Identification of the amino acid residues responsible for cold tolerance in *Flaveria brownii* pyruvate,orthophosphate dikinase

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Abstract Pyruvate,orthophosphate dikinase (PPDK), an enzyme important in C_4 photosynthesis, is typically a cold-sensitive enzyme. However, a cold-tolerant form of the enzyme has been isolated from the leaves of *Flaveria brownii*. Using an *E. coli* expression system and the PPDK cDNAs from *F. brownii* (cold-tolerant), *F. bidentis* (cold-sensitive) and maize (intermediately cold-tolerant), site-directed mutagenesis studies indicated that as few as three amino acids residues (of 880 residues) strongly influence the cold sensitivity of *Flaveria* PPDK. Gel filtration analysis of the PPDK expressed in *E. coli* showed that subunit association and cold tolerance are closely linked.

Key words: Pyruvate,orthophosphate dikinase; C_4 photosynthesis; Chilling; *Flaveria*

1. Introduction

Pyruvate,orthophosphate dikinase (PPDK, EC 2.7.9.1) catalyzes the synthesis of phosphoenolpyruvate, the primary inorganic carbon acceptor in C_4 photosynthesis [1,2]. The enzyme, encoded by a nuclear gene, is synthesized as a precursor protein which is processed during transport into the chloroplast [3]. The fact that PPDK activity is lowest among the C_4 acid cycle enzymes (and close to the observed maximum rates of photosynthesis) suggests that the enzyme may be closest to rate limiting for C_4 photosynthesis (see [4]). In maize, PPDK is inactivated by cold temperatures losing activity below about 10°C [5–7]. Cold inactivation of PPDK is due to the dissociation of the active tetrameric form to inactive monomers and dimers which can reassociate upon warming [6]. A recent investigation of the cold sensitivity of PPDK in a variety of plants revealed that the enzyme from *Flaveria brownii* is very cold-tolerant [5]. Bacterial expression studies with the PPDK from *F. brownii*, *F. bidentis* and maize PPDKs showed that the cold sensitivity of plant PPDKs is an intrinsic property of the protein and is the result of neither factors produced in the plant nor post-translational modification [8]. In this paper we describe results of experiments in which chimeric constructions of the cDNAs which encode the mature forms of PPDK in maize, *F. bidentis* and *F. brownii*, together with site-directed mutagenesis experiments, were used to determine those amino acid residues which affect the cold sensitivity of PPDK in plants.

2. Materials and methods

2.1. Plant material

Flaveria brownii, *F. bidentis* and maize were grown in the greenhouse in soil under natural illumination between 16 and 25°C.

2.2. Construction of E. coli expression vectors

Isolation of *F. brownii*, *F. bidentis* and maize PPDK cDNAs and construction of *E. coli* expression vectors pKK-brownii, pKK-bidentis and pKK-maize using the pKK233-2 Expression kit (Pharmacia-LKB) were as described previously [8]. Chimeric constructs were made by ligating together isolated fragments from specific plant cDNA clones.

2.2.1. Chimeras 001, 110, 011 and 100. *Nde*I-*Hin*dIII fragments of pKK-brownii and pKK-bidentis were reciprocally replaced with the corresponding fragments to produce pKK-001 (brownii/bidentis chimera) and pKK-110 (bidentis/brownii chimera). Similarly *Eco*RI-*Hin*dIII fragments of pKK-brownii and pKK-bidentis were reciprocally replaced to produce pKK-011 (brownii/bidentis chimera) and pKK-100 (bidentis/brownii chimera). Using *Eco*RI and *Nde*I sites on the plasmid pKK233-2, vector fragments were prepared by partial digestion in the above procedure.

2.2.2. Chimeras 1101 and 1110. A *Xho*I-*Hin*dIII fragment of pKK-110 was replaced with the corresponding fragment of pKK-bidentis to produce pKK-1101. A *Xho*I-*Hin*dIII fragment of pKK-bidentis was replaced with the corresponding fragment of pKK-brownii to produce pKK-1110.

2.2.3. Chimeras X01 and X10. For fragment exchange between shorter stretches of DNA, the linking PCR method [9] was used. A pair of primers, link-F: GCAGAGATGATGTTGGCAAG and link-R: CTTGCCAACATCATCTCTGC, which have complementary sequences to each other, were synthesized in the region where nucleotide sequences are identical in both *Xho*I-*Hin*dIII fragments. Using *Xho*I-*Hin*dIII fragments of pKK-brownii or pKK-bidentis subcloned into pBluescript II as templates, PCR was first conducted with two primer pairs link-F and M13 RV and M13 M4 and link-R. Four kinds of amplified fragments were gel purified. The M13 M4/link-R fragment of pKK-brownii and link-R/M13 RV fragment of pKK-bidentis were combined, and M4/link-R fragment of brownii and link-R/RV fragment of bidentis were combined, respectively. Using these as templates a second PCR was conducted with M13 M4 and M13 RV primers to link the fragments which cover the 5′- and the 3′-fragments. Amplified fragments were digested with *Xho*I and *Hin*dIII and subcloned into the corresponding sites of pKK-bidentis to produce pKK-X01 (brownii/bidentis) and pKK-X10 (bidentis/brownii).

2.2.4. Chimeras X101, X110. A *Pst*I site located between the linking point by PCR and *Hin*dIII site was utilised for recombination. A *Xho*I-*Pst*I fragment of pKK-X10 and a *Pst*I-*Hin*dIII fragment of pKK-bidentis were subcloned into the vector fragment of *Xho*I and *Hin*dIII digested pKK-bidentis in a three fragment ligation reaction to produce pKK-X101. Similarly a *Xho*I-*Pst*I fragment of pKK-bidentis and a *Pst*I-*Hin*dIII fragment of pKK-brownii were subcloned into pKK-bidentis to produce pKK-X110.

2.2.5. Chimeras of maize and F. brownii PPDK. PCR was performed to introduce the *Nde*I restriction site into maize PPDK cDNA with forward primer PPDK-F: CTCACTGTTCGAAGA-

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GAAGC and reverse primer mNde: CATATGCTCTGTCCGGCATAATC using pKK-maize as template. Amplified fragment was subcloned into pCRII (Invitrogen) and re-excised with *Sac*I and *Nde*I. The fragment was ligated into *Nde*I-blunt-ended *Hin*dIII fragment of pKK-brownii and *Sac*I-*Sma*I digested vector fragment of pKK-maize (three fragment ligation) to produce pKK-m/b(N). Similarly for introduction of a *Xho*I restriction site into maize PPDK cDNA, PCR was performed with forward primer PPDK-F and reverse primer mXho: CTCGAGGGATCTCAATCATTG using pKK-maize as template. Amplified fragment was subcloned into pCRII and re-excised with *Sac*I and *Xho*I. The fragment was ligated to *Sac*I-*Xho*I digested vector fragment of pKK-m/b(N) to produce pKK-m/b(X).

2.2.6. Point mutations. Site-directed mutagenesis was performed by Kunkel's method [10] using a Mutan-K kit and a Megalabel kit (Takara, Japan). *Xho*I-*Hin*dIII fragments of pKK-brownii and pKK-bidentis were subcloned in pBluescript II and single-stranded DNAs were prepared from *E. coli* strain CJ236. Oligonucleotides used for brownii to bidentis type mutations were 800PQ: GAAAGATAAATCTGCAAAAACTTG, 804SA: GCCTTGAGCAAGATAAATC, 816LI: TTCTGGTCAATAACCTCAAATG and 883VI: GCTTAAACAATGACTTGTGC. Oligonucleotides used for bidentis to brownii type mutations were 800QP: CAAGATAAATCGGCAAAAACTTG, 804AS: GAATGCCTTGAGAAAGATAAATC, 816IL: CTTTCTGGTCAAGAACCTCAAATG and 883IV: GCTTAAACAACGACTTGTGC. Mutated bases were confirmed by DNA sequencing and the fragments were re-excised with *Xho*I and *Hin*dIII and recloned into the corresponding sites of pKK-1110 (brownii to bidentis mutants) or pKK-bidentis (bidentis to brownii mutants). Point mutation of maize PPDK was performed by PCR mutagenesis. A fragment amplified by PCR in the presence of forward primer PPDK-F and reverse primer mLV: GATATCAGACAACCACCTGAGCTG from pKK-maize template was subcloned into pCRII. A *Sma*I-*Eco*RV fragment was excised from pCRII and inserted into *Sma*I site of pKK-maize to produce pKK-mLV.

2.3. Bacterial expression studies

Extracts from 10 ml bacterial cultures were prepared and PPDK activity and the cold inactivation of PPDK was assayed as described previously [8]. To eliminate interference from bacterial lactate dehydrogenase (LDH), kinetic constants were determined using PPDK which was partially purified on an hydroxyapatite column (1×10 cm) eluted with a phosphate gradient from 10 to 50 mM. Different forms of PPDK eluted at different P_i concentrations but all forms eluted prior to elution of LDH.

2.4. Gel filtration

Pyruvate, P_i dikinase was extracted from 0.6 g of leaves with 1 ml of extraction buffer (50 mM HEPES-KOH, pH 7.5, 10 mM $MgSO_4$, 2 mM phosphate, 1 mM pyruvate, 1 mM EDTA, 5 mM DTT, 0.5% (w/v) sodium isoascorbate and 2% (w/v) polyclar AT). The extract was passed through two layers of Miracloth and the filtrate was centrifuged at 10000×*g* for 15 min at 4°C. The supernatant was loaded onto a column (1.0 cm×4.0 cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden) equilibrated with gel filtration buffer (50 mM HEPES-KOH, pH 7.0, 2 mM phosphate, 10 mM DTT) containing 10 mM $MgCl_2$ and 2 mM EDTA (A) or gel filtration buffer containing 10 mM EDTA (B), and eluted with the same buffer. The eluate (100 μl) was loaded onto a Superose 6 column (Pharmacia) equilibrated with gel filtration buffer (A) or (B) and washed with the same buffer. The flow rate was 0.4 ml/min and the fraction size was 0.4 ml. A portion (7.5 μl) of each fraction was subjected to SDS-PAGE and PPDK was detected by Western blot analysis as described previously [8]. The column was calibrated with a mixture of thyroglobulin (670 kDa), gamma-globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). The m/b(N) chimera enzyme was produced from 10 ml bacterial cultures as described previously [8]. Buffer exchange, gel filtration and Western blot analysis were carried out as described above.

3. Results

3.1. Chimeric constructions

Alignment of the deduced amino acid sequences of mature forms of *F. brownii* (cold-tolerant) and *F. bidentis* (cold-sensitive) PPDKs indicate that the two proteins are highly homologous with no gap in the alignment (Fig. 1). *F. brownii* PPDK is eight residues longer than the *F. bidentis* protein at the N-terminal end due to the different processing site of the transit peptide [8]. The amino acid sequences of the PPDKs from the two species differ at 40 residues with few differences in the central region of the protein which contains the active site of the enzyme (Fig. 1). It is unlikely that all 40 amino acid substitutions influence the cold sensitivity of the protein. In an attempt to identify the amino acid residues important in controlling the cold stability of *F. brownii* PPDK, in vivo expression studies were conducted using chimeric constructions of *F. brownii* and *F. bidentis* PPDK cDNAs. In initial experiments we utilized *Eco*RI and *Nde*I restriction sites which are conserved in both sequences to divide the cDNAs into three regions: N-terminal, central and C-terminal (Fig. 1). Four kinds of chimeric constructs were made by exchanging these fragments (Fig. 2) and these were expressed in *E. coli*. In all cases PPDK activity was detected in crude extracts and the products, which react with antibody raised against maize PPDK, were confirmed on Western blots to have a molecular mass of about 94 kDa (data not shown). The cold stability of each chimeric protein was assayed by measuring the rate of inactivation on ice (Fig. 3A). Enzymes containing the C-terminal region of *F. brownii* (chimeras 100 and 110) demonstrated cold tolerance comparable to the original *F. brownii* PPDK. Conversely, enzymes possessing the C-terminal region of *F. bidentis* (chimeras 011 and 001) were cold-labile. The C-terminal regions of *F. bidentis* and *F. brownii* PPDK cDNA differ by 16 amino acid residues of which only three are non-conservative changes. In subsequent experiments the C-terminal region was divided into two parts utilizing a unique *Xho*I site (conserved in both cDNAs) and a pair of chimeric constructs were made (Fig. 2). Chimera 1110, which has the rear part of the C-terminal region carrying seven amino acid substitutions, exhibited cold tolerance while chimera 1101, which has the front part of the C-terminal region, did not (Fig. 3B). To investigate whether the C-terminal region of *F. brownii* PPDK can confer cold stability on PPDK of a different plant species, other chimeric constructs were made using the PPDK cDNA of maize, a C_4 monocot species. The amino acid sequence homology between *F. brownii* and maize PPDK is 77% with amino acid substitutions scattered along the sequence. As with *F. bidentis* there is no gap in the alignment except at its N-terminal processing site [8]. *Nde*I and *Xho*I restriction sites were introduced at the corresponding positions of the maize cDNA sequence and constructs were made which express chimeric enzymes possessing the C-terminal region or its rear part of the *F. brownii* PPDK. Products were fully active and as cold-tolerant as native *F. brownii* PPDK (Fig. 3C).

K_m values in the direction of PEP formation were determined for *F. brownii* and *F. bidentis* PPDKs and some of the chimeric PPDKs expressed in *E. coli*. In general K_m values for pyruvate, phosphate and ATP were slightly lower for enzymes expressed in *E. coli* compared to enzyme extracted from leaf material (Table 1) indicating that the affinity for the substrates was not impaired by chimerization of the protein.

3.2. Identification of the amino acid residues responsible for cold tolerance

The rear part of the C-terminal region of PPDK was further divided into two parts carrying three and four amino acid substitutions, respectively, and examined in a background of

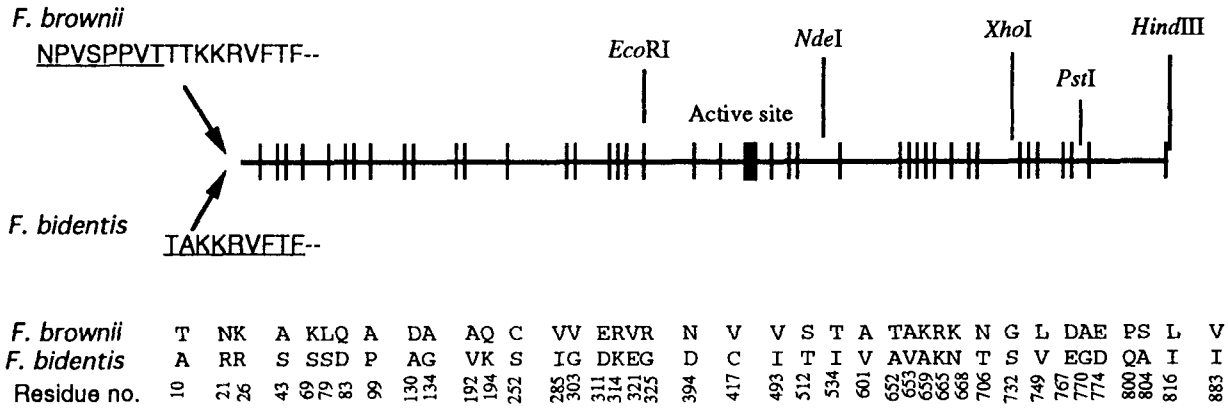

Fig. 1. A schematic comparison of *F. brownii* and *F. bidentis* PPDK cDNA showing the location of amino acid differences (vertical lines on the diagram and single letters below). The amino acid residues determined by N-terminal sequencing of the mature forms of PPDK are underlined and the restriction enzyme sites used in the construction of cDNA chimeras are indicated. The residue numbers for the non-conserved amino acids are shown below the alignment of non-conserved amino acids.

F. bidentis PPDK. The results show that the most C-terminal part of *F. brownii* PPDK cDNA was sufficient to confer cold tolerance on *F. bidentis* PPDK (Fig. 4, chimera X10). On the other hand, although chimera X01 (containing the penultimate C-terminal part of PPDK) was cold-labile it exhibited enhanced cold tolerance compared with native *F. bidentis* PPDK, suggesting that amino acid residues contained in chimera X01 may also affect cold lability of PPDK. The four amino acid substitutions contained in the most C-terminal part were examined more precisely using point mutations. First, brownii to bidentis form mutations were introduced into a chimera 1110 background. All four mutants exhibited cold tolerance (data not shown), indicating that more than two amino acid residues are involved in conferring cold tolerance. Secondly, bidentis to brownii mutations were introduced into a *F. bidentis* PPDK background and the cold tolerance was assayed (Fig. 4). The 804AS mutant was as cold-sensitive as the original *F. bidentis* PPDK, and the 800QP mutant acquired cold tolerance a little lower than *F. brownii* PPDK. Combination of the 804AS and the 800QP mutations was only as effective as the 800QP mutation in influencing the cold tolerance of PPDK. The 816IL mutant showed enhanced cold tolerance, and in combination with the 883IV mutation gave more tolerance, which was a little lower than X10. From these results, we concluded that three residues 800P, 816L and 883V are involved in conferring cold tolerance on *F. brownii* PPDK. In maize PPDK, two of the three residues identified above (800P and 816L) are the same as found in *F. brownii* PPDK and its cold stability is intermediate to the two *Flaveria* enzymes. The leucine residue at amino acid 883 of maize PPDK was converted to a valine residue and the cold tolerance of the expressed PPDK was assayed. The expressed protein was as cold-sensitive as the non-mutated form (Fig. 4).

3.3. Subunit association in F. brownii PPDK

The cold sensitivity of maize PPDK has been well characterized with the dissociation of the active tetramer to inactive dimers and monomers associated with a decrease in enzyme activity [6,11]. It has also been demonstrated that PPDK requires Mg^{2+} ions for tetramer formation [7,12]. We compared the subunit association/dissociation of both cold-tolerant and cold-sensitive types of PPDKs by gel filtration at room temperature and at 4°C. At room temperature the elution patterns of the cold-tolerant and the cold-sensitive PPDKs were the same. When cold-tolerant PPDK (e.g. *F. brownii* or m/b(X)) was subjected to gel filtration at 4°C the elution pattern was the same as that obtained at room temperature. However, the cold-labile enzymes (*F. bidentis* and maize PPDK) exhibited a change in elution pattern at the lower temperature with an apparent molecular weight about half of that observed at room temperature, indicating dissociation of the enzyme into dimers. The effect of EDTA on the molecular weight of PPDK was also tested by gel filtration at room temperature. The cold tolerant PPDKs exhibited the same elution profile in both the presence and absence of 10 mM EDTA. In contrast,

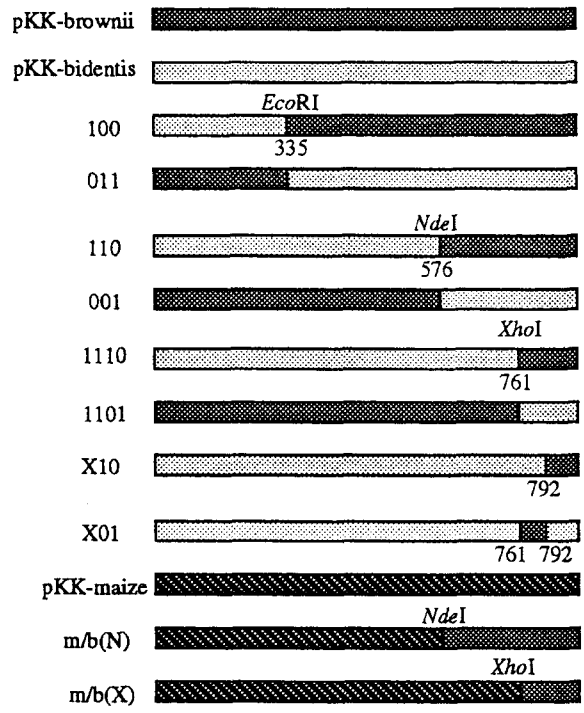

Fig. 2. Schematic diagram of chimeric PPDK cDNAs. Chimeric cDNAs were constructed from *F. brownii* (0) and *F. bidentis* (1) cDNA using the restriction enzymes indicated. Chimeras were also constructed using maize PPDK cDNA and the 3′-region of *F. brownii* PPDK cDNA. Numbers below the line indicate the location of the amino acid residue at which chimeric constructions were made.

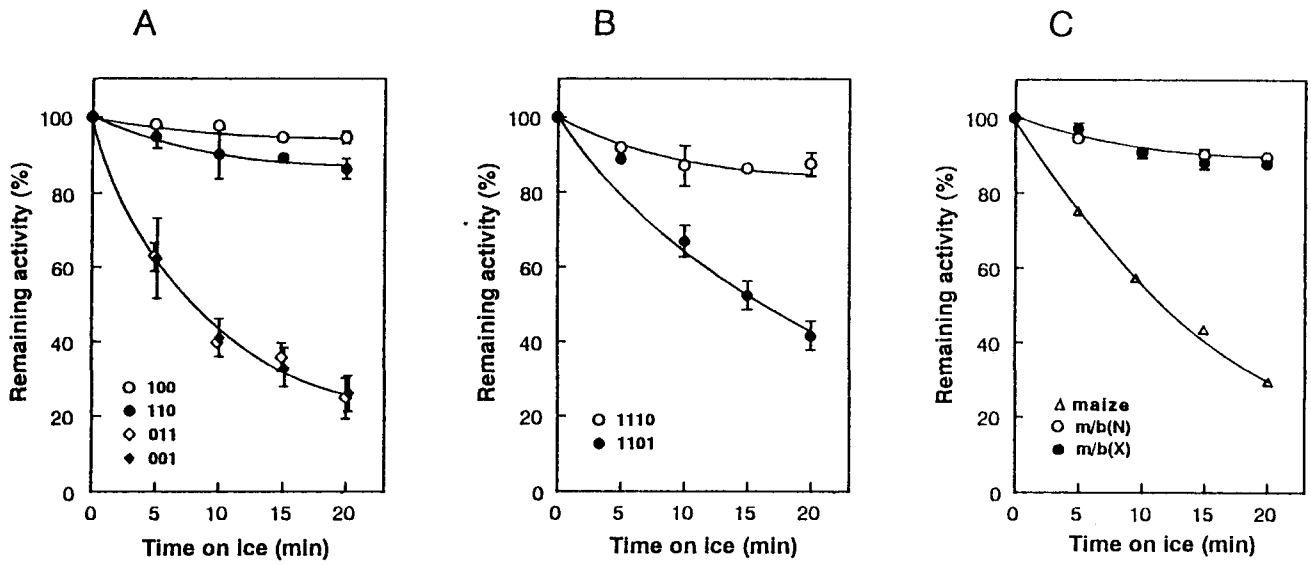

Fig. 3. Cold inactivation of chimeric PPDKs. Chimeric PPDK cDNA constructs were expressed in *E. coli* and the stability of the expressed enzyme determined at 0°C. The notations used to describe the chimeras are the same as those used in Fig. 2. The cold inactivation of maize leaf PPDK is also included.

the cold-sensitive forms of PPDK dissociated into dimers in the presence of 10 mM EDTA. These data indicate that cold-tolerant PPDK does not dissociate either in cold temperatures or in the presence of 10 mM EDTA.

4. Discussion

There are a group of proteins which become less active at cold temperatures and this loss of activity is often correlated with dissociation of multimeric proteins into smaller units [13]. As a general rule PPDK from plants generally fall into this group of proteins losing activity due to the cold-dependent dissociation of the active tetrameric form. However, the PPDK from *F. brownii* appears to be remarkably resistant to cold inactivation. Gel filtration studies showed that *F. brownii* PPDK does not dissociate either at low temperatures or in the presence of EDTA. These results are consistent with the suggestion that the cold sensitivity of PPDK is influenced by the affinity of the subunits for the central chelating Mg^{2+} ion and/or for each other, and that the amino acid residues involved in conferring cold tolerance are involved either directly or indirectly in subunit association. Two out of the three amino acid residues shown to be involved in conferring cold tolerance on plant PPDKs are hydrophobic residues and changes of these residues have great effect on the cold sensitivity of the enzyme despite conservative substitutions; this might suggest that hydrophobic interactions also play an important role in subunit association of plant PPDKs.

Plant PPDKs require Mg^{2+} ions for the formation of the active tetrameric enzyme [7]. Our data show that the cold-tolerant form of PPDK is not only tolerant to cold temperature but it is also resistant to inactivation with a chelating agent. It is not clear how Mg^{2+} ions are involved in subunit association. One possibility is that the four PPDK monomers are arranged around a central chelating Mg^{2+} ion, and that the cold sensitivity of PPDK is affected by the affinity of the monomeric subunits for the Mg^{2+} ion. In contrast to plants, bacterial PPDK is active as a dimer and does not require Mg^{2+} ions for enzyme stability.

It has been shown previously that the rate of inactivation of maize PPDK at 0°C varies with Mg^{2+} concentration and that the cold-induced inactivation of PPDK is completely prevented by pyruvate and phosphoenolpyruvate [6]. A recent report, that the pyruvate binding domain of bacterial PPDK resides within the C-terminal domain of the PPDK molecule [14], is consistent with the involvement of the C-terminal region of plant PPDK being critically involved in cold sensitivity since the bacterial and plant PPDKs share a considerable level of homology.

The physiological significance of the cold inactivation of PPDK in C_4 plants is yet to be fully understood. Most of the *Flaveria* C_3-C_4 intermediate species are commonly found

Table 1
Apparent K_m values for pyruvate, ATP and P_i for *F. brownii*, *F. bidentis* and maize PPDK expressed in *E. coli* and extracted from leaf material

Enzyme source	K_m (μM)					
	Pyruvate		ATP		P_i	
	Leaf	*E. coli*	Leaf	*E. coli*	Leaf	*E. coli*
F. brownii	67	32	88	50	341	256
F. bidentis	73	59	25	49	118	138
Maize	158	65	95	47	408	134
1/3 bidentis+2/3 brownii	–	73	–	73	–	159
2/3 maize+1/3 brownii	–	101	–	121	–	69

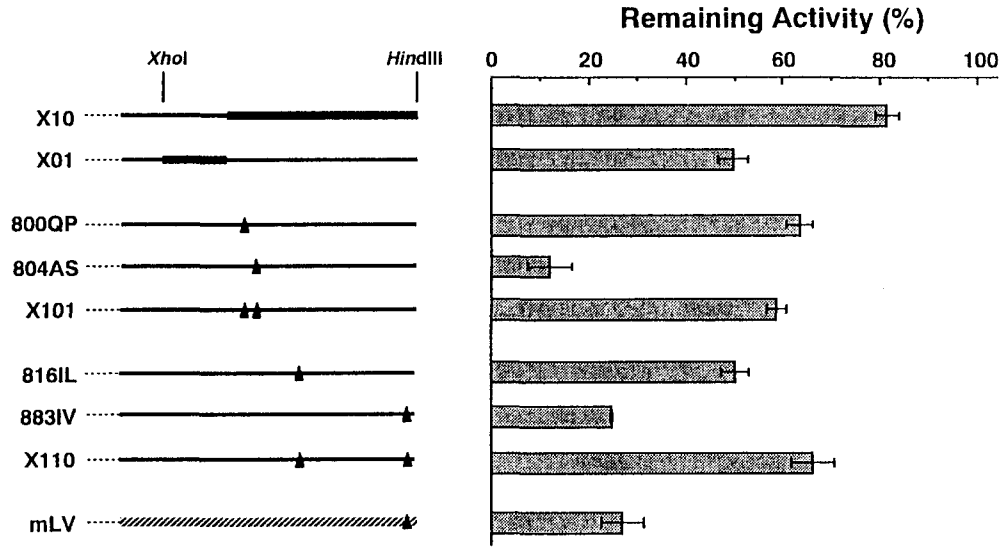

Fig. 4. The relative cold sensitivity of chimeric constructs and point mutations of plant PPDKs. Chimera X10 contained the C-terminal part of the *Xho*I/*Hin*dIII section of *F. brownii* PPDK in a *F. bidentis* PPDK background; chimera X01 contained the N-terminal end of the *Xho*I/*Hin*dIII section of *F. brownii* PPDK in a *F. bidentis* PPDK background. Single point mutations were constructed in a *F. bidentis* PPDK background.

in the coastal regions of Florida and Texas and in the arid tropical regions of Mexico [15]. It is therefore unlikely that *F. brownii* PPDK has evolved a cold-tolerant form of PPDK in response to the selective pressures of a cold climate. Since the kinetic properties of PPDK appear to be unaffected by amino acid substitutions within the C-terminal region of the enzyme, amino acid substitutions which confer cold tolerance on the protein may have accumulated in the PPDK gene without any deleterious effect. The fact that the maize enzyme into which a brownii-type point mutation was introduced showed no improvement in the cold tolerance of the enzyme reveals the importance of the surrounding amino acid sequence.

Data presented in this paper indicate that the C-terminal region of the PPDK subunit strongly influences the interaction of the subunits, their association into a tetramer and the cold tolerance of plant forms of the enzyme. Future experiments will be conducted to assess the effect amino acid changes introduced to confer cold tolerance on PPDK have on the kinetic and regulatory characteristics of the modified protein. At the same time the protein will be crystallized and the effect of amino acid substitution on the three-dimensional structure of the protein will be determined. This should provide information about how the PPDK subunits interact.

References

[1] Slack, C.R. and Hatch, M.D. (1967) Biochem. J. 103, 660–665.
[2] O'Leary, M.L. (1982) Annu. Rev. Plant Physiol. 33, 297–315.
[3] Aoyagi, K. and Nakamoto, H. (1985) Plant Physiol. 78, 661–664.
[4] Edwards, G.E., Nakamoto, H., Burnell, J.N. and Hatch, M.D. (1985) Annu. Rev. Plant Physiol. 36, 255–286.
[5] Burnell, J.N. (1990) Plant Cell Physiol. 31, 295–297.
[6] Hatch, M.D. (1979) Aust. J. Plant Physiol. 6, 607–619.
[7] Sugiyama, T. (1973) Biochemistry 12, 2862–2868.
[8] Usami, S., Ohta, S., Komari, T. and Burnell, J.N. (1995) Plant Mol. Biol. 27, 969–980.
[9] Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 61–68.
[10] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367.
[11] Shirahashi, K., Hayakawa, S. and Sugiyama, T. (1978) Plant Physiol. 62, 826–830.
[12] Hatch, M.D. and Slack, C.R. (1968) Biochem. J. 106, 141–146.
[13] Bock, P.E. and Frieden, C. (1978) Trends Biochem. Sci. 3, 100–103.
[14] Carroll, L.J., Xu, X., Thrall, S.H., Martin, B.M. and Dunaway-Mariano, D. (1994) Biochemistry 33, 1134–1142.
[15] Powell, A.M. (1978) Annu. Missouri Bot. Gard. 65, 590–636.