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CONTROL OF THE ACTIVATION/INACTIVATION OF PYRUVATE,Pi DIKINASE FROM THE C_4 PLANT MAIZE BY ADENYLATE ENERGY CHARGE, PYRUVATE, AND ANALOGS OF PYRUVATE*

H. Nakamoto and G. E. Edwards †

Botany Department and Institute of Biological Chemistry
Washington State University, Pullman, Washington 99164-4230 U.S.A.

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SUMMARY: Pyruvate,Pi dikinase, which is localized in the mesophyll chloroplasts of C_4 plants, requires a high adenylate energy charge for conversion of the enzyme from the inactive to the active form. The inactivation process is favored by a low energy charge, being maximal at values below 0.7. Pyruvate and analogs of pyruvate, oxamate and oxalate, strongly inhibit the inactivation process at millimolar levels. The results suggest that light activation of the enzyme in vivo may be mediated by an increased adenylate energy charge in the chloroplast. Pyruvate may allow a higher steady-state level of activation to be achieved in vivo by inhibiting inactivation.

Pyruvate,Pi dikinase (EC 2.7.9.1; PPDK), a key enzyme in C_4 photosynthesis, is located in the mesophyll chloroplasts of C_4 plants and catalyzes the conversion of pyruvate to PEP via the following reaction: pyruvate + ATP + Pi $\rightleftarrows$ PEP + AMP + PPi. It is activated in the light and inactivated in the dark (1). Based on studies in vitro, a protein factor is required for activation/inactivation and may serve as a catalyst for interconversion between the two forms of the enzyme (2, 3, 4, 5). ADP and very low levels of ATP (less than 1 μM) are required for inactivation of the enzyme; Pi is required for activation (3, 4). In the present study, we find that activation/inactivation of PPDK is controlled by adenylate energy charge, and further influenced by pyruvate, oxamate and oxalate. The results are discussed in relation to what changes may occur in the chloroplast during dark/light transitions to mediate enzyme activation.

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†To whom correspondence should be addressed.
Abbreviations: PPDK, pyruvate,Pi dikinase; PEP, phosphoenolpyruvate; DTE, dithioerythritol; BSA, bovine serum albumin.

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METHODS

Stromal extracts were obtained from maize mesophyll chloroplasts after their isolation from leaves of two week old plants of Zea mays, basically as previously described (6). In order to obtain most of the PPDK in its active form in stromal extracts, chloroplasts were isolated from illuminated leaves. Immediately after rupturing chloroplasts through freeze-thawing, 2 mM K_2HPO_4 was added and the extract centrifuged at 15°C for 15 min at 30,000g. The supernatant solution was treated on Sephadex G-25 columns pre-equilibrated with the buffer containing 20 mM Tris-HCl, pH 8.0, 5 mM DTE, 5 mM $MgCl_2$ and 0.2 mM EDTA. In order to obtain PPDK in its inactive form in chloroplast extracts, chloroplasts were prepared from darkened leaves (dark-treatment for two and one half hours). Immediately after rupturing chloroplasts through freeze-thawing, 1 mM ADP was added and the stromal extracts were treated on Sephadex G-25 as described above. Studies on the inactivation or activation of the enzyme from these preparations were initiated by the addition of stromal extracts to assay media. Pyruvate,Pi dikinase activity was determined as described previously (6).

Establishment of adenylate energy charge in the reaction media was carried out by incubation of ATP and AMP in a molar ratio giving the desired energy charge in a solution containing 20 mM Tris-HCl pH 8.0 and 12 mM $MgCl_2$, with 2 units of adenylate kinase/100 µl of incubation mixture for 30 min at 23°C prior to addition of K_2HPO_4, oxamate and the stromal extract.

RESULTS AND DISCUSSION

Pyruvate,Pi dikinase (PPDK) was extracted in an active form from chloroplasts which were isolated from preilluminated plants of maize. After desalting the extract with Sephadex G-25, the influence of the addition of ATP, ADP and AMP on inactivation was determined (Fig. 1). There was no inactivation in the presence of ATP or AMP, even after a long incubation period. The addition of ADP caused inactivation of the enzyme with a half-time of about 25 min. Since adenylate kinase is present in high activity in the stroma of maize chloroplasts (7), the ADP will be partially converted to AMP and ATP (at equilibrium the energy charge is approximately 0.5). These results are consistent with previous reports which indicate that ADP, plus low levels of ATP, are required for inactivation of the enzyme (3).

The effect of a number of factors on the inactivation of PPDK was determined (Table I). Pyruvate was a strong inhibitor of inactivation as previously shown (8). Oxalate and oxamate, analogs of pyruvate, also inhibited the inactivation of the enzyme. As shown with oxalate, this inhibition is not due to a chelation effect, since 4 mM oxalate inhibited similarly in the presence of 4 mM or 20 mM $MgCl_2$. PEP (5 mM) caused partial

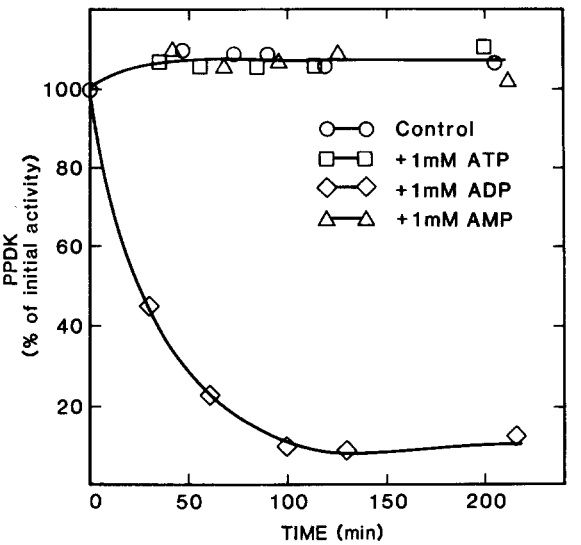

Fig. 1. The influence of addition of ATP, ADP, and AMP on the state of activation of PPDK in stromal extracts from maize mesophyll chloroplasts. Maize plants were preilluminated prior to isolation of chloroplasts in order to obtain the enzyme in an activated state. For the control treatment the reaction medium contained 16 mM Tris-HCl, pH 8.0, 4 mM DTE, 4 mM $MgCl_2$, 0.16 mM EDTA and 0.32% BSA. The medium was incubated at 23°C. In the treatment containing 1 mM ATP, 2 mM creatine phosphate and 5 units of creatine phosphate kinase/500 µl assay were added in order to recycle ADP to ATP in the event some hydrolysis of ATP occurred during the treatment.

inhibition of inactivation, while pyridine nucleotides (5 mM) had little effect. Previously, in testing a number of metabolites or cofactors on the activation of PPDK, it was found that AMP, ADP and PPi inhibited the process. Pyridine nucleotides had little effect on activation of the enzyme (4). The above results suggest that the relative level of adenylates and pyruvate might be major factors in controlling the activation of PPDK *in vivo*.

Oxalate, a structural analog of enolpyruvate, inhibits the reaction catalyzed by PPDK from *Bacteroides symbiosus* (9), green immature grains of *Triticum aestivum* (10) and leaves of *Digitaria sanguinalis*, a C_4 plant (Nakamoto and Edwards, unpublished), and the inhibitions are competitive with respect to pyruvate. Oxamate is also a competitive inhibitor of PPDK from *D. sanguinalis* with respect to pyruvate (Nakamoto and Edwards, unpublished).

To evaluate how inactivation of PPDK may be effected by the adenylate energy charge, stromal extracts were obtained from maize mesophyll chloroplasts after their isolation from illuminated (to obtain the active

Table I. The effect of various metabolites on the degree of inactivation of PPDK in stromal preparations from maize chloroplasts. The active enzyme was obtained from maize mesophyll chloroplasts isolated from preilluminated plants. The medium for inactivation contained 0.5 mM ADP, 16 mM Tris-HCl pH 8.0, 4 mM DTE, 4 mM $MgCl_2$, 0.16 mM EDTA and 0.32% BSA. After addition of metabolites, the reaction mixtures were incubated for 90 min at 23°C. The reactions were terminated by passing the mixtures through Sephadex G-25 columns pre-equilibrated with the buffer containing 20 mM Tris-HCl, pH 8.0, 5 mM DTE, 5 mM $MgCl_2$ and 0.2 mM EDTA. The influence of a given metabolite on the degree of inactivation, as a percentage of the ADP dependent inactivation, was calculated as:

$$\frac{\text{Activity}_{(-\text{ADP})} - \text{Activity}_{(+\text{ ADP + metabolite added})}}{\text{Activity}_{(-\text{ ADP})} - \text{Activity}_{(+\text{ ADP})}} \times 100.$$

Metabolite added	Concentration mM	Degree of inactivation % of control
NAD	5	92
NADH	5	100
NADP	5	94
NADPH	5	79
$MgCl_2$	20	83
Oxalate	4	21
Oxalate (4 mM) + $MgCl_2$	20	21
Oxalate	16	13
Oxalate	40	10
Phosphoenolpyruvate	5	59
PPi	5	84
Pyruvate	5	9
Oxamate	5	38

enzyme) leaves of maize. The total concentration of adenylates used was 0.5 mM, which is similar to that found in C_4 mesophyll chloroplasts (11). As shown in Fig. 2, at an adenylate energy charge of 0.3 to 0.6, there was maximal degree of inactivation of PPDK (measured after 60 min). Above an energy charge of 0.6, there was a rapid drop in the degree of inactivation of PPDK up to an energy charge of 1.0. The Km for ADP for inactivation is about 55 μM, while the requirement for ATP is less than 1 μM (3). Therefore, even at low energy charge values there will be sufficient ATP for inactivation, whereas at high energy charge values ADP may become limiting. In the

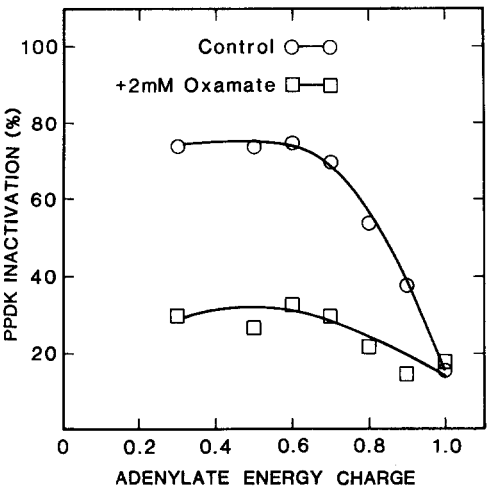

Fig. 2. The influence of adenylate energy charge and oxamate on the inactivation of PPDK. Stromal extracts containing active PPDK were obtained from maize mesophyll chloroplasts isolated from preilluminated plants. The incubation mixture contained 16 mM Tris-HCl pH 8.0, 4 mM DTE, 4 mM $MgCl_2$, 0.16 mM EDTA, 0.32% BSA and 2 mM K_2HPO_4. When added, the concentration of adenylates was 0.5 mM. After addition of Pi, adenylates, and oxamate as appropriate, the reaction mixtures were incubated for 60 min at 23°C. The reactions were terminated by passing the mixtures through Sephadex G-25 columns pre-equilibrated with the same buffer as used in Table I. The percentage inactivation of PPDK by adenylates was calculated as:

$$\frac{\text{Activity}_{(+\ Pi)} - \text{Activity}_{(+\ Pi\ +\ \text{adenylates})}}{\text{Activity}_{(+\ Pi)}} \times 100$$

In the presence of oxamate the percentage inactivation of the enzyme was calculated as:

$$\frac{\text{Activity}_{(+\ Pi\ +\ \text{oxamate})} - \text{Activity}_{(+\ Pi\ +\ \text{adenylates}\ +\ \text{oxamate})}}{\text{Activity}_{(+\ Pi\ +\ \text{oxamate})}} \times 100$$

presence of 2 mM oxamate there was inhibition of the degree of inactivation of PPDK throughout the energy charge range of 0.3 to 1.0 (Fig. 2).

The influence of energy charge on activation of PPDK was studied using the inactive form of the enzyme obtained from stromal extracts of maize mesophyll chloroplasts isolated from predarkened leaves. Figure 3 shows that activation of the enzyme is dependent on adenylate energy charge in an opposite manner to that of inactivation. The degree of activation of PPDK (after 60 min) increased with increasing energy charge from 0.5 up to a value of 1.0. The degree of activation in the presence of adenylates is expressed as a percentage of that in the absence of adenylates. Even with an energy charge of 1.0, with all of the adenylates in the form of ATP, the degree of

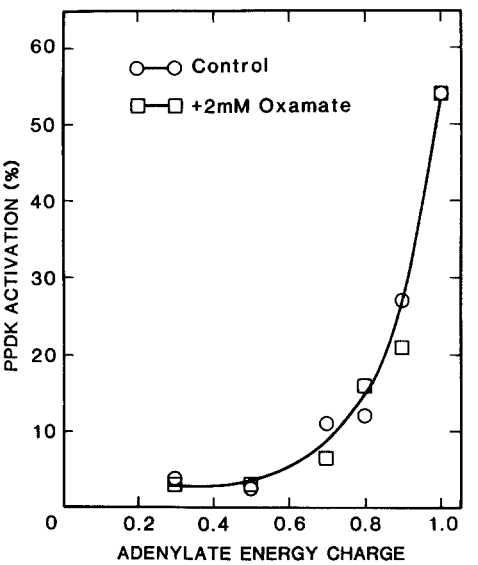

Fig. 3. The influence of adenylate energy charge and oxamate on the activation of PPDK. Stromal extracts containing inactive PPDK were obtained from maize mesophyll chloroplasts isolated from predarkened plants. The incubation mixture was the same as used in Fig. 2 for analysis of inactivation. After appropriate additions, the reaction mixtures were incubated for 60 min at 23°C. The reactions were terminated by passing the mixtures through Sephadex G-25 columns pre-equilibrated with the same buffer as used in Table I. In each treatment, the activation in the presence of Pi was subtracted from a corresponding treatment without Pi. Since in the absence of Pi there was little or no activation, the correction was minor. The percentage activation of PPDK in the presence of adenylates, relative to that without adenylates, was calculated as:

$$\frac{\text{Activity}_{(+\ Pi\ +\ \text{adenylates})}}{\text{Activity}_{(+\ Pi)}} \times 100$$

In the presence of oxamate the percentage activation of the enzyme was calculated as:

$$\frac{\text{Activity}_{(+\ Pi\ +\ \text{adenylates}\ +\ \text{oxamate})}}{\text{Activity}_{(+\ Pi\ +\ \text{oxamate})}} \times 100$$

activation was less than that in the absence of ATP. ADP, AMP and to a lesser extent, ATP, when considered individually, all have an inhibitory effect on the activation of PPDK (4). When considered together, obviously the adenylates are most inhibitory at low energy charge values (Fig. 3).

Oxamate had no effect on activation of $\bar{P}$PDK (Fig. 3), which is in contrast to its strong inhibition of inactivation (Fig. 2). This is consistent with a previous report which shows that pyruvate had no effect on *in vitro* activation of PPDK (4). Oxamate and oxalate, analogs of pyruvate, may bind to the active form of the enzyme and prevent the protein mediated

inactivation. Perhaps the inactive form of the enzyme cannot bind the substrate pyruvate, or its analogs, and thus the recognition of the enzyme by the activating protein factor would not be altered. However, the mechanism of interconversion of the enzyme between its active and inactive form needs to be elucidated in order to understand whether various effectors are modifying the enzyme or the protein factor.

It is clear from the present results that activation of the enzyme will be favored *in vivo* when the energy charge in the chloroplast is high, as is expected during illumination; and that pyruvate, a substrate of the enzyme, may facilitate a higher degree of activation over a range of energy charge values by inhibiting the inactivation process. In this respect, it is of interest that the levels of pyruvate in maize leaves when measured over a 24 h period is much higher in the light than in the dark (R. Kanai, personal communication).

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