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Purification and Characterization of Phosphoenolpyruvate Carboxylase from a Cyanobacterium

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Phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31) was purified 100-fold from the cyanobacterium *Coccochloris peniocystis* with a yield of 10%. A single isozyme was found at all stages of purification, and activity of other β-carboxylase enzymes was not detected. The apparent molecular weight of the native enzyme was 560,000. Optimal activity was observed at pH 8.0 and 40°C, yielding a V_{max} of 8.84 μmol/mg of protein per min. The enzyme was not protected from heat inactivation by aspartate, malate, or oxalacetate. Michaelis-Menten reaction kinetics were observed for various concentrations of PEP, Mg^{2+}, and HCO_3^-, yielding K_m values of 0.6, 0.27, and 0.8 mM, respectively. Enzyme activity was inhibited by aspartate and tricarboxylic acid cycle intermediates and noncompetitively inhibited by oxalacetate, while activation by any compound was not observed. However, the enzyme was sensitive to metabolic control at subsaturating substrate concentrations at neutral pH. These data indicate that cyanobacterial PEP carboxylase resembles the enzyme isolated from C_3 plants (plants which initially incorporate CO_2 into C_3 sugars) and suggest that PEP carboxylase functions anapleurotically in cyanobacteria.

In the photoautotrophic cyanobacteria, carbon dioxide fixation is mediated by the Calvin-Benson cycle. It has been found, however, that during short-term exposures of cyanobacterial cells to ^{14}C-bicarbonate in the light, both 3-phosphoglyceric acid (3-PGA) and C_4 acids are rapidly labeled, but only the ^{14}C from 3-PGA is transferred to other compounds (7). Although C_4 acids are never the sole initial products of photosynthetic CO_2 fixation in the cyanobacteria, they may account for 10 to 20% of the total fixed carbon during steady-state photosynthesis. These data indicate that two separate carboxylation reactions occur in cyanobacteria in the light, since dark fixation never exceeded 5% of the observed photosynthetic rate (7), and that a significant amount of carbon is continuously flowing into C_4 acids during photosynthesis.

The principal C_4 acid labeled during cyanobacterial photosynthesis in $^{14}CO_2$ is aspartate, which is labeled almost entirely in the β-carboxyl carbon, indicating that it originates from the amination of oxalacetic acid (OAA) produced by the β-carboxylation of phosphoenolpyruvate (PEP) (8, 12). The enzyme catalyzing the formation of PEP in cyanobacteria has generally been referred to as PEP carboxylase (PEP-case) (11, 14), although the enzyme has not been purified and characterized and in only one case has a cyanobacterial β-carboxylase been shown to be PEP-case (1).

OAA formation by the β-carboxylation of PEP is known to be catalyzed by three enzymes in bacterial and plant cells: PEP-case (EC 4.1.1.31), PEP carboxykinase (PEP-CK; EC 4.1.1.32), and PEP carboxytransphosphorylase (PEP-CTrP; EC 4.1.1.38). The reactions catalyzed by these enzymes have essentially the same stoichiometries and differ only in the inorganic phosphate acceptor utilized by each. Therefore, precise identification of the β-carboxylase enzyme is only possible if the stoichiometry of the reaction is known (32).

The three enzymes also have different physiological functions. PEP-CK is widely distributed in nature, and its function is the production of PEP used in gluconeogenesis or the generation of CO_2 in certain C_4 plants (plants which initially form C_4 acids). The reaction uses ADP as the P_i acceptor and is freely reversible. It therefore performs a number of partial reactions (28). PEP-CTrP also catalyzes a freely reversible reaction, utilizing P_i as the P_i acceptor, but has only been detected in the propionic acid bacteria and in one *Amoeba* species. The enzyme generally functions anapleurotically in vivo, although it catalyzes net carbon fixation in propionic acid bacteria, and in this case PEP is supplied by the action of pyruvate orthophosphate dikinase (32).

In contrast, PEP-case catalyzes the irreversible carboxylation of PEP, utilizing HCO_3^- to form OAA and P_i, and does not catalyze any partial reactions (22). PEP-case activity was originally detected in spinach leaf extracts by Bandurski and Greiner (2) and has since been found in most bacteria, algae, and vascular plants, but has not been detected in yeast, fungi, or animal tissue, in which its function is served by pyruvate carboxylase (32). Consequently, it has been assumed that PEP-case functions anapleurotically, supplying OAA to the tricarboxylic acid (TCA) cycle in bacteria and C_3 plants (plants which initially incorporate CO_2 into C_3 sugars). However, PEP-case also catalyzes the primary CO_2 fixation reaction in some higher plants (C_4 plants) which have a high apparent affinity for CO_2, and in some crassulacean acid metabolism (CAM) plants which fix CO_2 in the dark. These reactions are not mediated by a single isozyme, and in some investigations a number of PEP-case enzymes have been identified whose different kinetic properties can be correlated with specific physiological functions (30).

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The identity and function of the cyanobacterial β-carboxylase are unclear, and the aim of the present study was to characterize the enzyme from *Coccochloris peniocystis* and to examine the kinetic characteristics of the purified enzyme in an attempt to clarify its role in cyanobacterial carbon metabolism.

MATERIALS AND METHODS

Culture of cyanobacterium. *C. peniocystis* Kutz (UTEX 1548) was obtained from the University of Texas Culture Collection and grown axenically in batch culture as described previously (23). Cells were harvested in the late logarithmic phase of growth by centrifugation at 4,000 × *g* for 7 min at room temperature and then washed twice with fresh growth medium.

Preparation of cell extracts. Cell extracts of *C. peniocystis* were obtained by osmotic lysis of spheroplasts produced by incubating harvested cells in an osmoticum containing 30 mM potassium phosphate buffer, pH 7.5, 0.55 M sorbitol, 0.06% (wt/vol) lysozyme (egg white; Sigma Chemical Co.) and 2% (wt/vol) meicelase, at a concentration of 30 μg of chlorophyll *a* (Chl *a*) per ml (10^9 cells per ml or 2.31 mg [dry weight] of cells per ml) for 2 h at 30°C in the dark. The spheroplasts were recovered by centrifugation at 4,000 × *g* for 7 min, gently washed in an osmoticum containing buffer, and lysed by incubating the suspended cells (150 μg of Chl *a* per ml) in lysis buffer containing 50 mM potassium phosphate buffer, pH 8.0, 0.1 mM $MgCl_2$, 1% (wt/vol) bovine serum albumin (BSA), and 0.08% (vol/vol) 2-mercaptoethanol for 10 min at 0°C. The suspension was then centrifuged at 10,000 × *g* for 10 min at 0°C, the supernatant was decanted and stored on ice, and the lysis procedure was repeated on the pelleted material. The combined supernatants were incubated with insoluble polyvinylpolypyrrolidone (PVPP, 5% [wt/vol]) on ice for 10 min. PVPP was then removed from the suspension by centrifugation at 10,000 × *g* for 10 min at 0°C, and the supernatant was used immediately for enzyme isolation. Osmotic lysis of cells provided a higher yield of enzyme activity than did sonication or mechanical grinding with glass beads and therefore was the method of choice for cell breakage. Similar observations have been reported previously (10).

Cell extracts of leaves of greenhouse-grown plants of *Ananas comosus* were obtained by cutting 1 to 2 g (fresh weight) of fully expanded leaves into 1-cm strips and grinding the leaf strips in a medium containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-NaOH buffer, pH 7.0, 5 mM dithiothreitol, 1 mM $MgCl_2$, and 0.5% (wt/vol) Triton X-100 at 4°C under an N_2 atmosphere. The extract was filtered through two layers of cheesecloth and centrifuged at 10,000 × *g* for 5 min at 0°C, and the supernatant was used immediately for enzyme assay.

Purification of PEP-case. All procedures were carried out at 0 to 4°C. Solid ammonium sulfate was added slowly to the cell extract of *C. peniocystis*, which was stirred constantly, to a final concentration equal to 20% saturation. After 1 h, the suspension was centrifuged at 10,000 × *g* for 15 min, and the supernatant was decanted and raised to 40% saturation by the addition of solid ammonium sulfate. Precipitated material was recovered by centrifugation as described above, suspended in a minimal volume of extraction buffer (lysis buffer without BSA), and desalted by centrifugation through a Sephadex G25 column (1.5 by 5 cm) at 1,000 × *g* for 2 min.

The desalted material was applied to a DEAE-cellulose column (1.5 by 16 cm) equilibrated with extraction buffer and eluted with the same buffer until phycocyanin had been completely eluted. Elution was continued with a 300-ml linear gradient of 0 to 0.5 M NaCl in extraction buffer. Fractions containing PEP-case were pooled, and solid ammonium sulfate was added to 50% saturation. The precipitate was collected by centrifugation, suspended in 1 ml of extraction buffer, applied to the top of a Sephacryl S-300 superfine gel filtration column (1 by 80 cm) equilibrated with extraction buffer, and eluted with the same buffer. Fractions eluted from this column, which had high PEP-case activity, were pooled, and ammonium sulfate was added to 50% saturation. The resulting precipitate was collected by centrifugation, suspended in 2 ml of extraction buffer, and stored at 0 to 4°C.

Enzyme assays. (i) PEP-case. The standard assay mixture for PEP-case contained 50 mM Tris hydrochloride buffer, pH 8.0, 5 mM $MgCl_2$, 5 mM monosodium glutamate, 2 mM 2-mercaptoethanol, 2.5 mM PEP, and 25 mM $NaH^{14}CO_3$ (specific activity, 50 μCi/mmol) and 0.4 ml of the enzyme preparation (10). Unless otherwise stated, assay mixtures, without PEP, were incubated at 30°C for 10 min. The reaction was then initiated by the addition of PEP and terminated after 5 min by the addition of 0.5 ml of 0.15 M phenylhydrazine in 4 M HCl. Residual $H^{14}CO_3^-$ was removed by sparging the mixture with CO_2 for 30 min. Aqueous Counting Scintillant (10 ml; Amersham Corp.) was then added to the mixture, and ^{14}C incorporation into acid-stable products was determined with a Packard Tricarb 300C liquid scintillation counter. The rate of PEP-case-mediated OAA formation was calculated from the rate of ^{14}C incorporation, taking into account the specific activity of the added $NaH^{14}CO_3$ and the absolute amount of dissolved inorganic carbon in the assay mixture as determined by a sensitive gas chromatographic technique (4).

PEP-case activity was also determined spectrophotometrically by monitoring the rate of NADH oxidation at 340 nm in 3 ml of standard assay mixture containing 0.128 mM NADH and 10 U of malic dehydrogenase but without $NaH^{14}CO_3$.

In both assays, one unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of OAA per min.

(ii) PEP-CK. PEP-CK activity was estimated either by measuring the rate of the ATP-dependent $^{14}CO_2$ exchange reaction between OAA and PEP (28) or spectrophotometrically by measuring the rate of the ATP-dependent decarboxylation of OAA (16).

(iii) Pyruvate carboxylase. The ATP-dependent carboxylation of pyruvate in the presence of $H^{14}CO_3^-$ to form ^{14}C-OAA, catalyzed by pyruvate carboxylase (EC 6.4.1.1), was assayed by coupling this reaction with glutamic-oxalacetic transaminase (Sigma) to produce ^{14}C-aspartate in the presence of glutamate (18).

(iv) PEP-CTrP. PEP-CTrP activity was estimated by measuring the ATP-dependent rate of $^{14}CO_2$ exchange between OAA and PEP and the decarboxylation of OAA as described above for PEP-CK except for the addition of PP_i (0.5 mM), which facilitates PEP-CTrP activity (32).

Stoichiometry of the PEP-case reaction. The stoichiometry of the β-carboxylase reaction catalyzed by the enzyme from *C. peniocystis* was determined by simultaneous measurement of the rates of three reactions: NADH oxidation; ^{14}C incorporation from $H^{14}CO_3^-$ into acid-stable products; and the release of P_i into the reaction medium. The reaction was carried out as described for the spectrophotometric assay of PEP-case except that $H^{14}CO_3^-$ was added to give a specific

TABLE 1. Summary of the purification of PEP-case from *C. peniocystis*

Purification step	PEP-case activity (U)[a]	Protein (mg)	Sp act (U/mg)	Yield (%)	Purification (fold)
Crude supernatant					
Without PVPP	14.24	156.7	0.091	100	
With PVPP	13.44	154.1	0.087	94.4	
Ammonium Sulfate (20–40%)	10.85	50.4	0.22	76.2	2.4
DEAE-cellulose chromatography	2.96	0.77	3.85	20.8	42.2
Sephacryl S-300 gel filtration	1.95	0.22	8.87	13.7	97.4

[a] One unit of activity is defined as 1 μmol of OAA produced per min.

activity of 50 μCi/mmol. The reaction was initiated by the addition of PEP. Samples were removed at timed intervals for assay of P_i and for determination of ^{14}C incorporation into acid-stable products.

Molecular weight determination. The molecular weight of the native enzyme was estimated by gel filtration on a Sephacryl S-300 superfine column (1 by 80 cm) equilibrated with extraction buffer. The proteins used for calibrating the column were thyroglobulin (660,000), ferritin (440,000), catalase (232,000), and aldolase (158,000).

Protein concentration was determined by the TCA precipitation method of Bensadoun and Weinstein (3) with BSA (fraction V; Sigma) as the standard. P_i was determined by the method of Lowry and Lopez (21) with KH_2PO_4 as the standard.

Cyanobacterial Chl *a* was determined spectrophotometrically after extraction with methanol (4). The chlorophyll content of higher-plant extracts was also determined spectrophotometrically in 90% acetone with the formulas for Chl *a* and *b* of Jeffery and Humphrey (19).

RESULTS

Purification and stability of the enzyme. The scheme for PEP-case purification from *C. peniocystis* is outlined in Table 1. The enzyme was purified 100-fold with an overall yield of 10 to 15%. The elution profile from a DEAE-cellulose column indicated the presence of a single enzyme species which eluted between 0.25 and 0.35 M NaCl. A single peak of activity of a high-molecular-weight enzyme was also eluted from a Sephacryl S-300 superfine column. At no time during the purification was a lower-molecular-weight isozyme species observed. The molecular weight of the native enzyme, as estimated by gel filtration, was 560,000. At this stage of purification the enzyme could be stored at −80°C as a concentrated, undesalted solution containing 33% (vol/vol) glycerol for at least 6 weeks without detectable loss of activity.

Substrate requirements. The reaction was dependent on the presence of PEP and divalent cations. The PEP requirement could not be replaced by a combination of pyruvate, ATP, and acetyl coenzyme A (acetyl-CoA), and the reaction was not affected by avidin. Magnesium was the preferred cation cofactor, although manganese, and to a lesser extent cobalt and zinc, supported some activity (80, 60, and 60% of full activity, respectively).

The enzyme did not catalyze any partial reactions; that is, it did not catalyze the decarboxylation of OAA in the presence of ATP or an ATP-dependent exchange reaction between ^{14}C-bicarbonate and OAA, as would be expected if the reaction were catalyzed by PEP-CK (32). However, cell extracts of *A. comosus*, known to contain PEP-CK, catalyzed both decarboxylation (21.7 ± 2.4 μmol of OAA per mg of Chl per h) and the exchange reaction (443 ± 2.8 μmol of C per mg of Chl per h); these rates are similar to those reported previously (13). These two reactions were also not catalyzed by the *C. peniocystis* enzyme in the presence of PP_i (0.5 mM), conditions known to facilitate the activity of PEP-CTrP (32).

Activities of PEP-CK, PEP-CTrP, and pyruvate carboxylase were also not detectable in crude cell extracts obtained by lysing *C. peniocystis* cells, indicating that PEP-case is the only β-carboxylase present in this cyanobacterium.

The results of a typical experiment to determine the stoichiometry of the reaction with respect to NADH oxidation, ^{14}C-bicarbonate incorporation into acid-stable products, and the appearance of P_i in the reaction medium are shown in Table 2. Linear regression analysis of these data indicated that the three reactions occurred at equal rates, confirming that the reaction is phosphate-acceptor independent, as phosphate and not pyrophosphate is a product of the reaction.

Reaction kinetics. The enzyme catalyzed the incorporation of ^{14}C into acid-stable products at a linear rate with respect to both time, up to 15 min, and protein concentration, up to 75 μg/ml.

Maximum enzyme activity was observed at pH 8.0, although the rates obtained over the range pH 7.6 to 8.6 were relatively constant, and activity decreased markedly above and below this range. A sharp temperature optimum occurred at 40°C, with a calculated activation energy of 23 kJ/mol. The enzyme was rapidly inactivated at temperatures of 55°C or higher and was not protected from heat inactivation by aspartate, malate, or OAA at final concentrations of 10 mM.

The K_m values of the enzyme for PEP, HCO_3^-, and Mg^{2+}, determined under optimal conditions, were 0.6, 0.27, and 0.8 mM, respectively. Hyperbolic Michaelis-Menten reaction kinetics (Fig. 1) yielding linear, double-reciprocal Lineweaver-Burke plots were observed during all K_m determinations. Determination of the K_m for Mg^{2+} was complicated by the rapid, irreversible inactivation of the enzyme when the Mg^{2+} concentration was reduced below 20 μM.

Effectors of enzyme activity. The effect of a number of metabolites and other compounds on cyanobacterial PEP-case activity was determined under optimal conditions with

TABLE 2. Stoichiometry of the *C. peniocystis* PEP-case reaction

Reaction assayed	Compound concn (nmol/ml) at sampling time (min):							Linear regression (nmol/min)	Correlation (*r*)
	0	5	10	15	20	25	30		
NADH oxidation	0	73	142.6	204.3	—[a]	—	—	$Y = 13.65X + 2.6$	0.999
P_i formation	0	—	216.0	245.0	274.0	352.0	420.0	$Y = 13.03X + 34.1$	0.978
OAA formation	0	—	142.0	215.0	279.0	371.3	387.0	$Y = 14.6X - 3.0$	0.998

[a] —, Not determined.

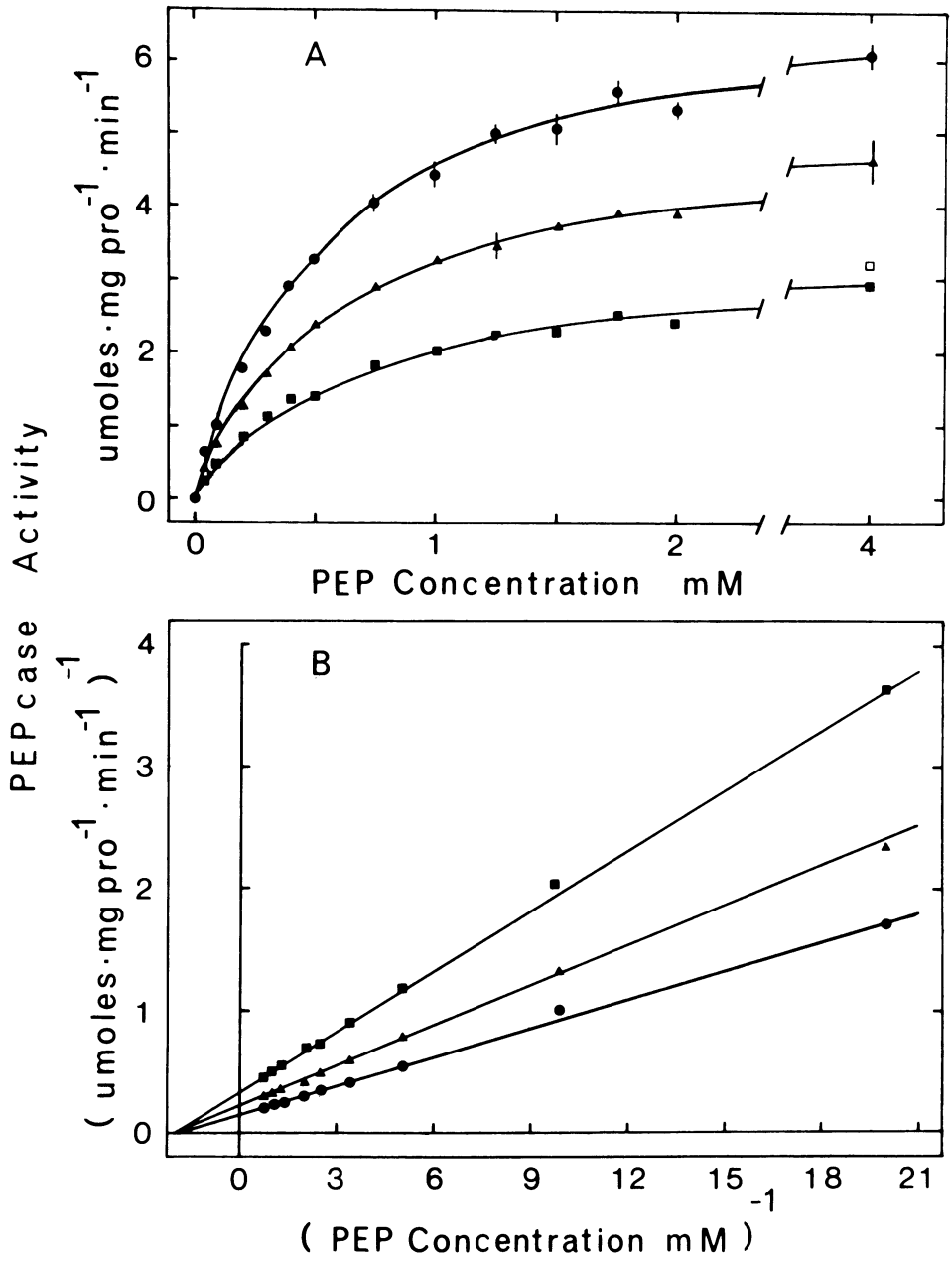

FIG. 1. (A) *C. peniocystis* PEP-case activity as a function of various PEP concentrations at OAA concentrations of 0 mM (●), 2.5 mM (▲), and 6.0 mM (■). The reaction rate at 30 mM PEP and 6.0 mM OAA is also shown (□). Vertical bar, Standard deviation from the mean. (B) Double-reciprocal plot of PEP-case activity as a function of PEP concentration at three concentrations of OAA. Symbols are as for panel A. pro, Protein.

final effector concentrations of 10 mM. The compounds tested were those known to affect PEP-case isoenzymes isolated from other sources and included glycolytic pathway and TCA cycle intermediates, amino acids, phosphorylated sugars, di- and trinucleotides, and pyridine carboxylic acids.

Although three compounds, pyruvate, 3-PGA, and NADPH, were found to activate the cyanobacterial PEP-case, the effect was marginal under optimal assay conditions and resulted in only a 10% enhancement of activity. The enzyme was severely inhibited by OAA (70%) and maleate (90%) and to a lesser extent by citrate (55%), isocitrate (30%), α-ketoglutaric acid (20%), ATP (50%), aspartate (25%), malonate (20%), and phosphate (20%). Inhibition by ATP resulted from metal chelation, as the addition of excess Mg^{2+} reduced the inhibition to 15%, but citrate inhibition was not reversed by Mg^{2+}. Pyridine carboxylic acids, including picolinic acid (70%), quinolinic acid (35%), and α-hydroxy-2-pyridylmethanesulfonate (85%), severely inhibited the reaction. A number of other compounds, including malate, glycine, fructose-1,6-diphosphate, acetyl-CoA, glucose 6-phosphate (G-6-P), GTP, 3-PGA, NaCl, SO_4^{2-}, avidin, and fluoride, which are known to affect the activity of PEP-case isolated from other sources, had no effect on the *C. peniocystis* enzyme.

Further investigation of the inhibition by OAA indicated that it interacts allosterically with respect to PEP, as it inhibits the enzyme in a noncompetitive manner (Fig. 1a). Hyperbolic reaction kinetics were observed at all OAA concentrations tested (0 to 8 mM; Fig. 1a). Lineweaver-Burke plots of these data were linear and indicate that OAA inhibition resulted from a reduction in V_{max}, while the K_m of PEP was not affected (Fig. 1b). Dixon plots of these data indicate a K_i for OAA of 5 mM. OAA inhibition was not relieved by a substrate concentration five times that of the inhibitor (Fig. 1a).

More detailed investigation of the effects of NaCl, PO_4^{3-}, and SO_4^{2-} on the enzyme indicated activation by NaCl between 25 and 200 mM to a maximum of 14% at 75 mM, while PO_4^{3-} and SO_4^{2-} inhibited the enzyme in a linear manner up to 15 mM, at which point inhibition was 25 and 15%, respectively.

PEP-case activity at pH 7.0 and 8.0 at nonsaturating PEP concentrations (0.5 mM) in the presence of 10 mM G-6-P, aspartate, malate, or OAA is reported in Table 3. At pH 8.0 results similar to those obtained at saturating PEP concentrations were observed. However, at pH 7.0, G-6-P activated the enzyme by 16%, reversing the pH inhibition, while the inhibitory effect of aspartate and malate was enhanced (Table 3).

TABLE 3. Effect of selected metabolites on *C. peniocystis* PEP-case activity at pH 7.0 and 8.0 and nonsaturating PEP concentration[a]

Assay mixture[b]	Relative enzyme activity (%)[c] at pH:	
	7.0	8.0
Complete	80	100
Plus G-6-P	96	107
Plus aspartate	26	70
Plus malate	58	97
Plus OAA	26	29

[a] Final PEP concentration was 0.5 mM.
[b] Final effector concentrations were 10 mM.
[c] Enzyme activities are expressed as a percentage of the rate observed in the complete reaction mixture at pH 8.0 (100% = 3.06 U/mg of protein).

DISCUSSION

The results of this study indicate that the β-carboxylase enzyme present in *C. peniocystis* is PEP-case and that PEP-case is the only β-carboxylase present in this organism. Stoichiometric and substrate requirement analyses indicate that the reaction is PEP and divalent cation dependent but nucleotide independent and that OAA and P_i are the products of the reaction. In addition, the purified enzyme does not catalyze any partial reactions and exhibits irreversible reaction kinetics under all assay conditions. Furthermore, PEP-case is the only β-carboxylase present in this organism, since crude cell extracts did not catalyze reactions consistent with PEP-CK, PEP-CTrP, or pyruvate carboxylase activities.

A single isozyme form of the enzyme was found to be present at all stages of purification. A single isozyme of PEP-case is similarly found in the green tissue of C_3 plants (25), whereas in green algae (6, 27) and in the tissues of C_4 plants (15) and CAM plants (5) two isozymes are readily distinguishable after DEAE-cellulose chromatography.

Kinetically, the *C. peniocystis* enzyme most closely resembles the enzyme found in C_3 plants and the etiolated tissue of C_4 plants. In these tissues a single isoenzyme species occurs which has a low V_{max} and a low K_m for Mg^{2+}, is not protected from heat inactivation by OAA, malate, or aspartate, and is inhibited but not activated by a number of compounds. On the other hand, the *C. peniocystis* enzyme exhibited two characteristics of the C_4 enzyme in that it had high K_m values for PEP and HCO_3^-. The high K_m for HCO_3^- is probably a reflection of the ability of the cyanobacteria to actively accumulate bicarbonate in the light, which results in dissolved inorganic carbon concentrations of at least 4 mM within the cell (9). A low K_m for HCO_3^- is therefore not required for the operation of the enzyme in this organism in the light, but a high K_m for HCO_3^- may be a factor regulating PEP-case activity in the dark when the internal inorganic carbon concentration is markedly reduced (24). The high K_m for PEP may also be a factor in controlling *C. peniocystis* PEP-case activity in the dark, as PEP concentrations have been found to decrease substantially in the dark in C_4 and CAM plants (17, 20).

Although cooperativity with respect to PEP binding has been observed with PEP-case enzymes from a number of sources (26), sigmoidal kinetics were not observed during either K_m or K_i determinations at pH 8.0 (Fig. 1). However, the noncompetitive inhibition of the *C. peniocystis* enzyme by OAA (Fig. 1) suggests that the enzyme responds allosterically to this compound. Similar results have been reported for all PEP-case isozymes (except those from CAM plants) with respect to OAA, malate, and aspartate, but the nature and number of allosteric sites are not known (26). The physiological significance of the OAA inhibition is also unknown, since OAA concentrations in vivo are usually quite low (26).

The enzyme was not activated to a significant extent by any of the compounds tested, but was inhibited by a number of compounds (Table 3). TCA cycle intermediates were the most potent inhibitors, especially those closest to OAA in the cycle. However, aspartate was a more effective inhibitor than malate.

C. peniocystis PEP-case is very sensitive to metabolite control by inhibitors at low pH and at nonsaturating PEP concentrations (Table 3). Under these conditions aspartate and malate are effective inhibitors of the enzyme. This may be a significant factor in the control of PEP-case activity in the dark. It is known that the internal pH of cyanobacteria decreases during a light-to-dark transition (9) and that aspartate concentrations remain high in the dark (17, 20). Therefore, a combination of reduced pH and high inhibitor concentration in the dark could significantly reduce the level of enzyme activity and account for the low levels of dark fixation observed in vivo (7). In the light, aspartate is the principal C_4 acid produced by cyanobacteria (8), and it appears that the enzyme can be inhibited by the accumulated product of its own activity in a manner similar to that observed in C_4 plants, where the major C_4 acid produced is the most effective inhibitor of PEP-case (31).

The results indicate that the β-carboxylation observed to occur in *C. peniocystis* (7, 8) is catalyzed by PEP-case and that this enzyme is the only β-carboxylase present in this cyanobacterium. Kinetically the cyanobacterial PEP-case most closely resembles those found in C_3 plants, suggesting that the cyanobacterial enzyme performs an anapleurotic role. However, since it is only active in the light and is not implicated in the initial photosynthetic fixation of inorganic carbon, it may serve a secondary role to augment the flow of carbon into specific synthetic pathways in the light.

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