Effect of Diethylpyrocarbonate on the Allosteric Properties of Phosphoenolpyruvate Carboxylase from *Crassula argentea*

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**ABSTRACT**

Phosphoenolpyruvate carboxylase from the Crassulacean acid metabolism plant *Crassula argentea* was substantially desensitized to the effects of regulatory ligands by treatment with diethylpyrocarbonate, a reagent which selectively modifies histidyl residues. Desensitization of the enzyme to the inhibitor malate and the activator glucose 6-phosphate was accompanied by the appearance of a peak in the ultraviolet difference spectrum at 240 nanometers, indicating the formation of ethoxyformylhistidyl derivatives. Hydroxylamine reversed part of the spectral change under native conditions, and almost all of the change under denaturing conditions, but failed to restore sensitivity to effectors. The pH profiles of desensitization to malate and glucose 6-phosphate indicated the involvement of groups on the enzyme with $pK_a$ values of 6.8 and 6.4, respectively. Under denaturing conditions, a total of 15 histidine residues per subunit were modified by diethylpyrocarbonate, whereas for the native enzyme nine histidines were modified per subunit. Effector desensitization occurs after the modification of two to three histidyl residues per subunit. The presence of malate reduced the apparent rate constant for desensitization by 60%, suggesting that the modification occurred at the malate binding site. Diethylpyrocarbonate treatment also eliminated the kinetic lag caused by malate. Glucose 6-phosphate did not protect the enzyme against diethylpyrocarbonate-induced desensitization.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) catalyzes the formation of oxaloacetate and inorganic phosphate from PEP and bicarbonate (1). The enzyme is found in bacteria, algae, and plants (1, 23), and is of special importance in plants which utilize C4 pathway of photosynthetic carbon fixation and in plants which exhibit CAM (1, 16, 19, 27). PEP carboxylase in CAM plants is a regulatory enzyme which is activated by G6P and inhibited by malate, the end product of the carboxylation pathway in vivo (20, 25, 28). This enzyme exists in both a day form and a night form, which have different kinetic properties with regard to the substrates and the effectors. G6P appears to be a mixed type activator for the day form of the enzyme, causing both decreased $K_m$ for PEP and an increased $V_{max}$, but the night form shows only an increase in $V_{max}$ in presence of G6P (25, 28). Inhibition by malate is of the mixed type for the day form of the enzyme, increasing $K_m$ for PEP and reducing $V_{max}$. In the presence of malate, the night form of the enzyme shows an increased $K_m$ for PEP with little effect on $V_{max}$ (28, 29).

In the last few years, studies using group selective chemical modifiers and PEP analogs have provided considerable insight into the catalytic site domain of the enzyme (1, 10), whereas the nature and biological function of residues in the effector binding sites have received little attention. Recently, chemical modification studies have established the participation of arginyl residue in the malate regulatory site (21). Also, kinetic studies of the response of PEP carboxylase from CAM leaves to the activator G6P as a function of pH have suggested the involvement of histidine and cysteine groups in activation by G6P (25).

In the present study we have further investigated the identity of the amino acids involved in the effector binding sites of PEP carboxylase from CAM plant *Crassula argentea*. For our studies, the malate-sensitive day form of the enzyme was purified and the effect of chemical modification of PEP carboxylase by diethylpyrocarbonate, a reagent selective for histidine (14, 15), was investigated.

**MATERIALS AND METHODS**

**Materials**

DEPC, G6P, l-malic acid, PEP, NADH, hydroxylamine, porcine muscle malic dehydrogenase, and lactic dehydrogenase were purchased from Sigma Chemical Co. All other chemicals were analytical reagent grade and were obtained from commercial sources.

**Enzyme Purification**

*Crassula argentea* was grown in a field plot and mature leaves were harvested after 4 h of light for enzyme extraction. PEP carboxylase was purified as described by *Wedding et al.* (21). The purified enzyme had a specific activity of 19 to 20 units/mg protein at 25°C and pH 7.0. The enzyme was stored at $-70°C$ and desalted on Bio-Rad, Bio-Spin 6 chromatography columns, as necessary, before dilution in appropriate buffer for subsequent assay or modification. The protein...
concentration was determined by using the Bio-Rad protein microassay, a dye-binding technique based on the method of Bradford (3). Enzyme molarity was calculated on the basis of the 400 kD tetramer form of PEP carboxylase.

**Enzyme Assays**

The enzyme activity was determined by the measurement of NADH disappearance at 340 nm in a coupled reaction system with malate and lactate dehydrogenase (13), using a Gilford response spectrophotometer with a cell chamber maintained at 25°C. Assays to test for activation of the enzyme by G6P were performed with 0.089 mM PEP, in the presence or absence of 5 mM G6P. Assays to test for inhibition of the enzyme by malate were performed with 1.5 mM PEP. The enzyme was preincubated, for 10 min, in assay mix with or without 10 mM malate, prior to starting the reaction by the addition of PEP. All assays were performed in 50 mM Hepes buffer (pH 7.5), with 4 mM MgCl₂, 4 mM NaHCO₃, 0.1 mM NADH, 36 IU of malate dehydrogenase, and 5.5 IU of lactate dehydrogenase.

To generate a lag, the enzyme was preincubated with 10 mM malate for 10 min in standard assay mix except for the presence of 0.15 mM NADH, 50 mM Mes buffer (pH 5.9), and no PEP. The reaction was initiated by the addition of 1.5 mM PEP. The assay was run for at least 15 min to ensure that a steady-state rate was obtained. The lag time was determined by the method of Neett and Ainslie (17), as described previously (18).

**Enzyme Modification**

PEP carboxylase was incubated with varying concentrations of DEPC at 25°C in 100 mM Mes buffer (pH 5.7 to 6.6) or Mops (pH 7.0). Unless otherwise stated, modification was carried out in the presence of 5 mM phosphoglycolate and 5 mM Mg²⁺ in order to protect active site residues and ensure modification of allosteric sites only. DEPC stock solutions were prepared in absolute ethanol. The reaction was stopped by addition of 10 mM histidine, and 20 µL aliquots of the modified enzyme were assayed for activity. Controls were run identically, except that 1% ethanol was added instead of DEPC. Pseudo first-order rate constants of the desensitization process were obtained from the slope of the plots of the log of percent inhibition or activation versus time. For protection studies, the enzyme was preincubated with effectors for 5 min at pH 6.6, and the modification reaction was initiated by the addition of DEPC.

**Reaction with Hydroxylamine**

PEP carboxylase (0.55 µM) was incubated with 0.5 mM DEPC for 6 min as described above. The reaction was stopped by addition of 10 mM histidine and then the samples were diluted with an equal volume of 0.8 M NH₄OH and incubated at room temperature. Aliquots were removed and assayed for the restoration of sensitivity toward G6P and malate.

**Spectroscopic Studies**

A sample of PEP carboxylase (0.79 µM) in 100 mM Mes (pH 6.6) in the absence or presence of 5 mM MgCl₂ and 5 mM phosphoglycolate was divided equally between the sample and reference microcuvettes and the absorbance base line was recorded. DEPC was added to the sample cuvette, and an equivalent volume of ethanol to the reference cuvette. Difference spectra were recorded at various time intervals after the addition of the reagent. For denaturation studies, the enzyme (0.55 µM) was incubated with 1% SDS and 8 M urea for 30 min, at room temperature, prior to DEPC treatment. The stoichiometry of modified histidine residues was calculated from the increase in absorbance at 240 nm based on a molar extinction coefficient of 3200 M⁻¹ cm⁻¹ (14).

**pH Studies**

PEP carboxylase (0.26 µM) was prepared in 100 mM Mes buffer pH 5.7 to 6.6 or Mops buffer pH 7.0, in the presence of phosphoglycolate and MgCl₂, and treated with 0.2 mM DEPC as described above. Pseudo first-order rate constants of the desensitization toward malate inhibition and G6P activation were determined as described above and their reciprocals were plotted against [H⁺].

**RESULTS AND DISCUSSION**

**Desensitization of PEP Carboxylase by DEPC**

Treatment of the enzyme with 0.2 mM DEPC progressively desensitized the enzyme to both the activating effects of G6P and the inhibitory effects of malate (Fig. 1). The desensitization process obeyed pseudo first-order kinetics. Observed velocities in assays containing the activator G6P decreased as a result of the DEPC treatment (Fig. 2A) and rates obtained in the presence of the inhibitor malate increased after treatment with DEPC (Fig. 2B). In both cases the control assays run in the absence of effectors showed either no effect of DEPC or a slight increase in rate, indicating that DEPC-induced desensitization was not occurring as a side effect of activity loss.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Time course of effector desensitization and absorption changes at 240 nm. The enzyme (0.26 µM) in 100 mM Mes buffer (pH 6.6), was incubated with 0.2 mM DEPC, and at designated times, aliquots were removed and assayed for sensitivity toward G6P (○), and malate (●) as described in "Materials and Methods." Time course of absorption changes at 240 nm measured, under the same conditions, with 0.79 µM enzyme (■).
DESENSITIZATION OF PEP CARBOXYLASE BY DIETHYLPYROCARBONATE

![Graph A](image1)

**Figure 2.** Effect of DEPC on PEP carboxylase activity. A, Effect of incubation with 0.2 mM DEPC (pH 6.6), on PEP carboxylase activity (0.26 μM) assayed with 0.089 mM PEP with (○) or without (●) 5 mM G6P; B, effect of incubation with 0.2 mM DEPC (pH 7.0), on PEP carboxylase activity (0.26 μM) assayed with 1.5 mM PEP with (○) or without (●) 10 mM malate.

Because malate is known to cause slow kinetic transients under some assay conditions, the effect of desensitization on the hysteretic behavior of PEP carboxylase was investigated. Incubation of unmodified enzyme with the inhibitor malate (10 mM) in assay mix at pH 5.9, prior to starting the reaction by the addition of PEP, resulted in a kinetic lag of 3.2 min. No lag was induced when the enzyme had been modified by 0.2 mM DEPC for 10 min before preincubation with malate, suggesting the involvement of a histidine residue in the subunit dissociation-association process that is responsible for the lag (18).

**pH Studies**

Further evidence for the involvement of histidine(s) comes from the study of the pH dependence of the desensitization process (Fig. 3). The reciprocals of the pseudo first-order rate constants of desensitization were plotted as a function of hydrogen ion concentration, according to the method of Cousineau and Meighen (6),

\[
\frac{1}{k_{\text{obs}}} = \left( \frac{1}{k_2 \cdot [\text{DEPC}]} \right) + \left( \frac{[H^+]}{k_1 \cdot K_a \cdot [\text{DEPC}]} \right)
\]

where \( k_{\text{obs}} \) is the pseudo first-order rate constant of desensitization, \( K_a \) is the apparent dissociation constant of the acidic form of the target residue, and \( k_2 \) is the second-order rate constant of modification of the unprotonated residue. From the x-axis intercept, the apparent \( pK_a \) values for the groups responsible for malate inhibition and G6P activation were found to be 6.8 and 6.4, respectively. These values are in the range typical of histidyl residues in proteins (5, 9, 14). Although the \( pK_a \) values were relatively close to one another, the pseudo first-order rate constants used to obtain these values were significantly different, suggesting that two distinct histidine residues with different reactivities are involved in the enzyme's response to malate and G6P. This study was limited to pH values between 5.7 to 7.0 because DEPC is known to modify amino acids other than histidine at alkaline pH values (15, 26).

**Protection Studies**

To determine whether the modification was occurring at the effector binding sites, the effects of malate and G6P on the rate of DEPC-induced desensitization were examined. The presence of 27 mM malate during the DEPC treatment reduced the apparent rate constant for modification from 2.3 \( \times 10^{-3} \) s\(^{-1}\) to 9.3 \( \times 10^{-4} \) s\(^{-1}\), indicating that DEPC modification of the enzyme occurs at the malate binding site. In a similar experiment, no protection against the DEPC desensitization was observed when the modification was done in the presence of 9.8 mM G6P. Desensitization of the enzyme to G6P appears to be more complex than the desensitization to malate, since at the low PEP and DEPC concentrations used to measure the G6P response, DEPC slightly increased the control rate while it decreased the rate in the presence of G6P (Fig. 2A). The lack of protection by G6P and the DEPC-induced activation of the enzyme suggest that DEPC may be

![Graph B](image2)

**Figure 3.** pH dependence of the rate of desensitization of PEP carboxylase to G6P (●) and malate (○) by DEPC.
modifying the protein near the activator site in a way that mimics the effects of G6P binding.

Amino Acid Residues Modified by DEPC

Desensitization of the enzyme to the inhibitor malate and the activator G6P was accompanied by the appearance of a peak in the ultraviolet difference spectrum at 240 nm (Fig. 4), indicating the formation of ethoxyformyl histidyl derivatives (14). Figure 4 also shows that there was some decrease in absorbance in the range 270 to 300 nm during the reaction, suggesting the modification of tyrosine (14). Although the absorption change occurs over a wider spectral range than expected for tyrosine modification, this change of absorption at 270 to 300 nm could not be due to a conformational change, since treatment of a denatured sample of enzyme with DEPC also gave rise to an absorption change in that region. However, given the observed pKₐs of the target groups it would appear unlikely that derivatization of tyrosine could be responsible for the loss of enzyme's sensitivity toward the effectors. Moreover, the absorption change in the range of 270 to 300 nm occurred within the first minute of incubation with DEPC with almost no further change (not shown). In contrast, the increase in absorbance at 240 nm was comparatively slow and showed a good correlation with the time course of effector desensitization (Fig. 1).

Reaction with Hydroxylamine

It has been suggested that desensitization of the enzyme toward the effectors may be correlated with the modification of a histidyl residue if hydroxylamine restores sensitivity (12). Enzyme modified by DEPC was, therefore, tested for reversibility by treatment with hydroxylamine. Addition of 0.4 mM hydroxylamine to DEPC-modified enzyme, however, caused no restoration of sensitivity throughout a 24 h period, although the spectral changes induced by DEPC were partially reversed (Fig. 4A). Similar results have been reported for several other enzymes (4, 7, 22). Three possible interpretations have been proposed to account for the irreversible loss of sensitivity after DEPC modification. First, DEPC may have reacted with amino acids other than histidine. Although DEPC reacts preferentially with histidine residues on most proteins, reaction with other amino acids has occasionally been observed (12, 14). Of the residues modified by DEPC, only histidine and tyrosine can be regenerated by treatment with hydroxylamine (12, 14). Second, the failure to regain full restoration of enzyme sensitivity to the effectors with hydroxylamine treatment and complete loss of the ethoxyformyl moiety may be partially due to the reaction of two equivalents of DEPC per equivalent of histidine followed by a Bamberger reaction to open the imidazole ring of histidine (2, 11). Third, the N-carbethoxyhistidine residues may be protected from access to the hydroxylamine nucleophile in solution. The last explanation seems more plausible than the first two in explaining the present data because the difference spectra show that a significant fraction of the absorbance change at 240 nm is not reversed by hydroxylamine under native conditions, but is reversed when the enzyme is denatured (Fig. 4). Analysis of the spectral change at 240 nm, after correction for the twofold dilution, indicates that approximately three of the modified histidines in the native enzyme are not accessible to hydroxylamine. Since effector desensitization is also not reversed by hydroxylamine, this spectral analysis suggests that as many as three histidyl groups may be involved in the response to effectors.

Stoichiometry of Modification by DEPC

On the basis of spectral changes observed with native enzyme in the absence of any protective agent, approximately nine histidine residues per subunit were modified by DEPC.
When the enzyme was modified in the presence of phosphoglycolate, only seven histidine residues per subunit were derivatized. These data suggest that two out of the nine available histidine residues per subunit may be at the active site, because they are protected from modification by the competitive inhibitor phosphoglycolate. This is in accord with the results reported by Iglesias and Andreo (10) on PEP carboxylase from maize leaves.

Maximal effector desensitization occurred after the modification of two to three out of the nine available histidine residues per subunit (Fig. 5). This estimate is in good agreement with the estimate derived from the spectral studies of hydroxylamine reversal (three residues). It should also be noted that the effector desensitization followed a similar time course as the absorption changes at 240 nm, indicating that the histidines which are modified during effector desensitization are among the first to be modified and thus among the most reactive and/or accessible histidines present on the enzyme (Fig. 1). Spectral studies performed in the presence of 60 mM malate indicate that malate protected close to one (0.9) histidyl residue from modification during the first 250 s of DEPC treatment, the normal time frame for maximal malate desensitization (Fig. 1). In these studies, Mg$^2+$ and phosphoglycolate were present to protect the active site histidines and to ensure that the observed protection by malate was restricted to allosteric sites. Thus, of the two to three histidines modified during the period of desensitization, one of these appears to be at the malate binding site.

It was not possible to achieve complete desensitization, even after 1 h of treatment with DEPC. The lack of complete desensitization to malate may relate to the fact that malate, as a mixed-type inhibitor, probably exerts part of its effect by direct competition with PEP at the catalytic site. Some residual malate sensitivity may thus be unavoidable if the catalytic site is fully functional, and in our experiments phosphoglycolate was included to protect the histidines in the PEP binding domain of the catalytic site. The inability to completely desensitize the enzyme to G6P may stem from the possibility that the target histidine(s) is near, but not part of the G6P binding site. Modification of a residue in such a position may interfere with, but not completely block, the binding of G6P. This model would also be consistent with the lack of protection by G6P, as noted earlier. Regardless of these complexities, the fact that we can substantially desensitize the enzyme to G6P and malate while retaining catalytic activity suggest that these effectors are binding at true allosteric sites that are distinct from the active site.

Under denaturing conditions (1% SDS and 8 M urea), the total number of histidines was found to be 15 residues per subunit, on the basis of absorbance increases at 240 nm. In comparison, the amino acid sequence deduced from the gene sequence of a CAM specific isoform of PEP carboxylase from Mesembryanthemum crystallinum (8) has revealed the presence of 20 histidine residues per subunit. The deduced sequence of the maize PEP carboxylase also includes 20 histidines per subunit (30), although a direct analysis of the amino acid composition of maize PEP carboxylase determined the existence of only 12 histidine residues per subunit (24). Given the probable interspecific sequence differences and the inherent limitations of estimates based on chemical modification

Figure 5. Correlation between sensitivity toward G6P or malate, and histidine residues modified by DEPC. The sample cuvette contained the same solution as described for Figure 4, except that 0.2 mM DEPC was used instead of 1 mM. A reference sample contained no DEPC. At various times, the absorbance change at 240 nm and the enzyme's sensitivity to the effectors were determined. (— —) indicates the maximal effector desensitization measured after 1 h of incubation with DEPC.
techniques, the value of 15 histidines/subunit for the Crassula enzyme is in reasonable agreement with the values reported for other higher plant PEP carboxylases.

LITERATURE CITED