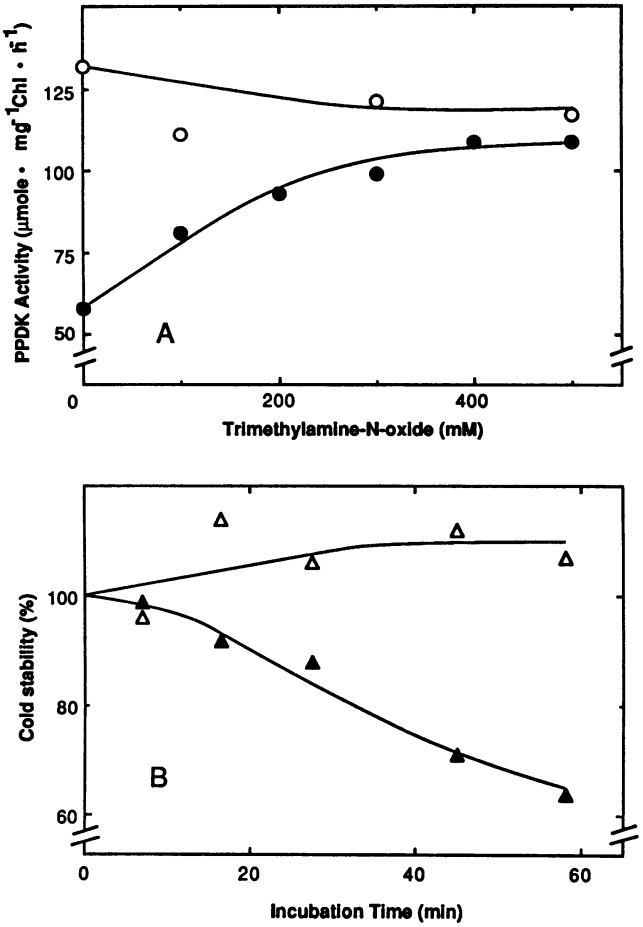

Figure 3. Influence of the concentration of TMAO on the cold lability of pyruvate,Pi dikinase of maize in the presence of 5 mM $MgCl_2$. A, Following desalting, leaf extracts of maize were incubated for 30 min with the indicated concentrations of TMAO at room temperature (○——○) or at 0°C (●——●) for 30 min. B, Cold stability (activity following incubation for the indicated time at 0°C divided by activity following incubation at room temperature times 100) without TMAO (▲——▲) and with 280 mM TMAO (△——△).

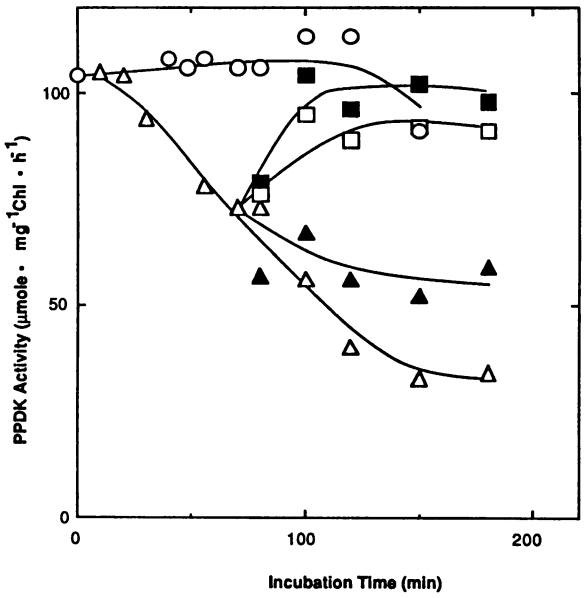

Figure 4. Activity of maize leaf dikinase following treatment of desalted leaf extracts (5 mM $MgCl_2$ was included in the incubation medium). Incubation at room temperature (○——○). Following incubation for 60 min at 0°C (△——△), aliquots were incubated further at 0°C (△——△), at 0°C with 310 mM TMAO (▲——▲), at room temperature (□——□), and at room temperature with 310 mM TMAO (■——■).

Table III. *Effect on the Activity of Dikinase by Incubation of Leaf Extracts of Maize with Various Concentrations of $MgCl_2$ and TMAO at Room Temperature or 0°C*

$MgCl_2$ (mM)	45 min at Room Temperature			45 min at 0°C		
	TMAO (mM)			TMAO (mM)		
	0	300	600	0	300	600
	μmol mg⁻¹ Chl h⁻¹					
Experiment 1						
0	108	109		3.9 (4)	55.4 (51)	
1	131	121		20.4 (16)	103 (85)	
5	139	133		96.3 (70)	122 (91)	
Experiment 2						
0	123	129	129	7.4 (6)	44.5 (35)	110 (90)

lability. However, there was some protection with $(NH_4)_2SO_4$ or K_2SO_4, indicating that the sulfate ion gives some stability. This is consistent with the Hofmeister Series Phenomena, where certain ions like sulfate have been found empirically to favor maintenance of some proteins in a native state whereas others are destabilizing (29). However, as the study with α-chymotrypsin shows, there is not always a positive correlation between salting out and maintaining quaternary structure (2).

Bock and Frieden (3) note that cold lability of enzymes is generally related to dissociation of oligomeric structure. Further, this might occur due to a decreased stability of hydrophobic bonds or a temperature dependent change in the pK value of ionizable groups which control association-dissociation. When dissociation is pH dependent, as dikinase was found to be in the present study, the temperature dependence of inactivation might be accounted for (at least in part) by pK changes on ionizable groups which control the protein quaternary structure. Protection by glycerol and some dependence of cold lability on pH suggest that both ionic and hydrophobic interactions may be involved in the quaternary structure of dikinase. Further studies are required to confirm this.

In the present study representative compatible solutes (glycerol, proline, glycinebetaine, and TMAO) were found to increase the cold stability of dikinase from maize. TMAO could substitute for Mg^{2+} as a protective agent. Although the mechanism of action of solutes on proteins is poorly understood, the consensus view is that the solutes affect protein conformation through modification of the structure and concomitant physical properties of the solvent, rather than acting on the protein directly. Polyhydric alcohols may stabilize proteins by decreasing the capacity of the solvent to disrupt hydrogen bonds (10). This would be applicable to those oligomeric proteins held together by hydrogen bonds between subunits. Alternatively, Gekko and Timasheff (9) demonstrated that an aqueous glycerol solution increases the pref-

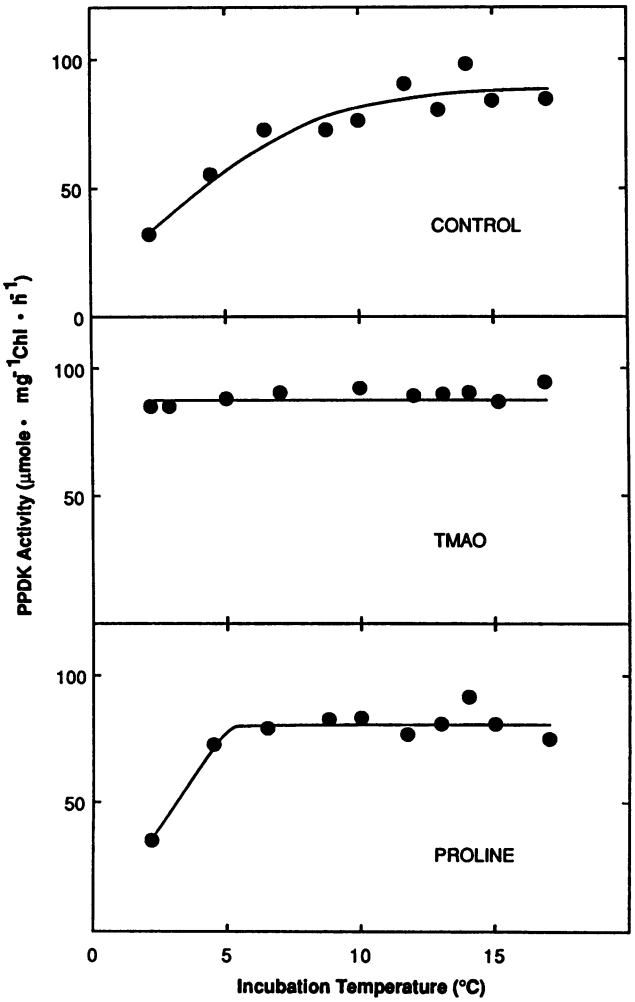

Figure 5. Effect of incubation temperature on dikinase in desalted leaf extracts of maize. Incubation was for 30 min with 3 mM $MgCl_2$. The concentration of TMAO was 450 mM and the concentration of proline was 300 mM.

erential hydration of the protein. Since this is thermodynamically unfavorable, the protein molecule minimizes the contact area with the water by adopting a more compact conformation. Presumably, oligomers would be stabilized by glycerol if the total hydrophobic contact area between protein and solution were less in the associated state. There is evidence that compatible solutes stabilize proteins through a mechanism outlined by the exclusion volume theory. Several studies suggest that a number of organic compounds including glycerol, betaine, proline, and polyethylene glycol are excluded from the protein domain and increase protein solvation through their interaction with water (21). If contact between the protein and solute is unfavorable, the protein may be confined to a fraction of the total volume.

The physiological significance of the present study relative to the possible protection of enzymes in plants against cold lability is uncertain. There is some evidence that dikinase is labile in vivo when leaves are exposed to low temperatures. This might be due to enzyme dissociation (see introduction). Factors which could influence the association-dissociation equilibrium of the enzyme in vivo are concentration of the protein, pH, concentration of divalent cations, and levels of protective organic solutes. The concentrations of divalent cations and solutes used in the present study are not necessarily unphysiological since, for example, the level of glycinebetaine in spinach chloroplasts approaches 300 mM under conditions of salt stress (19) and some C_4 species have high levels of proline or betaine under water stress (1, 28).

Compatible solutes increase in certain species when exposed to low temperature (1, 14, 20). In some cases these may act as cryoprotectants. For example, addition of proline to maize callus cultures dramatically lowers the temperature at which freezing injury occurs (26) and certain compatible solutes protect thylakoid membranes during freeze/thaw treatments (6). However, it is also possible that plants having tolerance to chilling temperatures (*e.g.* 10–12°C) synthesize organic solutes which protect cold labile enzymes or membranes as one, among several, means of preventing loss of metabolic functions.

In maize the proline level increases several fold in response to drought and salinity, but to a lesser extent than in some species (1); and there is limited glycinebetaine production in maize under saline conditions (28) or water stress (13). However, maize plants exposed to drought are better able to tolerate subsequent exposure to −5°C for 2 h (15), suggesting specific solutes may accumulate during drought which increase tolerance to low temperatures. Also, in studies with maize callus, treatments which increase accumulation of proline increase the ability of the tissue to survive when stored at 4°C (7). There is growing evidence that a number of environmental stresses, including salinity, drought, high temperature and low temperature, can trigger the synthesis of compatible solutes. Whether some chilling tolerant races or species of C_4 plants can synthesize compatible solutes which protect against chilling injury upon exposure to low temperatures needs to be considered.

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LITERATURE CITED

1. **Aspinall D, Paleg LG** (1981) Proline accumulation: physiological aspects. *In* LG Paleg, D Aspinall, eds, The Physiology and Biochemistry of Drought Resistance in Plants. Academic Press, Sydney, pp 205–241
2. **Aune KC, Goldsmith LC, Timasheff SN** (1971) Dimerization of α-chymotrypsin. II. Ionic strength and temperature dependence. Biochemistry **10:** 1617–1622
3. **Bock PE, Frieden C** (1978) Another look at the cold lability of enzymes. Trends Biochem Sci **4:** 100–103
4. **Burnell JN, Hatch MD** (1985) Regulation of C_4 photosynthesis: purification and properties of the protein catalyzing ADP-mediated inactivation and Pi-mediated activation of pyruvate,Pi dikinase. Arch Biochem Biophys **237:** 490–503
5. **Burnell JN, Hatch MD** (1985) Light-dark modulation of leaf pyruvate,Pi dikinase. Trends Biochem Sci **10:** 288–291
6. **Coughlan SJ, Heber U** (1982) The role of glycinebetaine in the protection of spinach thylakoids against freezing stress. Planta **156:** 62–69
7. **Duncan DR, Widholm JM** (1987) Proline accumulation and its implication in cold tolerance of regenerable maize callus. Plant Physiol **83:** 703–708
8. **Edwards GE, Nakamoto H, Burnell JN, Hatch MD** (1985)

Pyruvate, Pi dikinase and NADP-malate dehydrogenase in C_4 photosynthesis: properties and mechanism of light/dark regulation. Annu Rev Plant Physiol **36:** 255–286

9. **Gekko K, Timasheff SN** (1981) Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. Biochemistry **20:** 4667–4676
10. **Gerlsma SY** (1968) Reversible denaturation of ribonuclease in aqueous solution as influenced by polyhydric alcohols and some other additives. J Biol Chem **243:** 957–961
11. **Hand SC, Somero GN** (1982) Urea and methylamine effects on rabbit muscle phosphofructokinase. Catalytic stability and aggregation state as a function of pH and temperature. J Biol Chem **257:** 734–741
12. **Hatch MD** (1979) Regulation of C_4 photosynthesis: factors affecting cold-mediated inactivation and reactivation of pyruvate,Pi dikinase. Aust J Plant Physiol **6:** 607–619
13. **Hitz WD, Hanson AD** (1980) Determination of glycine betaine by pyrolysis-gas chromatography in cereals and grasses. Phytochemistry **19:** 2371–2374
14. **Loescher WH, Fellman JK, Fox TC, Davis JM, Redgwell RJ, Kennedy RA** (1985) Other carbohydrates as translocated carbon sources: acyclic polyols and photosynthetic carbon metabolism. *In* R Heath, J Preiss, eds, Regulation of Carbon Partitioning in Photosynthetic Tissue. American Society of Plant Physiologists, Rockville, MD, pp 309–332
15. **Luhan RL, Wicks ZW** (1985) A method of screening for frost tolerance in corn (*Zea mays* L.) inbred lines. NCR-2 Report, p 16
16. **McWilliam JR, Ferrar PJ** (1974) Photosynthetic adaptation of higher plants to thermal stress. *In* RL Bieleski, AR Ferguson, MM Cresswell, eds, Bulletin 12. The Royal Society of New Zealand, Wellington, pp 467–476
17. **Paleg LG, Douglas TJ, van Paal A, Keech DB** (1981) Proline, betaine, and other organic solutes protect enzymes against heat inactivation. Aust J Plant Physiol **8:** 107–114
18. **Pollard A, Wyn Jones RG** (1979) Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta **144:** 291–298
19. **Robinson SP, Jones GP** (1986) Accumulation of glycinebetaine in chloroplasts provides osmotic adjustment during salt stress. Aust J Plant Physiol **13:** 659–668
20. **Sakai A, Larcher W** (1987) Frost Survial of Plants. Responses and Adaptation to Freezing Stress. Ecological Studies 62. Springer-Verlag, Berlin, Heidelberg
21. **Selinioti E, Nikolopulos D, Manetas Y** (1987) Organic cosolutes as stabilisers of phosphoenolpyruvate carboxylase in storage: an interpretation of their action. Aust J Plant Physiol **14:** 203–210
22. **Shirahashi K, Hayakawa S, Sugiyama T** (1978) Cold lability of pyruvate,orthophosphate dikinase in the maize leaf. Plant Physiol **62:** 826–830
23. **Sugiyama T** (1973) Purification, molecular and catalytic properties of pyruvate phosphate dikinase from the maize leaf. Biochemistry **12:** 2862–2867
24. **Sugiyama T, Schmitt MR, Ku SB, Edwards GE** (1979) Differences in cold lability of pyruvate,Pi dikinase among C_4 species. Plant Cell Physiol **20:** 965–971
25. **Taylor AO, Slack CR, McPherson HG** (1974) Plants under climatic stress. VI. Chilling and light effects on photosynthetic enzymes of sorghum and maize. Plant Physiol **54:** 696–701
26. **Withers LA, King PJ** (1979) Proline: An novel cryoprotectant for the freeze preservation of cultured cells of *Zea mays* L. Plant Physiol **64:** 675–678
27. **Wyn Jones RG** (1984) Phytochemical aspects of osmotic adaptation. *In* BN Timmerman, C Steelink, FA Loewus, eds, Recent Advances in Phytochemistry, Vol 13. Phytochemical Adaptations to Stress. Plenum Press, New York, pp 55–78
28. **Wyn Jones RG, Storey R** (1981) Betaines. *In* LG Paleg, D Aspinall, eds, Physiology and Biochemistry of Drought Resistance in Plants. Academic Press, Sydney, pp 171–204
29. **Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN** (1982) Living with water stress: evolution of osmolyte systems. Science **217:** 1214–1222