The Effect of Adenine Nucleotides on Purified Phosphoenolpyruvate Carboxylase from the CAM Plant Crassula argentea

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ABSTRACT

The effects of adenine nucleotides on phosphoenolpyruvate carboxylase were investigated using purified enzyme from the CAM plant, Crassula argentea. At 1 millimolar total concentration and with limiting phosphoenolpyruvate, AMP had a stimulatory effect, lowering the $K_m$ for phosphoenolpyruvate, ADP caused less stimulation, and ATP decreased the activity by increasing the $K_m$ for phosphoenolpyruvate. Activation by AMP was not additive to the stimulation by glucose 6-phosphate. Furthermore, AMP increased the $K_m$ for glucose 6-phosphate. Inhibition by ATP was competitive with phosphoenolpyruvate. In support of the kinetic data, fluorescence binding studies indicated that ATP had a stronger effect than AMP on phosphoenolpyruvate binding, while AMP was more efficient in reducing glucose 6-phosphate binding. As free Mg$^2+$ was held constant and saturating, these effects cannot be ascribed to Mg$^2+$ chelation. Accordingly, the enzyme response to the adenylate energy charge was basically of the “R” type (involving enzymes of ATP regenerating sequences) according to D. E. Atkinson’s (1968 Biochemistry 7: 4030–4034) concept of energy charge regulation. The effect of energy charge was abolished by 1 millimolar glucose 6-phosphate. Levels of glucose 6-phosphate and of other putative regulatory compounds of phosphoenolpyruvate carboxylase were determined in total leaf extracts during a day-night cycle. The level of glucose 6-phosphate rose at night and dropped sharply during the day. Such a decrease in glucose 6-phosphate concentration could permit an increased control of phosphoenolpyruvate carboxylase by energy charge during the day.

Investigations of AdN$^3$ effects on crude extracts of PEPC (EC 4.1.1.31) from C$_4$ plants have previously shown the potential relationship between energy charge and enzyme activity (5, 11, 19). In their original paper, Wong and Davies (19) showed a decrease in $K_m$ for PEP of the enzyme in the presence of AMP and an inhibition of the enzyme by ATP. Although these authors used a fully saturating concentration of metal cations (16.7 mM MgCl$_2$), their work was subsequently reinterpreted (5) as showing the effect on PEPC of metal chelation by AdN. Coombs et al. (5) clearly established that due to the low $K_m$ of AdN for Mg$^2+$, nucleotides could indeed nonspecifically inhibit the enzyme by metal chelation. In an attempt to overcome this side effect of nucleotides, these latter authors used a Mg$^{2+}$-buffered medium consisting of a mixture of glycerol 1-phosphate (50 mM) and MgCl$_2$ (30 mM) supposed to provide an 8 mM free Mg$^{2+}$ solution in the presence of 5 mM ATP (3) and did not observe any major effect of the AEC on enzyme activity. However, as sugar phosphates strongly interfere with the regulatory properties of PEPC (14), the presence of 50 mM glycerol 1-phosphate casts doubt on this latter observation. In addition, the use of high Mg$^{2+}$ levels (16.7–30 mM) leads to a very rapid rate of nonenzymatic oxaloacetate decarboxylation and thus to an underestimation of the true rate of enzyme activity as measured by a standard coupled assay with MDH or by $^1$CO$_2$ fixation (13, 18). More recently, Lavergne and Champigny (11) have provided new evidence for a true control of PEPC from Pennisetum americanum L. and from corn and sorghum by AEC at low PEP concentrations and saturating levels of Mg$^{2+}$. In addition, they found that a crude preparation of PEPC from C$_4$ plants (Triticum aestivum L., Spinacia oleracea L., Pism sativum L.) did not respond to AEC.

The scarcity of information on an effect of adenine nucleotides on PEPC from CAM plants and the availability of computer programs permitting calculation of the composition of reaction media maintaining constant free levels of metal and anions (7) and of a new assay for PEPC activity allowing the use of high Mg$^{2+}$ levels during experimentation (13) were an incentive to investigate the effect of AEC on purified PEPC from the CAM plant, Crassula argentea.

MATERIALS AND METHODS

Enzyme Preparation. PEPC was purified from leaves of Crassula argentea Thunb. as previously described (13). The purified preparation was found to have a specific activity of 18 IU/mg, showing one band on an SDS-PAGE gel. Protein concentration was determined by the method of Bradford (4).

PEPC Assay. The activity of PEPC was measured in a 1-mL cell and was thermostated at 25°C, by monitoring (with a Beckman DU-50 spectrophotometer) the decrease in absorbance of NADH at 340 nm in an assay system coupled with added MDH and LDH (13). Assay mixtures included 50 mm ACES (pH 7.0), 5 mm free Mg$^{2+}$, 5 mm NaHCO$_3$, 0.16 mm NADH, and 6 IU of MDH plus 2.5 IU of LDH. Mg$^{2+}$ was from MgACES rather than from MgSO$_4$ as previously used (13), since high concentrations of SO$_4^{2-}$ were found to inhibit PEPC. Assays were based on constant levels of free Mg$^{2+}$ and free anions (except as indicated). Free ion concentrations were calculated using a computer program previously described (7). Reactions were started by the
addition of PEPC. Rates were calculated during the first 1.5 min and were found to be linear with correlation coefficients greater than 0.995. At PEPC concentrations below 0.03, the time used for calculations was reduced to 1 min with correlation coefficients being greater than 0.99. Kinetic parameters were determined by fitting the rates obtained at various substrate concentrations to the Michaelis-Menten equation modified to provide estimates of the Hill number as well as $V_{max}$ and $K_m$ (21).

**Fluorescence Studies.** Changes in PEPC conformation due to the binding of PEPP, G 6-P, and nucleotides were studied by monitoring the changes in the fluorescence emission of ANS in the presence of purified PEPC upon addition of these reagents (10). Changes in fluorescence emission were measured with a SPEX fluorimeter (Model DM1B) in a 3-mL cell magnetically stirred and thermostated at 25°C. ANS was excited at 350 nm (slit width: 4 nm), and emission was measured at 490 nm (slit width: 2 nm). The reaction medium (2.5 mL) was similar to the one used for PEPC assay, except that ACES concentration was reduced to 15 mM. The ANS added was 50 nm and the protein concentration used in the assay was about 50 nm, assuming a molecular weight of 400,000 (tetramer form) for PEPC. For fluorescence studies, all additions during the time course of the experiment were buffered solutions containing 5 mM free Mg$^{2+}$.

**Enzymic Determination of Organic Acids and G 6-P.** Leaves (15 g, about 10 leaves) were rapidly disrupted in 15 mL of 20% HClO$_4$. After centrifugation and elimination of the protein pellet (20,000g for 15 min), the pH of the supernatant liquid was raised to pH 8.5 using 1 M K$_2$CO$_3$. After stirring on ice for an additional 15 min, the supernatant was filtered and titrated back to pH 7.4 with 1 N HCl. Enzymic determination of organic acids and G 6-P present in the supernatant fraction was carried out spectrophotometrically at pH 7.4 by following oxidation or reduction of pyridine nucleotides using, respectively, NADP$^+$ malic enzyme (EC 1.1.1.40) for malate, NADP$^+$ isocitrate dehydrogenase (EC 1.1.1.42) for isocitrate, LDH (EC 1.1.1.27) for pyruvate, pyruvate kinase (EC 2.7.1.40) plus LDH for PEPC, and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) for G 6-P.

**Enzymes and Substrate.** Pig heart MDH, hog muscle LDH, and rabbit muscle pyruvate kinase were purchased from Boehringer Mannheim; pig heart isocitrate dehydrogenase and bakers' yeast G 6-P dehydrogenase were purchased from Sigma Chemical Company. All reagents were of the highest purity commercially available.

**RESULTS**

Table I shows the effect of AdN on PEPC activity at low and high PEPC concentrations. The $K_m$ of *C. argentea* PEPC for PEPC was found to be about 0.1 mM. At a low PEPC concentration (0.01 mM), 1 mM AMP led to a very high stimulation of activity (+428%). Under similar conditions, ADP caused less stimulation (+70%), whereas the presence of 1 mM ATP had the opposite effect, reducing the activity by about 45%. It should be noted that the concentration of Mg$^{2+}$-complexed nucleotides differed greatly according to the nucleotide considered (Table I, numbers between brackets). However, at concentrations of PEPC below the $K_m$, concentrations of AMP up to 10 mM (corresponding to about 1.45 mM Mg$^{2+}$-complexed AMP) still stimulated enzyme activity by about 500%, whereas a concentration of 1 ATP (0.03 mM) corresponding to about the same concentration of Mg$^{2+}$-complexed ATP (1 mM) inhibited enzyme activity by about 50%. It followed that the respective effects of AMP (stimulation) and ATP (inhibition) were still observed when concentrations of Mg$^{2+}$-complexed nucleotides were used rather than total concentrations of nucleotides. In addition, as all of these experiments were carried out in a medium containing a constant concentration of 5 mM free Mg$^{2+}$, the observed effects of AdN cannot be ascribed to their ability to bind Mg$^{2+}$.

**Table 1. Effect of Adenine Nucleotides on Purified PEPC from *C. argentea***

<table>
<thead>
<tr>
<th>Condition</th>
<th>PEPC activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 mM PEPC</td>
<td>1 mM PEPC</td>
</tr>
<tr>
<td>Control (−AdN)</td>
<td>0.60</td>
</tr>
<tr>
<td>+1 mM AMP (0.145 mM)$^b$</td>
<td>3.17 (+428%)</td>
</tr>
<tr>
<td>+1 mM ADP (0.91 mM)</td>
<td>1.02 (+70%)</td>
</tr>
<tr>
<td>+1 mM ATP (0.97 mM)</td>
<td>0.34 (~43%)</td>
</tr>
</tbody>
</table>

* All values are the means of at least two determinations.

Under similar conditions, but at a higher PEPC concentration (1 mM), none of the nucleotides induced any significant change in activity (less than 5%) (Table I). At such a PEPC concentration, only very high concentrations of AdN were found to cause changes in PEPC activity. AMP at 15 mM inhibited the reaction 39%, whereas the same concentration of ATP caused a 61% inhibition.

Figure 1 shows the effect of AdN on the $K_m$ of *C. argentea* PEPC. At concentrations of AMP, ADP, and ATP up to 6 mM, these nucleotides caused no significant changes in $V_{max}$. Therefore, from their effects on the $K_m$ of PEPC for PEPC (Fig. 1), it follows that AMP is a K-type activator of PEPC and ATP a K-type inhibitor. ADP affected the $K_m$ for PEPC only slightly.

Detailed investigation of the kinetics of PEPC at low PEPC concentrations (below $K_m$ value) indicated a slight sigmoidicity of the isotherm obtained in the absence of AdN (Fig. 1, inset, line a). The Hill number calculated from the complete isotherm (up to 50 mM PEPC) of the same enzyme preparation was 1.38. The presence of ATP, although decreasing the rates, did not significantly modify the sigmoidicity of the isotherm (Fig. 1, inset, line b) and hence the value of the Hill number (1.41). In contrast, AMP completely abolished the sigmoidicity (Fig. 1, inset, line c).
in cooperativity with the apparent when compared was previously shown (Table I and Fig. 1), whereas that, effect. On one hand, G 6-P could simply compete with ATP for a binding site on the enzyme and hence reduce its effect. On the other hand, G 6-P, being a very potent K-type activator, could reduce the K-type inhibitory effect of ATP by lowering the apparent K. However, as the effect of G 6-P is only slightly altered by ATP concentrations up to 6 mM (+9.13 nmol min⁻¹ in the presence of 1 mM G 6-P alone [control] and +7.01 nmol min⁻¹ in the presence of 1 mM G 6-P and 6 mM ATP), it is apparent that the competition of ATP with G 6-P is rather small when compared to the potent competition of ATP with PEP (see Fig. 1).

In contrast, AMP, which stimulated the activity of PEP in the absence of G 6-P, became an inhibitor when supplied in the presence of G 6-P. When compared to its effect in the absence of AMP (control, +9.13 nmol min⁻¹), G 6-P appears to have had a strongly reduced effect in the presence of AMP, with virtually no stimulation (+0.4 nmol min⁻¹) detectable at the highest AMP concentration used (17 mM). Further investigations of the effect of AMP on the stimulation of PEP by G 6-P have shown that, whereas the calculated Kₐ for G 6-P in the absence of AMP was repeatedly found to be below 0.01 mM, a value higher than 0.25 mM was obtained in the presence of 15 mM AMP, supporting the hypothesis of a competitive effect of AMP with G 6-P.

Using the decrease of the fluorescence emission of ANS as an extrinsic probe of the conformational changes (10) of PEP resulting from PEP or G 6-P binding to the enzyme, the effects of ATP and AMP on the binding of PEP and G 6-P to the protein were investigated (Fig. 2). It was found that compared to the control (Fig. 2, trace a), PEP binding as shown by fluorescence changes was reduced to a greater extent in the presence of ATP (Fig. 2, trace c, −77%) than in the presence of AMP (−37%). In contrast, compared to the control condition (Fig. 2, trace d), the G 6-P binding as indicated by fluorescence changes was reduced to a greater extent in the presence of 1 mM AMP (Fig. 2, trace f, −66%) than in the presence of ATP (−45%). Using the changes in ANS fluorescence, estimated Kₐ values of 0.06 mM and 0.17 mM for PEP and G 6-P, respectively, were found. Unfortunately, modifications of these values induced by the presence of AdN could not be quantified by using this approach. Since binding of AdN caused a similar type of change in ANS fluorescence, it was not possible to discriminate between changes due to G 6-P, PEP, or AdN when two of these compounds were simultaneously present. Similarly, attempts made to use the intrinsic fluorescence of tyrosine residues of PEP, rather than the fluorescence of the extrinsic probe ANS, were unsuccessful due to the potent quenching effect of AdN at the excitation wavelength (280 nm) required to excite tyrosine and to the similar effect caused by the oxaloacetate produced in the presence of PEP. Nevertheless, along with the kinetic data, the above results suggest that the positive effect of AMP on PEP activity results from its binding at the G 6-P (activation) site due to its higher affinity for that site than for the active site (PEP site), whereas the effect of ATP was due to its higher affinity for the active site of the enzyme than for the activation site.

Whatever the mechanism of AdN effects, it follows that the activity of the enzyme from the CAM plant C. argentea could be controlled by AEC as was originally found with the enzyme extracted from the C₄ plant maize (19). Such a possible effect was investigated (Fig. 3). At a low PEP concentration (0.08 mM) (Fig. 3A, line a), the activity of the enzyme showed a response to energy charge basically similar to the “R” type of Atkinson (2). The enzyme activity was increased by low AEC values and inhibited by high values. As previously shown with the C₄ enzyme (11) and as can be inferred from the nature of the AdN effect (essentially on Kₐ for PEP), this effect of AEC was decreased by increasing PEP concentration (data not shown). In the presence of 5 mM malate, a well-known inhibitor of PEP (14), the effect of AEC is still observed (Fig. 3A, line b). When expressed as a percentage of the activity measured in the absence of AdN (Fig. 3B), the effect of AEC in the presence of malate (Fig. 3B, line b) was even higher than the effect observed in the absence of malate (Fig. 3B, line a). This indicates that the effects of AdN were

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Table II. Effect of ATP and AMP on PEP Activity in the Presence or Absence of G 6-P

Experimental conditions are described under “Materials and Methods.” PEP was 0.66 μg mL⁻¹. Concentrations of nucleotides are total concentrations.

<table>
<thead>
<tr>
<th>Condition</th>
<th>PEPC activitya</th>
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<tbody>
<tr>
<td></td>
<td>0.05 mM PEP</td>
</tr>
<tr>
<td></td>
<td>nmol min⁻¹</td>
</tr>
<tr>
<td>Control</td>
<td>3.07</td>
</tr>
<tr>
<td>+2 mM ATP</td>
<td>1.34 (−56%)</td>
</tr>
<tr>
<td>+4 mM ATP</td>
<td>1.13 (−63%)</td>
</tr>
<tr>
<td>+6 mM ATP</td>
<td>1.04 (−66%)</td>
</tr>
<tr>
<td>+17 mM ATP</td>
<td>0.77 (−75%)</td>
</tr>
<tr>
<td>+2 mM AMP</td>
<td>4.28 (+39%)</td>
</tr>
<tr>
<td>+4 mM AMP</td>
<td>4.07 (+33%)</td>
</tr>
<tr>
<td>+6 mM AMP</td>
<td>4.07 (+33%)</td>
</tr>
<tr>
<td>+17 mM AMP</td>
<td>3.96 (+29%)</td>
</tr>
</tbody>
</table>

a All values are the mean of at least two separate determinations.
b Percentage stimulation (+) or inhibition (−) of activity as determined in the absence of AdN (control).

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Fig. 2. Effect of AMP and ATP on PEP- and G 6-P-induced fluorescence changes of ANS bound to C. argentea PEPC. a, decrease of fluorescence emission of ANS upon PEP addition to PEPC; b, as a, but in the presence of 1 mM AMP; c, as a, but in the presence of 1 mM ATP; d, decrease of fluorescence emission of ANS upon G 6-P addition to PEPC; e, as d, but in the presence of 1 mM ATP; f, as d, but in the presence of 1 mM AMP. Experimental conditions are described in detail under “Materials and Methods.” Numbers along the recordings are ΔE × 10⁻³.
Fig. 3. Effect of adenylate energy charge on C. argentea PEPC activity. A, effect of AEC on the velocity of PEPC reaction: a, in the presence of 0.08 mM PEP; b, in the presence of 0.08 mM PEP and 5 mM malate; c, in the presence of 0.08 mM PEP and 1 mM G-6-P. B, same data expressed as percentage of the activity measured in the absence of AdN: a, in the presence of 0.08 mM PEP; b, as a plus 5 mM malate; c, as a plus 1 mM G-6-P. According to Atkinson’s concept (2), the AEC was defined as ([ATP]+0.5[ADP])/([ATP]+ADP)+[AMP]). The final concentration of AdN was 1 mM (total concentration). The charge series was obtained with calculated concentrations for each nucleotide. PEPC was 0.8 mg/mL. Other experimental conditions are described under “Materials and Methods.”

Fig. 4. Changes in metabolite concentrations in the leaves of C. argentea during a full day-night cycle. Enzymic determinations of metabolites were spectrophotometrically performed as described under “Materials and Methods” using plants grown in a growth chamber with night and day temperatures being 18°C and 30°C, respectively. FW, fresh weight.

distinct and additive to the effect of malate. On the contrary, it was found that the presence of 1 mM G-6-P ablated the effect of AEC on the enzyme (Fig. 3A and B, trace c). This effect of G-6-P, already observed with the C4 enzyme (11), suggested a potential mechanism of regulation of PEPC in C. argentea, by affecting the sensitivity of the enzyme to AEC during a day-night cycle. In order to support such a possibility, the changes in metabolite concentrations in the leaves of C. argentea were determined. Figure 4A shows the characteristic changes in malate concentration observed in this CAM plant, in which about 50% of the total malate pool was found to be depleted during the day under the growing conditions used. In comparison, as has been classically observed (9), the isocitrate pool appeared essentially stable during the full day-night cycle. In Figure 4B, changes in pyruvate, PEP, and G-6-P are shown. As observed in other NAD(P) malic enzyme-type CAM plants, pyruvate rises in the early morning hours presumably from malate decarboxylation by malic enzyme (6, 9). Changes in PEP concentration during the day-night cycle indicated a high rate of PEP utilization during the night. Such an observation is ascribed to the active carboxylation of PEP to OAA and further reduction to malate under the action of PEPC and MDH (6, 14). Finally, the changes in G-6-P concentration are indicated, and it is shown that, as in other CAM plants (9), a higher level of G-6-P was found during the night than during the light period in C. argentea.

DISCUSSION

The above results address two different aspects of the effects of adenine nucleotides on the activity of PEPC from C. argentea. The first is related to the mechanism that could account for the opposite effects of AMP and ATP on the enzyme, and the second is more related to the physiological regulation of PEPC in CAM plants during a day-night cycle. In good agreement with what has been established with PEPC extracted from C4 plants (11, 19), the effects of AdN on PEPC are not necessarily due to their ability to chelate Mg2+ as suggested by Coombs et al. (5). Under assay conditions where free Mg2+ was held constant at a fully saturating concentration (5 mM), ATP and AMP still exert their respective inhibitory and activating effects at low levels of PEPC (Table I and Fig. 1).

Kinetic studies on C. argentea PEPC clearly show that ATP acts essentially as a K-type inhibitor of the enzyme, competing with PEP at the PEP-binding site. A similar result was obtained with Pennisetum PEPC by Mares and Leblova (12). On the other hand, AMP essentially acts as a K-type activator of the enzyme (Fig. 1). The results reported here show that AMP can compete with G-6-P, leading to a considerable increase of the Kd value of PEPC for G-6-P. This was also supported by the fluorescence studies showing the different effects of ATP and AMP on postulated conformational changes of PEPC induced by PEP and G-6-P (Fig. 2). A full understanding of the reasons for the different affinities of the two closely related adenine nucleotides for the PEPC and G-6-P binding sites requires further study. However, it should be noted that ATP and PEP have a much higher free energy of hydrolysis than AMP and G-6-P; the ∆G” values in kilocalories per mole are 14.8, 7.3, 2.4, and 3.2, respectively, for PEP, ATP, AMP, and G-6-P. At a molecular level, a higher free energy corresponds, in part, to the presence of more negative charges adjacent to the terminal phosphate. One can speculate that the occurrence and repartition of these negative charges play a critical role in the binding of phosphate esters to PEPC, thus influencing the affinity of these closely related compounds for the PEP and G-6-P binding sites as previously suggested for the binding of inorganic phosphate and sulfate to PEPC (19).

As a result of the opposite effects of ATP and AMP on the Kd of PEPC for PEP (Fig. 1), it appears that the enzyme of C. argentea can be controlled by the AEC, as reported for the enzyme extracted from different C4 plants (11, 19). Its response to AEC is basically of the “R” type according to Atkinson’s concept of energy charge (2). From our data and those obtained with the C4 plant enzyme (5, 11, 12, 19), it appears that the sensitivity of the enzyme to AEC is controlled (a) by the level of AMP (nucleotides are K1 type effectors), (b) by the level of free Mg2+ (ATP having a higher Kd for Mg2+ than AMP), and (c) by the level of G-6-P (G-6-P strongly reduces the AEC response to nucleotides). It is well established that in CAM plants maximal turnover of PEP occurs during the dark period (6, 9), during which the level of G-6-P is simultaneously the highest (Fig. 4). This will contribute to reducing an effect of high AEC on the enzyme during a cycle. In contrast, the sharp decrease in G-6-P concentration during the light period will increase the susceptibility of the enzyme to AEC. The changes in the level of free Mg2+ in the cell of a CAM plant during the day-night cycle are not presently documented.

The status of the adenylate energy charge in leaves during a
dark-light transition is a matter of debate (16). Nevertheless, it is generally agreed that upon illumination a small increase in AEC does occur in C₃ chloroplasts (8, 17). The specific data available on energy charge in a CAM plant are restricted to the measurement of the AEC of the adenylate pool in whole leaf extracts (15). A decrease of AEC from about 0.8 during the night period to about 0.6 during the light period was found (15). This emphasizes the problem of the intracellular location of PEPC in a CAM plant. Depending on the CAM species studied, PEPC may be strictly located in the cytosolic fraction (14, 20) or found in association with the chloroplasts (1, 20). The decrease in the concentration of G 6-P during the day (Fig. 4) lends support for the concept of an increased control of PEPC by AEC, which supposedly increases in chloroplasts during the light period at least in C₃ plants (8, 17).

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