INVESTIGATION OF THE MECHANISM OF ENZYMATIC CARBOXYLATION OF PHOSPHOENOLPYRUVATE

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Although phosphoenolpyruvate (PEP) carboxylase isolated from various sources has been investigated (Bandurski and Greiner, 1953; Tchen and Vennesland, 1955; Suzuki and Werkman, 1958), the reaction mechanism has not been elucidated. The experiments to be reported clarify certain aspects of the PEP carboxylase-catalyzed reaction mechanism.

Highly-purified PEP carboxylase (350-fold purified; specific activity, 4 units per mg protein; 1 unit is equivalent to the carboxylation of 1 µmole of PEP per minute), isolated from germinating peanut cotyledons, was employed in these experiments. The properties and partial purification of this enzyme are reported elsewhere (Maruyama and Lane, 1962). Further purification of the enzyme was achieved by ammonium sulfate fractionation and hydroxyapatite chromatography. In order to be certain that the purified carboxylase was free of enzyme contaminants that might interfere in subsequent O^{18}-bicarbonate distribution experiments, it was necessary to verify the stoichiometry of the reaction. It had been previously demonstrated (Maruyama and Lane, 1962) that oxalacetate is the sole carboxylation product. In the stoichiometry experiment (see Table I) DPNH and malic dehydrogenase were added to the reaction mixture to circumvent non-enzymatic decarboxylation of oxalacetate. As shown in the table, purified PEP carboxylase coupled to malic dehydrogenase catalyzes the carboxylation of PEP to form stoichiometric quantities of orthophosphate and oxalacetate. Therefore, the enzyme preparation was considered suitable for use in the O^{18} distribution experiments to be described.

TABLE I

Stoichiometry of PEP Carboxylase-catalyzed Reaction

Incubation time	Orthophosphate formation	Malate formation[1]
(minutes)	(μmoles)	(μmoles)
5	0.21	0.23
10	0.42	0.47
15	0.69	0.70
20	0.89	0.90
30	1.30	1.30
40	1.70	1.80

Reaction mixture contained (in μmoles): Tris, pH 7.8, 80; $NaHC^{14}O_3$ (32,100 cpm per μmole), 10; PEP, 3.5; $MgCl_2$, 4; GSH, 5; DPNH, 5; excess malic dehydrogenase; and PEP carboxylase, 0.043 units (specific activity, 3 units per mg protein). Total volume, 1.0 ml. Temperature, 35°.

[1] Malate formation was determined from the C^{14}-activity fixed and the specific activity of C^{14}-bicarbonate.

The purified carboxylase exhibits an absolute requirement for Mg^{++} and a sulfhydryl compound (such as GSH), is reversibly inhibited by CMB, is insensitive to avidin or hydroxylamine (0.2 M), and does not catalyze $HC^{14}O_3^-$-oxalacetate exchange. The K_m value for both PEP and Mg^{++} is approximately 3×10^{-4} M. The fact that partial reactions or intermediates could not be demonstrated and the fact that the reaction is irreversible greatly increased the difficulty of carboxylase mechanism investigations. We considered the possibility that the bicarbonate anion, rather than molecular CO_2, as visualized by Tchen, Loewus and Vennesland (1955), is the active substrate species involved in the carboxylation reaction. It is proposed that the nucleophilic bicarbonate anion attacks the electrophilic phosphorus atom of PEP initiating the events depicted in Figure 1. This model was tested using O^{18}-carbonate (mainly O^{18}-bicarbonate at the reaction pH) as substrate and then analyzing the reaction products (orthophosphate and malate) for O^{18}. Due to the lability of oxalacetate, DPNH and malic dehydrogenase were included in the reaction mixture and malate was analyzed for O^{18} instead of oxalacetate. If the model is correct, one atom of O^{18} should be incorporated into orthophosphate for every two atoms of O^{18} incorporated into malate. Non-enzymatic O^{18}-bicarbonate-H_2O^{18} exchange was minimized by use of short incubation periods and a relatively alkaline final reaction pH

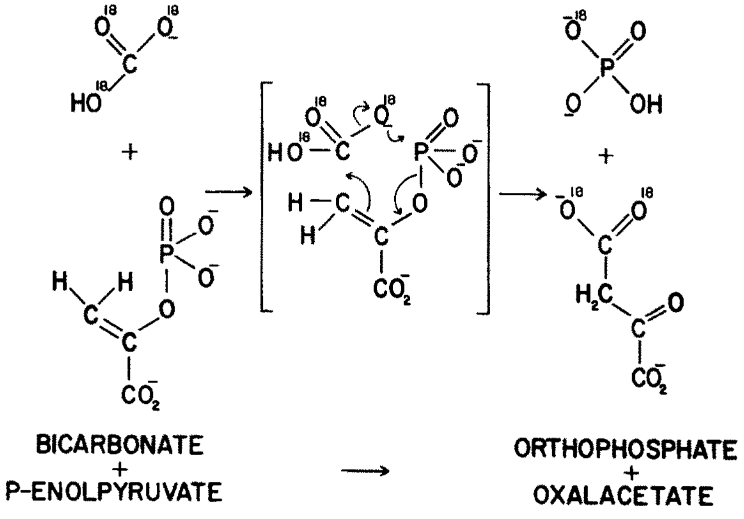

Figure 1. Proposed Mechanism of PEP Carboxylation Catalyzed by Peanut PEP Carboxylase

(pH 8.3). The proposed model reaction was confirmed as evidenced by the data from three experiments summarized in Table II. The incorporation of O^{18} into orthophosphate was considerably greater than could be accounted for by any process other than direct O^{18} transfer from bicarbonate (or carbonate) to the phosphoryl group of PEP. Moreover, the ratios of O^{18} incorporation into orthophosphate relative to malate were relatively close to the theoretical value. Despite the precautions

TABLE II

Incorporation of Bicarbonate (or Carbonate) O^{18} into the Enzymatic Reaction Products of PEP Carboxylation

Reaction product[1]	Quantity formed	Total oxygen content of product	O^{18} content of product		
			% Excess	Total incorporation	
				µatoms	Ratio $\frac{\text{phosphate}}{\text{malate}}$
	µmoles	µatoms			
Experiment 1					
P_i	40	160	4.7	7.5	-
Experiment 2					
P_i	40	160	6.9	11.0	-
Experiment 3					
P_i (a)	40	160	5.8	9.3	-
P_i (b)	40	160	5.7	9.1	-
Malate (a)	40	200	12.5	25.0	0.38 (theory, 0.5)
Malate (b)	40	200	10.7	21.4	0.43 (theory, 0.5)

Reaction mixture contained (in µmoles): Tris, pH 8.0, 914; PEP, 40; $MgCl_2$, 45; GSH, 57; DPNH, 60; malic dehydrogenase, 35 units; PEP carboxylase, 5, 6.5, and 7.2 units (specific activity, 4 units per mg protein) in Experiments 1, 2, and 3, respectively; and $K_2CO_3^{18}$ (59% $K_2CO_3^{18}$), 103. Final volume, 6.0 ml. Reaction was initiated by addition of crystalline $K_2CO_3^{18}$ to the cold (0°) reaction mixture followed immediately by mixing of the tube contents at the incubation temperature of 35°. Tubes were incubated (including thermal equilibration time) for 9, 6, and 5 minutes, respectively, in Experiments 1, 2, and 3. The O^{18} contents of carbonate reisolated from control tubes (PEP carboxylase omitted) after a 5- and 10-minute incubation at 35° were 21.5 and 18.5%, respectively.

[1] Orthophosphate was isolated as $MgNH_4PO_4$, purified, and converted to NaH_2PO_4 (Experiments 1 and 2) or $Ba_3(PO_4)_2$ (Experiment 3). NaH_2PO_4, $Ba_3(PO_4)_2$, and malic acid were analyzed for O^{18} by YEDA Research and Development Co., Ltd., Wiezman Institute of Science, Rehovoth, Israel.

taken considerable O^{18}-bicarbonate-H_2O^{18} exchange occurred as reflected by the O^{18} content of the residual carbonate after incubation compared to its original O^{18} content (see Table II, Legend and Footnote). This in no way effects the validity of the conclusions drawn. O^{18} incorporation into orthophosphate via H_2O^{18} could not have occurred to any significant extent since any H_2O^{18} generated would have undergone tremendous dilution with unlabeled H_2O. Water samples obtained at the end of the reaction contained no more than the natural abundance of O^{18} (0.21, 0.21, and 0.19% O^{18}).

The experiments described suggest that in the reaction catalyzed by PEP carboxylase: (1) the bicarbonate (or carbonate) anion, rather than CO_2, is the active substrate species involved and (2) nucleophilic attack by the bicarbonate (or carbonate) anion on the electrophilic phosphoryl phosphorus atom of PEP is involved (see Figure 1).

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References

Bandurski, R. S., and Greiner, C. M., J. Biol. Chem., 204, 781 (1953).

Maruyama, H., and Lane, M. D., Biochim. Biophys. Acta, In Press (1962).

Suzuki, I., and Werkman, C. H., Arch. Biochem. Biophys., 76, 103 (1958).

Tchen, T. T., and Vennesland, B., J. Biol. Chem., 213, 533 (1955).

Tchen, T. T., Loewus, F. A., and Vennesland, B., J. Biol. Chem., 213, 547 (1955).