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Malate Inhibition of Phosphoenolpyruvate Carboxylase from *Crassula*[1]

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ABSTRACT

Phosphoenolpyruvate carboxylase partially purified from leaves of *Crassula* and rendered insensitive to malate by storage without adjuvants can be altered to the form sensitive to malate inhibition by brief, 5-minute preincubation with 5 millimolar malate. The induction of malate sensitivity is reversible by lowering the malate^{2-} concentration. Of the reaction components only HCO_3^- increases the sensitivity to malate in subsequent assay. Phosphoenolpyruvate (PEP), which itself tends to lower sensitivity to subsequent malate inhibition, also reduces the effect of malate in the assay, as does glucose-6-phosphate. PEP isotherms showed that the insensitive or unpreincubated enzyme, responds to the presence of 5 millimolar malate during assay with a 3-fold increase in K_m, but no effect on V_{max}. Enzyme preincubated with malate shows the same effect of malate on K_m, but in addition V_{max} is inhibited 72%. It thus appears that both sensitive and insensitive forms of PEP carboxylase are subject to K-type inhibition by malate, but only the sensitive form also shows V-type inhibition. Preincubation with malate at different pH values showed that at pH 6.15, the inhibition by malate in subsequent assay at pH 7 was much lower than at pH 7 or 8. When the reaction is prerun for 30 minutes with increasing concentrations of PEP, subsequent assay with malate shows progressively less inhibition due to malate. When 0.3 millimolar PEP either alone or with 0.1 millimolar ATP and 0.3 millimolar NaF is present during preincubation, the effect of malate in a following assay is to activate the reaction. These results may indicate an effect of phosphorylation of the enzyme on sensitivity to malate.

In many CAM plants the evening task of fixing CO_2 into oxaloacetate to be converted to malate is assigned to PEPC,[2] EC 4.1.1.31. While in these plants it has a central role in a special kind of metabolic adaptation, the enzyme is widely distributed among both higher plants and microorganisms (13) so we conclude that the enzyme has a variety of roles as has been suggested by Latzko and Kelly (6). Among these it seems likely that in most tissues the anaplerotic role may be most important. The special roles in CAM and C_4 metabolism may be simply adaptations of this primary function.

Because of the need for avoiding a futile carboxylation/decarboxylation cycle, the means by which PEPC is regulated in CAM plants has been of particular interest, but it turns out that the enzyme from many non-CAM plants and microorganisms responds in a similar way to the effectors which are found to work with the CAM enzyme (6, 13), which is consistent with a primary role as a means of replenishing carboxylic acid reserves. In CAM plants the ligand most clearly associated with the diurnal variation in the activity of the enzyme has been malate (3, 9). There is a consensus that CAM PEPC probably is regulated by malate concentration alone or in some combination with G-6-P (9, 11, 12, 16). Malate inhibits the enzyme isolated from day leaves, by implication causing the enzyme to be turned off during the day when CAM plants are decarboxylating the malate accumulated overnight. The enzyme isolated from night leaves is usually resistant to inhibition by malate (16, 17) thus implying that the enzyme is free to carboxylate PEP and to cause accumulation of malate during the night. Malate is also an inhibitor of PEPC from C_4 plants and of the C_3 enzyme as well (6). Reports of interactions of malate with G-6-P are manifold (2, 4, 5, 9, 11, 12), although they include few examples of studies with even partially purified enzymes. Indeed, a major problem in understanding PEPC regulation has been the transient nature of the characteristics attributable to PEPC and the differing characteristics reported for the enzyme from different species (2, 6, 7, 12). The trend with the CAM enzyme appears to be loss during purification or storage of the sensitivity to malate associated with the day enzyme. The insensitive night enzyme generally remains intractably insensitive. A number of adjuvants, including malate itself, have proven useful in maintaining the day enzyme in the sensitive configuration (2, 7, 17).

Although the differences between the day, sensitive, and the night, insensitive, enzymes may include other features, it is clear that the sensitive enzyme is smaller than the insensitive one. We have found that the two exist in a dimer/tetramer relationship which changes diurnally, together with sensitivity to malate (17).

Knowledge of the quantitative regulation of PEPC in CAM plants has come mostly from studies with intact cells or rapidly prepared, crude extracts of the enzyme. Understanding of the way in which the enzyme responds to its effectors is uncertain and confused. In this study we have attempted to clarify this somewhat using external means of converting the enzyme from one form to another so that the responses of a relatively pure enzyme may be observed and associated with its physical state. The way we have chosen to do this is associated with our earlier observation that PEPC in the insensitive tetramer form was partially converted to dimer by treatment and chromatography with malate (18). That the enzyme might be converted by treatment with reaction components or effectors was also suggested by the fact that malate inhibition of the insensitive enzyme was greater when the reaction was started by adding PEP, *i.e.* when the enzyme was preincubated with the reaction mixture before the reaction was started (9, 17). These possibilities for switching the enzyme from one state to another have been explored and exploited in the present study.

MATERIALS AND METHODS

Plant Materials. The enzyme was isolated from leaves of *Crassula argentea* Thunb. Leaves were collected in late morning

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[2] Abbreviations used are: PEPC, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; ACES, (N-[2-Acetamido]-2-aminoethanesulfonic acid); G-6-P, glucose-6-phosphate.

from plants growing in the field. This schedule was intended both to minimize the malate content of the leaves and the pH change during grinding and to produce uniform enzyme for these studies. It is of interest that apparently identical preparations showed different levels of inhibition by malate, but the enzyme was concentrated and stored until the malate inhibition was minimal in all cases.

Enzyme Preparation. Leaves brought into the laboratory were chilled in an ice bath. A sample, usually 200 g, of leaves was cubed to 5 mm size with a razor blade. The tissue was disrupted by grinding in a Polytron homogenizer in a proportion of 1 g leaves to 2 ml of grinding medium. This medium consisted of 50 mM Hepes, 1 mM EDTA, 1 mM DTT (pH 7.2) with 1% Triton X-100. The grinding was done in three 6 s bursts at full speed, separated by 15 s cooling intervals. The homogenate was filtered through fine nylon mesh, and further disrupted by 3 min sonication at 5 amp in 30 s periods with 30 s cooling intervals between. The sonicated homogenate was then centrifuged 30 min at 30,000*g*. The supernatant liquid was fractionated by $(NH_4)_2SO_4$. A fraction precipitating at 0 to 35% saturation was centrifuged at 10,000*g* for 15 min and the precipitate discarded. The fraction precipitating between 35 and 55% saturation was dissolved in a minimal volume of 0.05 M Hepes (pH 7.2), with 1 mM EDTA and 5 mM DTT. This fraction was then desalted on a Sephadex G-25-40 column 2 × 20 cm, and applied to a 1 × 28 cm Fractogel TSK DEAE 650 M column in the same buffer. The enzyme was eluted from this column with a 100 ml 0 to 400 mM linear NaCl gradient. The specific activity of the PEPC at this point was about 4 IU/mg protein like that used previously (17) which was about 80% pure on the basis of SDS gels. The enzyme was stored at −70°C in the eluting buffer.

Assay Procedure. Assays were carried out in 1.0 ml of 0.05 M Aces (pH 7.2), 5 mM HCO_3^-, 5 mM Mg^{2+}, 0.15 mM NADH, and 0.5 IU of malate dehydrogenase as a coupling enzyme, using NADH while producing malate from the oxaloacetate produced by PEPC.

Various concentrations of PEP were used as indicated in the text. The oxidation of NADH was followed at 340 nm in a spectrophotometer cell held at 25°C. Where comparative rates with and without malate were measured the inhibited and control treatments were determined at the same time.

All components of the assays were added as free ions. For this purpose a computer program which writes assay protocols which maintain constant levels of free ligands (14) was used.

Preincubation. The PEPC, with a protein content of 2 to 3 mg/ml in storage, was diluted 1:2 with a diluting medium containing twice the desired concentration of ligands. After the preincubation was complete, 10 to 20 μl of the preincubated enzyme was added to assays and the rate measured for 3 to 5 min. In cases where the assay showed a lag, the final 1 to 2 min was taken for determining the rate. The time period for preincubation was 5 min, and was carried out at room temperature in 0.5 ml polyethylene centrifuge tubes. Aliquots of preincubated enzyme were added as rapidly as possible to a control assay and to an assay identical except for the presence of 5 mM malate. Most ligands were made up in 50 mM Hepes (pH 7.2).

HPLC Chromatography. The procedures used for HPLC separations of PEPC were as previously described (18) including the use of a Waters I-250 size exclusion column, except that 0.05 M Hepes buffer with 0.05 M NaCl at pH 7.0 and a flow rate of 0.5 ml/min was used for all separations.

RESULTS

The method used in these studies for conversion of the malate resistant form of PEPC to the malate sensitive form was based on the earlier observation (18) that when a resistant enzyme, shown by HPLC to be entirely in the tetrameric form, was chromatographed with a low concentration (2 mM) of malate, HPLC analysis indicated that part of the enzyme had been converted to dimer. In preliminary studies using as a criterion the percentage inhibition of PEPC activity by malate, we found that a fairly short preincubation with malate resulted in a pronounced increase in malate inhibition on subsequent assay. We settled on a 5 min preincubation as a standard procedure which gave a substantial effect of the treatment without resulting in complete loss of PEPC activity.

Malate Concentrations. The effect of increasing concentrations of malate in causing greater sensitivity to malate inhibition is illustrated in Table I, where several characteristics of the response to preincubation with malate are apparent. First, the activity of enzyme preincubated 5 min with increasing concentrations of malate is progressively diminished. This is not due to malate carried over from preincubation (the highest concentration of malate added with the preincubated enzyme was 50 μM) but rather represents progressive conversion of the enzyme to a less active form during preincubation. The velocity of the preincubated enzyme assayed in the presence of 5 mM malate is even more inhibited; that is, the preincubation with malate has resulted in a lower activity enzyme and assay in the presence of malate causes a further reduction of activity. The difference between the rates from assays with and without malate represents malate inhibition of the enzyme, and is represented by the second column of percent inhibition. The first percent inhibition column shows the progressive effect of increasing malate concentration on loss of enzyme activity during preincubation. Both sets of inhibition data show that enzyme activity is affected by preincubation with malate as well as the relative sensitivity of the residual activity to malate inhibition during assay. These results raise questions as to whether the inhibitory effect of malate is due to conversion of an active tetramer to a less active dimer as we (17, 18) have assumed, or whether malate induces the conversion of PEPC from an active form to one which is inactive.

Reversibility of Malate Sensitization. It seemed desirable to determine whether the increased sensitivity of PEPC to malate induced by preincubation with malate was reversible or whether the changes induced by malate were permanent. Acting on the assumption that the enzyme responds only to free $malate^{2-}$, for which we have some support from preliminary work, we have chosen to alter the $malate^{2-}$ concentration available during preincubation by adding Mg to complex the malate and reduce the concentration of $malate^{2-}$.

As can be seen in Table II, this strategy has produced evidence of the reversibility of the changes induced by preincubation with malate. Enzyme which without preincubation was inhibited only 15% by malate, shows an increase to 58% inhibition after 5 min preincubation with 5 mM malate. To the same enzyme, 10 mM

Table I. *Effect of Increasing Concentration of Malate on Activity of Phosphoenolpyruvate Carboxylase*

All treatments were preincubated 5 min with malate concentration indicated and then assayed at pH 7.2 in 50 mM Aces with 5 mM PEP, 10 mM HCO_3^-, 5.0 mM Mg^{2+}, and 0.15 mM NADH with 1 IU of malate dehydrogenase with and without 5 mM malate.

Malate Concentration	Velocity Control Assay	Assay + Malate	Inhibition	
			By preincubation	By malate in assay
mM	*nmol/min*		*%*	
0	4.78	4.18	0	13
1	4.06	3.07	15	24
5	3.32	2.21	31	33
10	2.70	1.02	44	62
20	2.33	0.80	51	65

Table II. *Reversibility of Sensitization to Malate Inhibition Using Mg^{2+} to Complex Malate during Preincubation*

Preincubated treatments were carried out in sequence on the same enzyme sample. The nonpreincubated treatment was an aliquot of the same enzyme. Assays as in Table I.

Treatment	Control Velocity	Velocity with 5 mM Malate	Inhibition
	nmol/min		%
No preincubation	3.90	3.29	15.6
Preincubate 5 min with 5 mM malate	3.29	1.39	57.8
Preincubate additional 5 min with added 10 mM Mg_2SO_4	3.26	2.40	26.4
Preincubate additional 5 min with added 5 mM malate	2.10	1.26	40.0

Table III. *Influence of Preincubation of PEPC with Reaction Components on Activity and Sensitivity to Malate Inhibition*

All preincubation treatments used 5 mM of the ligand indicated with a 5 min exposure prior to assay. Assays as in Table I.

Preincubation	Velocity		Inhibition
	Control	+5 mM malate	
	nmol/min		%
None	3.49	2.29	34
Mg^{2+}	2.63	2.04	22
Malate	2.72	0.63	77
Malate + Mg^{2+}	2.68	0.80	70
HCO_3	2.26	1.84	60
PEP	1.92	1.67	13
PEP + malate	2.27	1.02	55
G-6-P	3.21	2.07	36
G-6-P + malate	2.46	1.30	47

$MgSO_4$ (sufficient to reduce the free malate^{2-} from 5 mM to about 1 mM) was added. After 5 additional min, the enzyme is inhibited only 26% by 5 mM malate in the assay. When a further 5 mM malate is added and the enzyme preincubated 5 min before the final assay, the inhibition has increased to 40%, which tends to confirm the assumption that the decrease in percent inhibition brought about by adding Mg^{2+} was due to reducing the amount of free malate available to the enzyme. This also supports the assumption that the enzyme can bind only that form of malate.

While it is unlikely that desensitization of PEPC in the cell would be due to an increase in free Mg^{2+} like that used here, the fact that preincubation with Mg alone has little effect on subsequent inhibition by malate (see Table III) and that it has little effect on the inhibition due to malate when added in equal concentration (also Table III) gives further support to the conclusion that the effect of Mg^{2+} in Table II results from reduction of the concentration of uncomplexed malate in the preincubation.

Effect of Reaction Components. It was observed (18) that the presence of PEP during chromatography of PEPC with malate was capable of preventing the formation of dimeric forms of the enzyme in response to the presence of malate. Although it is believed that PEPC has a site for malate binding different from the active site (10), some of the data from the current and other studies (17, 18) indicate that PEP and malate are interacting in some way. The effect of preincubation with various constituents of the PEPC assay on the sensitivity of PEPC to malate inhibition in assay is summarized in Table III. From this it is apparent that the effect of Mg^{2+} may be to moderate the effect of preincubation in either buffer or buffer plus malate on malate sensitivity. Preincubation with HCO_3 appears to cause some increase in malate sensitivity, but this has not been pursued further. Preincubation with malate produces consistently high sensitivity to malate and preincubation with PEP moderates the effect of preincubation in either buffer or buffer + malate, as does preincubation with G-6-P. The PEP and PEP plus malate treatments have been replicated sufficiently to give statistical validity to the interaction of the two ligands. The figures shown for these treatments in Table III are means of four determinations, and SE indicate that the difference between malate and malate + PEP is significant at the 5% level. Thus, there is a significant reduction of malate inhibition when PEP is present during preincubation. This is not completely comparable with the HPLC results in which the dimer seemed to disappear completely when the enzyme was chromatographed with PEP and malate (18). The difference may be due to longer exposure to malate in the HPLC studies or it may as suggested later, indicate a lack of complete equivalence between malate inhibition and aggregation state.

Effect of Preincubation with Malate on Kinetic Parameters. Although it seems clear that preincubation with malate does produce some change in PEPC which results both in a decreased activity and an increased sensitivity to inhibition by malate, the nature of the changes produced is not apparent from studies of the type described above. Some clues as to the way in which PEPC is being affected by malate may be obtained from Figure 1 and Table IV. In Figure 1 are shown paired PEP isotherms of PEPC treated in three different ways. The upper solid line is with enzyme which has been given no preincubation. The enzyme was diluted one-half and assayed immediately. The dashed line just below this one is for the same enzyme run with 5 mM malate. As may be seen from Table IV, the effect of malate in this case is only to cause a >3-fold increase in K_m. There is no difference in the V_{max} values for the control and malate-treated samples. The Hill number is slightly reduced by malate. The two middle lines are produced with enzyme which has been diluted one-half and preincubated 5 min with buffer alone. In this case the total activity is reduced, the relative K_m values for control and malate-treated samples are similar to those from the same treatments

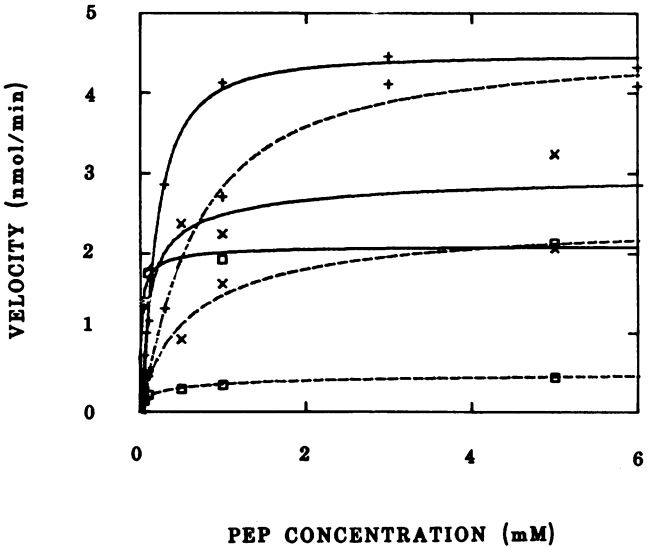

FIG. 1. Preincubation effects on the response of phosphoenolpyruvate carboxylase to phosphoenolpyruvate and malate. All assays used the standard composition with the concentration of PEP indicated. (+), Enzyme not preincubated; (——), control; (- - -), with 5 mM malate; (×), enzyme preincubated 5 min in buffer only; (——), control; (- - -), with 5 mM malate; (□), enzyme preincubated 5 min in buffer plus 5 mM malate; (——), control; (- - -), with 5 mM malate.

Table IV. *Kinetic Parameters of PEPC Assayed after 5 Min of Various Types of Preincubation*

Data obtained from fitting of lines of Figure 1.

Preincubation	V_{max}	K_m (PEP)	nH	Inhibition
	nmol/min	*mM*		%
None				
Control	4.49 ± 0.08	0.19 ± 0.15	1.36 ± 0.23	
5 mM malate	4.55 ± 0.12	0.65 ± 0.25	1.15 ± 0.17	−1
Buffer only				
Control	3.15 ± 0.05	0.08 ± 0.31	0.56 ± 0.31	
5 mM malate	2.48 ± 0.06	0.66 ± 0.21	0.85 ± 0.19	21
Buffer + malate				
Control	2.12 ± 0.04	0.02 ± 0.15	0.73 ± 0.23	
5 mM malate	0.59 ± 0.03	0.43 ± 0.14	0.47 ± 0.10	72

with the nonpreincubated sample, but malate in the assay causes a 20% decrease in V_{max}. Dilution and preincubation has caused a decreased activity and conditioned the enzyme for a greater inhibition of V_{max} by malate, but the effect of malate on K_m remains the same. The final pair of lines are produced by enzyme which has been preincubated 5 min with 5 mM malate before assay. The total activity is further reduced by this treatment and the K_m for PEP is again increased when malate is present in the assay. The most striking difference here is the decrease in V_{max} when malate is present, the enzyme is now inhibited 72% by 5 mM malate.

The presence of malate in the assay (or in the preincubation) has increased the K_m for PEP 3- to 10-fold and this effect is independent of whether or not the PEPC is 'sensitive' to malate as defined by reduced V_{max}. Malate under these circumstances is seen to be a mixed inhibitor, as has been reported (2–4, 16), only when the enzyme is sensitive to malate, but when resistant to malate, the inhibition by malate is purely competitive and thus appears to have been mostly unobserved in experiments using high levels of PEP. The decreased activity found when assaying with malate is interpreted as a decrease in V_{max}, but it may be due either to a decrease in the intrinsic activity of the enzyme by, *e.g.* converting it to a form with a slower reaction rate, or it may be a result of converting the enzyme to a form which is inactive so that the residual activity represents the amount of the original active form still present. It is very difficult to distinguish between these two possibilities, especially when it is assumed that either reaction is reversible.

pH Effects on Sensitization by Malate. It was of interest to learn the degree to which hydrogen ion concentration plays a role in the change in sensitivity to malate inhibition brought about by preincubation with malate. Preliminary experiments had shown that there were substantial differences in the response to preincubation with malate independent of pH and depending on which buffer ion was present. It was therefore decided to use a mixed buffer consisting of 50 mM glycyl-glycine and 50 mM Aces over the entire pH range studied from pH 6.15 to 8.18. This mixture has some buffering capacity over the whole range, although it is weak in some regions. To help with buffering below pH 7, the malate used was adjusted to the intended pH before addition to preincubation mixtures. All assays were run at pH 7.0, so changes in rate and sensitivity to malate inhibition are entirely due to preincubation effects.

Preincubation at pH values over the range from 6 to 8 has relatively little effect on subsequent velocity at pH 7.0 (Table V). However, preincubation with buffer only does show striking differences in the subsequent response to malate, ranging from 4 to 33% inhibition. When the enzyme is preincubated with malate the differences are even more striking, ranging from 6% inhibition at pH 6.15 to 69% at pH 7.11 and 42% at pH 8.18.

This response is quite different from the pH profile of the enzyme during assay, where the sensitive day enzyme was strongly inhibited at pH values below 7 and little inhibited at pH 8 (11).

Table V. *Effect of pH on Sensitivity of PEPC to Malate after Preincubation with Buffer Alone or Buffer Plus 5 mM Malate*

Buffer used at all pH values was 50 mM glycyl-glycine + 50 mM Aces. Assays at pH 7.2 as in Table I.

pH	Treatment	Control Rate	Rate with 5 mM Malate	Inhibition
		nmol/min		%
8.18	Buffer	2.73	1.84	32.6
8.18	Buffer + malate	1.52	0.88	42.3
7.11	Buffer	2.67	2.57	3.7
7.11	Buffer + malate	2.01	0.62	69.2
6.15	Buffer	2.64	2.36	10.4
6.15	Buffer + malate	1.36	1.28	5.7

Table VI. *Effect of Prerunning PEPC with Varying PEP Concentrations on Subsequent Inhibition by Malate*

Assays prerun 30 min, then transferred to another assay with or without 5 mM malate. (c) = control assay; (i) = assay with 5 mM malate.

Treatment	V_m (c)	K_m (c)	V_m (i)	K_m (i)	Inhibition
	nmol/min	*mM*	*nmol/min*	*mM*	%
None	8.11	0.21	6.85	0.65	16.4
0.05 mM PEP	5.14	0.15	4.70	0.23	8.6
0.1 mM PEP	5.22	0.11	4.84	0.22	7.3
0.3 mM PEP	5.00	0.12	5.41	0.28	−8.2
0.3 mM PEP + 0.1 mM ATP	5.25	0.08	5.97	0.22	−13.8
0.3 mM PEP + 0.1 mM ATP + 0.3 mM NaF	5.59	0.03	7.09	0.52	−26.8

Effect of Prerunning the PEPC Reaction on Sensitivity to Malate. In an attempt to evaluate the possibility that the enzyme might be changed by the turnover process in a way which would affect the response to malate, the data shown in Table VI were obtained. The reaction was prerun in a 1.0 ml volume with the PEP concentrations indicated in otherwise normal assays except that the enzyme concentration was 10-fold higher than that normally used. This was to ensure that all the added PEP was used up during the 30 min prerun, and following the assay indicated that this occurred within 10 min. The prerun assay then provided the enzyme (one-tenth for each assay) for assays at varying concentrations of PEP.

The data in Table VI show that prerunning in this way has resulted in some loss of activity and a decrease in K_mPEP in both the control PEP isotherms and those run with 5 mM malate. Most interesting is the progressive decrease in percent inhibition (calculated on the basis of V_{max}) as the amount of PEP used during the prerun increases, culminating in activation at 0.3 mM PEP. When the highest concentration of PEP is supplemented by 0.1 mM ATP, the activation by malate is even greater and the inclusion of 0.3 mM NaF, an inhibitor of phosphatases, further increases the level of activation. These treatments of an already "insensitive" PEPC produce a decrease in sensitivity which resembles the reported (1, 8) differences in phosphorylated and dephosphorylated PEPC, where the phosphorylated form is reported as malate insensitive.

Effect of Malate on Aggregation. The stock enzyme solution used in these studies was relatively dilute (1.5 mg protein/ml) which made it difficult to process by HPLC under conditions

like those used in the preincubation treatment. However, HPLC columns run at this concentration of enzyme using 200 μl samples permitted comparison of the untreated enzyme with the same enzyme preincubated for 5 min in 5 mM malate and chromatographed in the same buffer plus 5 mM malate. It was found that the untreated stock enzyme contained about equal quantities of the activity of the tetrameric and dimeric forms. The ratio of the integrated dimer activity peak to the tetramer peak was 1.21. When pretreated and chromatographed with malate, more of the enzyme activity was found in the smaller peak, corresponding to the dimer of the 100 kD monomeric form. Here the ratio of the dimer to the tetramer was 2.45. The malate treatment has approximately doubled the proportion of the enzyme present as dimer, although some tetramer still remains. This latter point is interesting in that it indicates that at least under these conditions, it is not possible to completely shift the equilibrium to the smaller form.

The malate treatment has also substantially reduced the total activity. Summing tetramer and dimer peaks and comparing control and malate-treated runs, shows an inhibition of 72.6%. This rather large increase in sensitivity due to preincubation and separation by HPLC associated with a doubling of the proportion of dimer raises questions about quantitative aspects of the linkage between the aggregational state of the enzyme and its inhibition by malate.

DISCUSSION

Although there is a general agreement that malate probably is a major factor in regulation of PEPC (5, 11, 12) and there are a number of papers in which malate has been shown to inhibit this enzyme from various sources (10–13), relatively little is known about the nature of the malate inhibition and the factors which affect it. The fact that short preincubation with relatively low concentrations of malate is capable of rendering PEPC more sensitive to malate inhibition has made it possible to clarify some aspects of malate inhibition, although of course the results raise more questions than they answer.

One point concerns the type of inhibition involved. Where studies have attempted to characterize this, the inhibition is usually classified as mixed, *i.e.* both an increase in K_m and a decrease in V_{max} are observed (3, 9, 17). This type of inhibition would be consistent with the idea that the malate site is different from the active site of PEPC (10).

However, some of our earlier results (17, 18) indicated that there is a consistent effect of malate on K_m regardless of how sensitive the enzyme may be to malate according to the usual definition (decreased activity at fixed levels of PEP). This has been confirmed here and we have shown that there is an essentially constant competitive effect of malate (increased K_m) regardless of the degree of malate inhibition of V_{max}. It therefore seems likely that malate does bind at the PEP site and reduces the affinity of the enzyme for its substrate. The effect on V_{max} also shown in Table IV can be altered by the type of preincubation, but this does not influence the K effect. The V effect may well be a result of binding of malate at another site, but it is of interest that the presence of PEP reduces the V inhibition due to malate so it may be that even that effect is due to malate binding at the active site. On the other hand, the fact that malate isotherms usually yield nonhyperbolic relationships with malate concentrations and Hill numbers in the vicinity of two (9, 17) may indicate two binding domains for malate close enough to be cooperative.

The additive effect of preincubation with malate and assay with malate does not seem consistent with inhibition of the enzyme by malate, but rather with a progressive conversion from an active to an inactive form when exposed to malate under the appropriate conditions. We are coming to accept the hypothesis that the V type inhibition is not a classic case of inhibition, but that it represents the conversion, under the influence of malate, of an active form of the enzyme to an inactive one. Similar suggestions have recently been made (14) with respect to maize leaf PEPC dissociated from tetramer to dimer by diethylpyrocarbonate. It was found that activity and tetramer level of aggregation could be restored by treatment with 0.4 M hydroxylamine. Even though reversible, of course, this dissociation resulting from modification of histidine residues may be quite different from that occurring *in vivo* or *in vitro* as a result of exposure to malate.

The reasons for suspecting that malate inhibition represents a loss of activity relate to the results we have obtained with enzyme preincubated with malate. The first effect of such preincubation is to decrease the activity of the enzyme. The amount of decrease is proportional to both preincubation time and malate concentration. Another effect of this preincubation is to render the insensitive enzyme susceptible to inhibition by malate present during assay. This inhibition is additional to that caused by malate during preincubation and again is proportional to concentration, although there is no significant increase in inhibition during at least relatively short assays of 5 to 16 min. This is consistent with the earlier (17) observation that the presence of all reaction components in the assay appears to prevent any change in inhibition over time which may also account for reports (2) that total activity is not altered diurnally.

There are several types of evidence which may appear to contradict this. For example, we have found that both tetramer and dimer can be detected on PAGE gels with an activity stain (17) and in HPLC chromatograms as in these studies, but it could be that the conditions used for assay encouraged reassociation of the dimer to tetramer and thus conferred activity on the enzyme which had separated as dimer. As another example, we and others (2, 5, 17) find that the usual concentration of malate added to assays causes only partial inhibition of sensitive PEPC. However, we have found that if the inhibition is determined as a function of malate concentration, a sufficiently high concentration of malate can result in complete inhibition (17).

The effect of pH during preincubation of PEPC with buffer or buffer plus malate is interesting in comparison with the pH profile of day and night enzyme and of their response to malate (17). Where the sensitive enzyme from day leaves shows strong inhibition at pH values below 7 and less at higher pH values, the preincubation with malate at low pH results in relatively little subsequent sensitivity to malate inhibition, while pH values of 7 and above produce enzyme which is quite sensitive to malate inhibition. This may suggest that the process leading to sensitizing the enzyme by preincubation occurs as a result of binding at a different location than the malate which causes the subsequent inhibition during assay and that this site either binds a different (dissociated) form of malate or that some component binding in this site has a quite different pK than that responsible for inhibition during assay.

The question of the degree to which covalent modification of CAM PEPC by phosphorylation is a factor in the aggregational and other changes which appear to constitute a major part of the regulatory process for this enzyme seems to us to still be open in spite of two reports (1, 8) that the enzyme is phosphorylated and dephosphorylated *in vivo*.

There seems little doubt that the enzyme can be phosphorylated, although the phosphorylation of both serine and threonine residues (1) casts some doubt on the specificity of the process. Of somewhat more concern is the report (1) that the phosphorylated, night, "resistant" enzyme was inhibited 66% by 4 mM malate at 8.5 mM PEP and that dephosphorylation by phosphatase, which is assumed to render the enzyme sensitive to malate, increased the inhibition only to 81%. We have tended to interpret an enzyme which is inhibited 66% by malate as a sensitive

enzyme regardless of whether it comes from day or night leaves. In fact we seldom find an enzyme from night leaves which shows more than 25% inhibition, even from 10 mM malate.

We have presented here evidence which could bear on the question of the role of phosphorylation in the activity of the enzyme. These data imply that phosphorylation may result from the natural turnover of the enzyme and thus that PEP may donate phosphate to the enzyme. The indications of the results with prerun enzyme are that the phosphorylated enzyme, if that is what is produced by prerunning, is resistant to malate inhibition. The additional change produced by including ATP or NaF in the assay during preruns brings the enzyme to a state in which malate actually acts as an activator. We have some confidence in this result because we often encounter "resistant" enzymes which are stimulated by malate without pretreatment of any type, and other preincubation treatments have sometimes produced an enzyme which is activated by malate. On the general question of the role of phosphorylation in sensitivity to malate, we are uncertain of how close such a relationship may be and are undertaking studies designed to illuminate the interactions of these phenomena.

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