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Comparison of Phosphoenolpyruvate-Carboxykinase from Autotrophically and Heterotrophically Grown Euglena and Its Role during Dark Anaerobiosis[1]

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ABSTRACT

***Euglena gracilis* (1224-5/9) contains phosphoenolpyruvate carboxykinase when grown autotrophic with CO_2 in the light. Its yield is higher when an additional carbon source like glucose has been added. The enzyme is lacking in cells provided with CO_2 alone and kept in the dark, whereas highest yields result if both glucose and CO_2 are provided together in the dark. The enzyme was purified by ammonium sulfate precipitation, gel filtration on Sephacryl S-300 and affinity chromatography on GMP-Sepharose. The latter step was most effective to protect the enzyme from inactivation. Its homogeneity was tested electrophoretically and immunologically. Enzymes from autotrophic and heterotrophically grown cells have identical pH optima and similar isoelectric points. The molecular weight was different: 761,000 for the enzyme from autotrophic and 550,000 for that from heterotrophic cells as determined by gel filtration. The subunit molecular weight of both enzymes is nearly the same. The kinetic data of the enzymes are slightly different. Glycolytic and tricarboxylic acid cycle intermediates are of limited influence on enzyme activity and inhibitory in unphysiological high concentrations. From Ouchterlony double immunodiffusion and enzyme-linked immunosorbent assay, it is evident that the enzyme is localized in the cytosol. With the latter quantification test the phosphoenolpyruvate carboxykinase protein content was found 10 times higher in heterotrophically grown cells than when cultivated under autotrophic conditions.**

Euglena cells accumulate waxmonoesters in a high yield at the expense of their carbohydrate storage when they are kept anaerobic (10, 24, 26). For autotrophic waxester formation see Rosenberg (22). The occurrence of odd-numbered fatty acids and long chain alcohols in the lipid fraction pointed to the methylmalonyl-CoA pathway being involved and to propionyl-CoA as an intermediate (24). Incorporation studies with $^{14}CO_2$, succinate, and propionate support this view as well as the sensitivity of this kind of fermentation against rotenone (25). Further, from anaerobically supplied [2-^{14}C]pyruvate no succinate was labeled (25). This implies that CO_2 fixation is not started from pyruvate. As Peak and Peak (19) failed to detect pyruvate carboxylase it seems rather sure that CO_2 fixation is started from PEP.[3] (For a general discussion on CO_2 fixation, see Levedahl [14].) This paper deals with enzymes involved in CO_2 incorporation into oxaloacetate with PEP as the second substrate. PEP-carboxytransphosphorylase (4.1.1.38) seems to be absent in Euglena as demonstrated by Ohman and Plhák (18) and Peak and Peak (19) for strain Z and by Fischer (8) for strain T. PEPC (4.1.1.31) and PEPCK (4.1.1.32), two other enzymes involved in PEP carboxylation, have been found in strain Z by Laval-Martin *et al.* (13) and by Peak and Peak (19). The latter authors and Miyatake *et al.* (17) failed to detect PEPCK activity in autotrophically grown cells. We found both enzymes in our strain T, PEPCK even in cells cultivated with CO_2 in light. The activities of these two enzymes strongly depend on growth conditions. In general PEPCK is involved in gluconeogenesis as shown by Briand *et al.* (3) for Euglena cells grown heterotrophically with lactate. PEPC is thought to function in anaplerotic CO_2 fixation in the dark in connection with amino acid biosynthesis (19), although a strict proof is still lacking.

A comparison of both enzyme activities in cells from autotrophic or heterotrophic growth in the dark showed that PEP-carboxylase could not be responsible for the high CO_2 incorporation rate described in our earlier papers. We therefore decided to follow up further PEP-carboxykinase only. Using autotrophically grown cells we followed the induction rate of PEPCK in the light and in the dark in the presence of either CO_2 alone or glucose and CO_2. Additionally we purified the enzyme from autotrophic and heterotrophic cells and compared the properties of both proteins.

MATERIALS AND METHODS

Growth. *Euglena gracilis* T, strain 1224-5/9 (SAG, Göttingen, FRG) was cultivated either autotrophically (with 4% CO_2 in air) or heterotrophically with glucose (1%) as carbon source. The medium used was similar to that of Cramer and Myers (5). Cells were harvested at the end of the logarithmic phase of growth and checked routinely for bacterial contamination. Finally the cells were kept frozen at −20°C until use for enzyme purification.

Purification. All purification steps were carried out at 4°C. The cells were thawed and resuspended in extraction buffer (0.1 M Tris [pH 7.5], 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM DTT, 25 mM $(NH_4)_2SO_4$ and 0.5% [w/v] PVP) (4) and immediately disrupted by sonication using a Branson sonifier (step 5, cycle 20%, 12 min). After centrifugation first at 3,000*g*, 5 min and secondly at 40,000*g*, 20 min yielding the crude extract a protamine sulfate precipitation (final concentration 0.2% w/v) was followed. The clear supernatant of this step was subjected to $(NH_4)_2SO_4$ precipitation which was carried out in two steps: after the first precipitation at 25% the enzyme was still in the supernatant fluid, but with the subsequent step of 45% the enzyme was pelleted. After suspending the precipitated enzyme in extraction buffer (the

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[3] Abbreviations: PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; ELISA, enzyme-linked immunosorbent assay.

same buffer as above mentioned without PVP) it was immediately put on a calibrated Sephacryl S-300 (Pharmacia) (ϕ 2.5 × 86 cm) column which was previously equilibrated with the same buffer. The column was run with a flow rate of 28 ml/h and fractions of 2 ml were collected. Protein and enzyme activity were checked. Fractions containing more than 50% of the maximal PEPCK activity were pooled and applied to the next column which was an affinity chromatographic step using GMP-Sepharose. This material was prepared similar to the method described by Lamed *et al.* (12) and March *et al.* (16). The column (ϕ 1.6 cm × 32 cm) equilibrated with 0.1 M Tris (pH 7.5) and 5 mM DTT was run with a flow rate of 21 ml/h. The pooled fractions from Sephacryl S-300 were applied to the column, washed with an appropriate amount of buffer, and eluted with a linear gradient of 0 to 3.75 mM $MnCl_2$ and ITP.

The GMP-Sepharose pool was directly used for kinetic analysis. For studying the homogeneity by gel electrophoresis the enzyme was previously concentrated by ultrafiltration.

Gel electrophoresis. Gel and buffer systems described by Davis *et al.* (6) were used for gel electrophoresis of native protein. SDS gel electrophoresis was done according to Studier (27) using acrylamide concentrations of 6 to 8% (w/v) acrylamide.

Protein. An aliquot of the samples was precipitated with TCA (0.5% w/v finally) and subsequently the protein was determined by the method of Lowry *et al.* (15) using BSA as a standard.

Preparation and partial purification of antiserum. Lyophilized enzyme protein (0.92 mg) was resuspended in complete Freund's adjuvant and finally injected intracutaneously into a rabbit. Injection was repeated after one week and subsequently at intervals of two to three weeks. A high titer of antibody was attained not earlier than six weeks after the first injection. The animal was bled to obtain approximately 30 ml of blood. The blood was left to clot for 2 h. Finally the antiserum was collected by centrifugation (40 min, 7700*g*), then $(NH_4)_2SO_4$ was added to a final concentration of 30%. The precipitate was centrifuged and it was redissolved in half the original volume of 0.15 M PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.2). The antiserum fraction was desalted on a PD-10 column (Pharmacia, Freiburg, FRG) previously equilibrated with 0.15 M PBS. Further purification of the serum was attained using a protein A-Sepharose column as described by the instruction manual "Affinity Chromatography" of Pharmacia (1).

Assay of enzyme activities. Following in principle the methods described by Briand *et al.* (3) both enzymes could be tested subsequently in the same vial. The reactions

$$PEP + HCO_3^- \xrightarrow{PEPC} oxaloacetate$$

and

$$PEP + HCO_3^- + GDP \xrightarrow{PEPCK} oxaloacetate + GTP$$

were coupled to the reaction

$$Oxaloacetate + NADH + H^+ \xrightarrow{MDH} malate + NAD^+$$

and could be followed spectrophotometrically at 360 nm.

In the direction of decarboxylation the labile substrate oxaloacetate was produced and was kept sufficiently constant by this auxillary reaction. Routinely in decarboxylation 0.1 M Tris (pH 8.0) was used as described by Briand *et al.* (3) and in carboxylation 0.1 M imidazole pH 7.5. DTT (10 mM) was added in both directions. L-Malate was 20 mM and NAD-malate dehydrogenase was present with 10 units. For testing PEPCK GDP (1.4 mM) or, for testing in the direction of decarboxylation, GTP (0.75 mM) was added. The reaction was started with the respective PEPC or PEPCK preparations.

Propionyl-CoA carboxylase activity was tested as described by Kaziro (11).

Immunoelectrophoresis. Immunoelectrophoresis (3.5 h; 100 V) was carried out in 1% agarose in 50 mM barbital buffer (pH 8.0). Better results were found if we used Tris-borate buffer (pH 8.5) (89 mM Tris, 89 mM borate, and 1 mM EDTA) instead of barbital buffer. Furthermore the presence of 0.1% Triton X-100 was necessary to facilitate diffusion of the enzyme.

ELISA. The test was carried out as described by Pönsgen and Betz (21) adapting it for the PEPCK of Euglena. The antigen as standard was used in the range of 15 to 60 μg per 200 μl.

RESULTS

PEPC and PEPCK Activities in Cells Grown Autotrophically and heterotrophically. In cells grown auto- as well as heterotrophically, PEPC and PEPCK could both be detected. The activities showed an inverse pattern (Table I). High PEPC activity could be observed during autotrophic growth, combined with low but clearly detectable PEPCK activity. However, enlarged GDP-dependent PEP-carboxylation was found during heterotrophy, where PEPC was low.

As PEPCK is obviously the enzyme responsible for the incorporation of CO_2 in fermenting cells its induction rate was followed further in autotrophic and heterotrophic growing cells.

Induction of PEPCK. When cells from autotrophic growth were transferred into the dark and supplied with glucose the activity of PEPCK rose continuously with a lag period of nearly 12 h (Fig. 1a). During the exponential phase of growth PEPCK was induced with a rate of 1.83 microunits h^{-1}. In the light with the same carbon source the rate of induction was similar but lower (81%, see Fig. 1b).

To determine whether proteins with PEPCK activity are identical in autotrophic and heterotrophic Euglena cells, purification was made to study their properties.

Purification. Enzymes from both sources were purified near homogeneity with high yields (Table II and Fig. 2). The extent of purification was different: the specific activity of the protein from autotrophic cells was considerably lower than that from heterotrophic cells. With preparations from heterotrophic cells the enzyme was purified routinely to 70- to 100-fold with a yield of 10%.

From Figure 2, A and B, it is evident that the enzymes migrated in SDS-gel electrophoresis as a single protein band with almost the same subunit mol wt of 240,000. As marker proteins phosphorylase *b* (97,400), myosin (205,000), and β-galactosidase (116,000) were used.

Immunological studies. Antibodies were raised in rabbits against purified enzyme from heterotrophic cells. Using immunoelectrophoresis we could show that the antibodies were pure and specific. Precipitin lines were found with both purified

Table I. *PEP-Carboxylating Enzymes in E. gracilis T*

Enzyme	Specific Activity	
	Heterotrophic	Autotrophic
	units/mg protein	
Crude extract		
PEPC	0.06	0.263
PEPCK		
Carboxylation	0.613	0.042
Decarboxylation	0.184	0.047
Purified enzyme		
PEPCK		
Carboxylation	32.6	23.8
Decarboxylation	16.0	9.6
Isotope exchange	ND[a]	162.3

[a] Not determined.

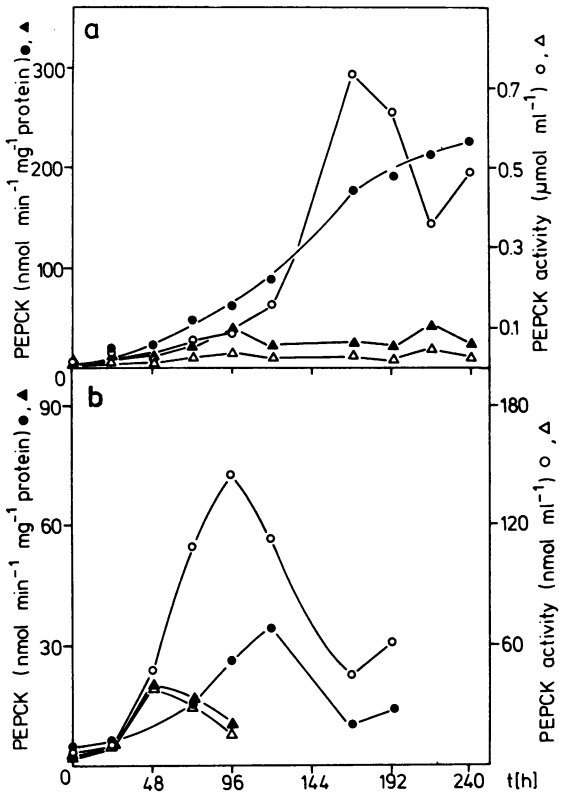

FIG. 1. a) Time course of PEPCK activity in darkness with glucose (1%) and CO_2 (2%) as carbon sources (○——○, ●——●). The control cells were supplied with CO_2 (△——△, ▲——▲) alone. b) Time course of PEPCK activity in light either with glucose and CO_2 (○——○, ●——●) or CO_2 alone (△——△, ▲——▲).

enzymes (Fig. 3). From these results it is evident that the two enzymes are not distinct immunologically. No precipitation was observed if control serum was used instead of antibodies.

In the immunoprecipitation test purified PEPCK was incubated with antibodies in various dilutions. The antigen-antibody complex was precipitated with protein A from *Staphylococcus aureus*. Figure 4 shows decreasing inhibition of enzyme activity with diluted antiserum. This result indicated an immunoreaction with the active protein.

In the ELISA test, crude extracts (supernatant of 40,000*g*) from autotrophically and heterotrophically grown Euglena cells were compared. The latter contained 48.8 ng PEPCK μg protein^{-1}, whereas only 2.5 ng/μg protein could be detected in autotrophic cells.

With protein fractions from mitochondria (containing 140–50 μg protein) from cells precultivated with malate-glutamate and finally grown in Koren-Hutner medium in the light no precipitin line could be detected in Ouchterlony double immunodiffusion.

Properties of PEPCK. Properties of both purified enzymes are summarized in Table III. Obviously they do not correspond in molecular size when determined on a Sepharose 4B gel filtration chromatography. On the other hand subunit mol wt as calculated by SDS-PAGE are nearly identical. The results of the size determination suggest that the heterotrophic enzyme is a dimer while the autotrophic one obviously consists of three equal subunits. The isoelectric points of the two enzymes are nearly identical. The same is true concerning the pH optimum of both: in the decarboxylation and in the presence of Mn^{2+} their values are exactly identical with a high value in the alkaline region; in the opposite direction(carboxylation) in the presence of either Mn^{2+} or Mg^{2+} the pH optima were also nearby identical (pH 7.5/7.6).

Most kinetic data of both purified enzymes are almost identical. However the K_m values for GDP and GTP are about 2-fold higher in the protein from heterotrophic cells. For HCO_3^- the K_m values of both enzymes differ only by a factor of 1.4. Nevertheless the K_m values for HCO_3^- are rather high. On the other hand both enzymes are characterized by low K_m values for oxaloacetate which indicates a strong binding of this substrate while the K_m for PEP is rather high in both proteins. In Table IV results of a series of experiments on the influence of metabolic intermediates, on the catalytic activity of PEPCK are summarized. It is surprising that the inhibitory effect of these organic acids is very low. Their role in regulating the enzyme activity cannot be important so much the more as high concentrations of presumed inhibitors have been used in this test. The limited but distinct effect of α-ketoglutarate both in decarboxylation and in carboxylation is probably due to the structural similarity between α-ketoglutarate and oxaloacetate. All other metabolites tested, including succinate, were of minor influence on PEPCK activity especially in the concentration range of physiological importance. Even in high concentrations (up to 20 mM), the inhibitory effect of these compounds was small.

With the exception of α-ketoglutarate the inhibitors summarized in Table IV were almost inefficient in the carboxylation reaction, too. The influence of α-ketoglutarate in different concentra-

Table II. *Comparison of Progress in PEPCK Purification between Cells either Grown Heterotrophic (A) or Autotrophic (B)*

A. $n = 6$ (representative example shown)

	Total Activity	Protein	Volume	Specific Activity	Yield	Purification
	units	*mg/ml*	*ml*	*units/mg protein*	*%*	*-fold*
Crude extract	196.6	14.7	72	0.186	100	1.0
Protamine sulfate	180.1	14.4	79	0.158	92	1.18
$(NH_4)_2SO_4$	89.6	31.7	11	0.255	46	1.4
Gel filtration	78.3	1.1	28	2.52	40	13.6
Affinity chromatography	21.8	0.181	6.0	20.02	11	107.6
B. $n = 4$ (representative example shown)						
Crude extract	36.67	17.7	181	0.011	100	1.0
Protamine sulfate	39.4	15.1	190	0.014	107	1.3
$(NH_4)_2SO_4$	15.9	41.2	20.5	0.019	43.3	1.7
Gel filtration	16.5	0.77	49	0.438	45	39.8
Affinity chromatography	2.6	0.13	10.9	3.75	7.1	340.9

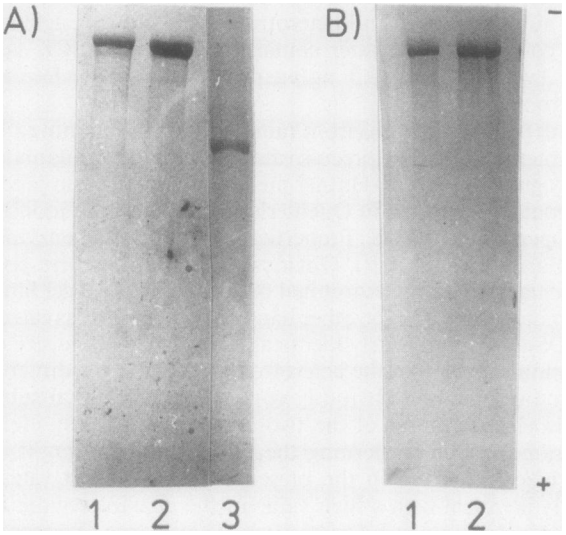

FIG. 2. SDS-gel electrophoresis of purified PEPCK derived from heterotrophically (A) and autotrophically (B) grown cells. Protein (in the concentration range of 0.05–0.12 mg ml^{-1}) from pooled peak fractions after affinity chromatography on GMP-Sepharose was applied. Electrophoresis was carried out with 6% (A) or 6 to 8% acrylamide (B).(A) Lane 1 and 2: protein denatured for 2 and 5 min in a 100°C bath. Lane 3: myosin as standard (10 μg). (B) Lane 1 and 2: protein denatured as described above.

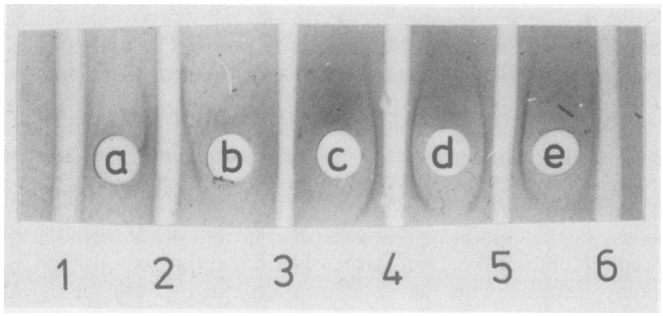

FIG. 3. Immunoelectrophoresis of purified PEPCK derived from cells grown heterotrophically (a and b, with 0.122 mg protein/ml) or autotrophically (c, d, and e, with 1.3 mg protein/ml). Enzyme extract (10 μl) was added to the wells and the channels 1, 2, 4, 5, and 6 were filled with antiserum (5.0 mg protein ml^{-1}) diluted 1:4 with PBS 0.15 M, whereas in channel 3 control serum was added.

tion of oxaloacetate or PEP. From the results summarized in Figure 5, a and b, α-ketoglutarate seems to be a competitive inhibitor with respect to both oxaloacetate and PEP.

DISCUSSION

In *E. gracilis* T (1224-5/9) PEPC and PEPCK could be detected after both autotrophic and heterotrophic growth. Their activities show an inverse pattern (Table I): PEPC is higher in autotrophic cells, whereas PEPCK is more abundant after heterotrophic growth. This finding is more remarkable as Miyatake *et al.* (17) could not detect any PEPCK activity in autotrophic cells of their strain Z. Our results are in accordance, however, with LAVAL-MARTIN *et al.* (13), working with the strains Z and ZR. They described decreasing activity of PEPCK and an increase in PEPC during the transition from heterotrophic growth on lactate to autotrophic conditions. This process was somewhat impeded in the presence of DCMU (25 μM). The authors' conclusion that PEPCK is bound to heterotrophic growth, whereas

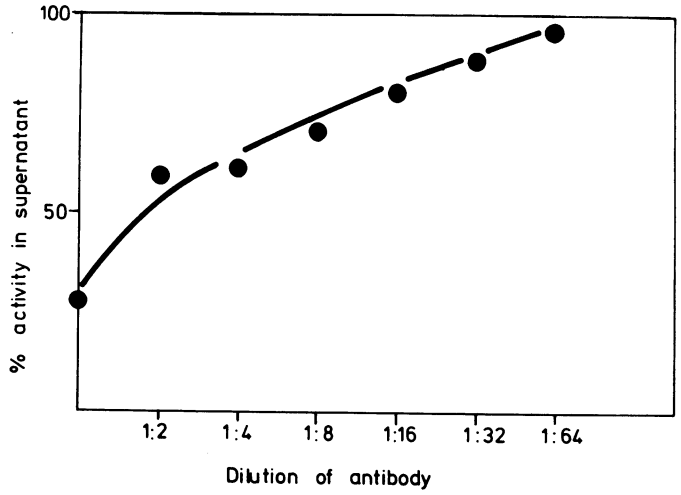

FIG. 4. Immunotitration of purified PEPCK from heterotrophically grown cells. Various dilutions of antibodies were incubated for 5 min with 50 μl of purified PEPCK at 25°C. After this incubation protein A (50 μl, 40 mg ml^{-1}) was added and incubated again for 5 min. Finally the mixture was centrifuged for 2 min and in the supernatant the remaining PEPCK activity was determined.

Table III. *Properties of Purified PEPCK from Autotrophically and Heterotrophically Grown Euglena gracilis T*

Activity was tested in the assay system described by Briand *et al.* (3) with the addition of 10 mM DTT. The carboxylation reaction was measured with GDP (1.4 mM) and 0.1 M imidazole pH 7.5 as buffer system. Standard errors are given for the mol wt, isoelectric point and K_m value for $KHCO_3$. The values are means of $n = 3$ to 4 determinations.

Properties	Growth Condition	
	Autotrophic	Heterotrophic
Mol wt[a]	761,000 ± 22,000	550,000
Subunit mol wt[b]	240,000	247,000
pH optimum:		
Decarboxylation	8.8	8.8
Carboxylation (Mg^{2+}; Mn^{2+})	7.5	7.6
Isoelectric point[c]	6.48 ± 0.035	6.46 ± 0.099
K_m values (mM) (Mn^{2+})		
PEP	0.8	0.95
GDP	0.056	0.12
$KHCO_3$	4.5 ± 0.76	7.0
Oxaloacetate	0.016	0.022
GTP	0.065	0.141

[a] Calculated by gel filtration (Sepharose 4B). [b] Calculated by SDS-PAGE. [c] Determined by isoelectric focusing.

PEPC is related to autotrophic conditions, is supported by our results. An inverse pattern of PEPC and PEPCK activity was further described for different growth phases of heterotrophic Euglena cells (on lactate) (3): PEPCK activity was high in the logarithmic phase and decreased when the cells became stationary. PEPC activity evolved just opposite.

Purification of PEPCK was accelerated by using affinity chromatography as the last step, which procedure additionally protected the enzyme from inactivation. Concerning yield and purification, our results correspond well with those described by Wicheanvonagoon and Arinze (29) for PEPCK from guinea pig mitochondria. They are rather different however from those described for yeast (28), and Ascaris (20).

After highest purification the specific activity of PEPCK from autotrophic Euglena is about 5 times lower than that from heterotrophic cells. This result fits well to that of immunoquan-

Table IV. *Influence of Metabolites on Activity of Purified PEPCK from Heterotrophically Grown Euglena Cells*

Inhibition is expressed in percent of maximum activity using the decarboxylation reaction of the enzyme with a final Mn^{2+} concentration of 0.75 mM. A constant oxaloacetate concentration of 66 μM was produced by adding 20 mM L-malate.

Substrate	Concentration (mM):		
	5	10	20
		%	
α-Ketoglutarate	36	55	74
Succinate	15	11	32
Fumarate	13	24	33
Glyoxylate	11	19	24
Malonate	13	16	35

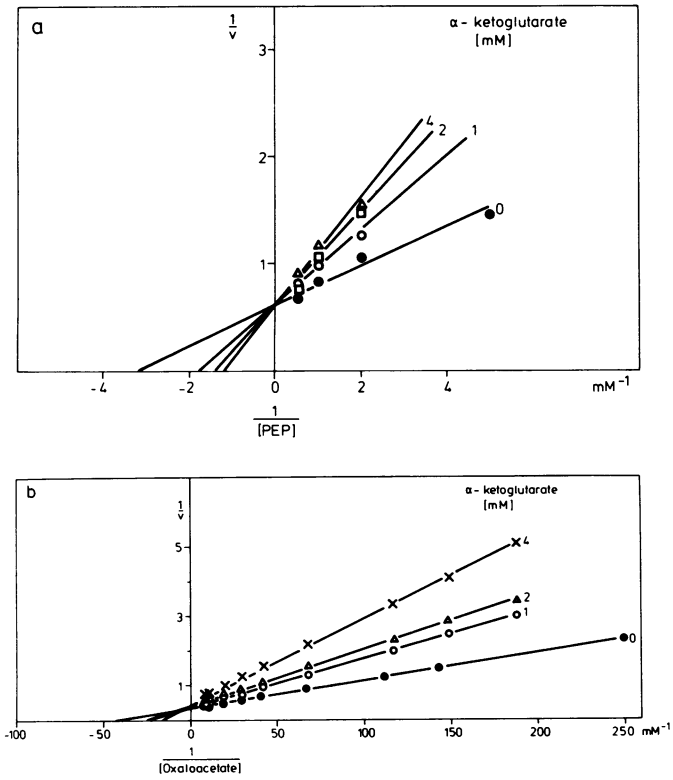

FIG. 5. Double reciprocal plot of inhibition of PEPCK activity by α-ketoglutarate at variable PEP concentrations (a) and variable oxaloacetate concentrations (b), respectively. In (a), the concentration of $MgCl_2$ was maintained at 5 mM and $MnCl_2$ at 1 mM. In (b), a $MnCl_2$ concentration of 0.75 mM was used.

tification tests which showed that 20-fold more PEPCK protein was synthesized in heterotrophic growth than in autotrophy. Both observations support the idea that this enzyme is related mainly to heterotrophy.

The mol wt of Euglena PEPCK was found distinctly higher than in the enzymes from yeast (28), Ascaris (20), or that from guinea pig liver mitochondria (29). The heterotrophic protein of Euglena can be split into two subunits, the autotrophic one in three with almost identical mol wt of the subunits (240,000–247,000) in both forms (Fig. 2, A and B). Their high values are most striking, but the similarity to the multienzyme complex described by Ernst-Fonberg and Wolpert (7) could be excluded, as no PEPC activity was present after affinity chromatography.

For optimal catalytic activity the presence of divalent cations is absolutely required. Most effective are Mn^{2+} and Mg^{2+}. When tested in the direction of carboxylation Mg^{2+} was 5 mM and Mn^{2+} 1 mM in the presence of 1.4 mM GDP in our experiments. Activation of PEPCK by a combination of Mn^{2+} and Mg^{2+} agrees with Satoh's (23) results with embryonal chicken liver. With our enzyme decarboxylation was tested with 0.75 mM Mn^{2+} only. In summary a common feature of all the PEPCKs purified so far from different sources is their stimulation by divalent cations.

Free sulfhydryl groups are essential for optimal catalytic activity of Euglena PEPCK. DTT was used in our assay system. Sulfhydryl binding compounds are strong inhibitors. Most effective were *p*-hydroxymercuriphenyl sulfonic acid and ethylmaleimide. The inhibitor concentration for more than 50% inhibition is variable from 10^{-3} to 2×10^{-6} M dependent on the age of the purified enzyme. DTT or glutathione failed to prevent this inhibitory effect.

The pH optimum for both directions of PEPCK activity was found in the alkaline region. This is in contrast to Miyatake *et al.* (17) who described a more acid optimum for carboxylation for his strain Z. The kinetic data of both enzymes are similar to those of proteins from vertebrate and invertebrate systems. Moreover they correspond with the kinetics determined from another strain of Euglena (Table V). Most striking among all these data is the difference in K_m for HCO_3^-. This value is extremely high in chicken liver enzyme (9) and surprisingly low in Euglena strain Z. The K_m values for the Ascaris enzyme and for our strain are intermediate.

From these K_m values a functional difference is evident between PEPCK from chicken, Ascaris, and Euglena (strain T). Its low affinity to HCO_3^- points to oxaloacetate decarboxylation in the enzyme from chicken liver. In Ascaris, carboxylation seems to be the preferred direction (20): the K_m for HCO_3^- is 3-fold lower than in the chicken enzyme and its affinity to PEP is higher than to oxaloacetate. Carboxylation is additionally favored by anaerobic conditions in the parasite's environment.

PEPCK from our Euglena strain seems well suited to catalyze anaerobic CO_2 fixation. We expect that it is the key enzyme for the methylmalonyl-CoA pathway yielding odd-numbered chains of fatty acids and alcohols by incorporation of propionyl-CoA into waxmonoesters in fermenting Euglena (24–26).

The enzyme from autotrophic cells has a K_m for HCO_3^- of 4 to 5 mM at pH 7.5. This corresponds to a CO_2 concentration of 0.3 to 1.2 mM in vivo (2). Rising CO_2 concentrations in anaerobic cells favour carboxylation (24). As calculated from the data of Schneider and Betz (26) odd-numbered fatty acid and alcohol chains are synthesized with about 0.016 mM min^{-1}. In earlier studies on CO_2 incorporation (26) into odd-numbered chains a rate of 0.22 nmol min^{-1} mg $protein^{-1}$ was found for propionyl-CoA formation. This corresponds well with an activity of propionyl-CoA carboxylase of 0.33 milliunit × mg protein as found in crude extract (data not shown). An activity of PEPCK in the direction of carboxylation of 42 milliunits mg protein (Table I) seems high enough to catalyze the first step in anaerobic CO_2

Table V. *Comparison of Kinetic Parameters of Purified PEPCK from Different Sources*

Substrates	K_m Values				
	Ascaris enzyme	Chicken liver	*E. gracilis* Z	*E. gracilis T*	
				Autotrophic	Heterotrophic
			mM		
HCO_3^-	7.1	20.0	0.066	4.0	7.0
PEP	0.12	0.155	0.13	0.8	0.95
GDP	0.022	0.051	0.059	0.056	0.12
Oxaloacetate	0.84		0.014	0.016	0.022
GTP			0.065	0.065	0.141

incorporation.

Our kinetic data on PEPCK fit well to the observations on the induction of the enzyme, as described in this paper, as well as to older data on $^{14}CO_2$ incorporation into waxesters in fermenting Euglena cells (25, 26) and to the results of Laval-Martin (13).

The problem of how PEPCK is controlled in living cells is still open. All metabolic intermediates so far tested are of minor efficiency. No significant influence can be expected under physiological conditions (Table IV). Only α-ketoglutarate was found slightly inhibitory in both directions, obviously in competition to oxaloacetate. For all PEPCKs purified thus far, no indications are known for control by glycolytic or tricarboxylic acid cycle intermediates. The differences between heterotrophic and autotrophic forms of PEPCK point to control at enzyme expression.

The localization of PEPCK in *E. gracilis* is still under discussion. Using antibodies against the heterotrophic form of the enzyme we found it exclusively in the cytosol of heterotrophic cells which had been raised in Koren-Hutner medium. Miyatake *et al.* (17) described the same for their strain Z with cells grown in the same medium under illumination. In contrast Briand *et al.* (3) postulated it to be localized in the mitochondrial matrix.

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LITERATURE CITED

1. Affinity Chromatography Principles and Methods August 1983 Pharmacia Fine Chemicals, Printed in Sweden by Ljiengföretagen AB, örebro
2. BAUWE H 1986 An efficient method for the determination of Km values for HCO_3^- of phosphoenolpyruvate carboxylase. Planta 169: 356–360
3. BRIAND J, R CALVAYRAC, D LAVAL-MARTIN, J FARINEAU 1981 Evolution of carboxylating enzymes involved in Paramylon synthesis (Phosphoenolpyruvate Carboxylase and Carboxykinase) in heterotrophically grown *Euglena gracilis*. Planta 151: 168–175
4. CALVAYRAC R, D LAVAL-MARTIN, J BRIAND 1983 Different functions of phosphoenolpyruvate carboxylase in Euglena depending on culture conditions. Physiol Veg 21: 835–844
5. CRAMER M, J MYERS 1952 Growth and photosynthetic characteristics of *Euglena gracilis*. Arch Mikrobiol 17: 384
6. DAVIS CH, LH SCHLISELFELD, DP WOLF, CA LEAVITT, EG KREBS 1967 Interrelationships among glycogen phosphorylase isozymes. J Biol Chem 242: 4824–4833
7. ERNST-FORNBERG ML, JS WOLPERT 1981 Multienzyme complex containing acetyl-CoA carboxylase from *Euglena gracilis*. Methods Enzymol 71: 60–72
8. FISCHER E 1969 Wechselseitige Beeinflussung von CO_2- und Acetatassimilation bei *Euglena gracilis* KLEBS. Dissertation, Göttingen
9. HEBDA CA, T NOWAK 1982 The purification, characterization, and activation of phosphoenolpyruvate carboxykinase from chicken liver mitochondria. J Biol Chem 257: 5503–5514
10. INUI H, K MIYATAKE, Y NAKANO, S KITAOKA 1983 Production and composition of wax esters by fermentation of *Euglena gracilis*. Agric Biol Chem 47: 2669–2671
11. KAZIRO Y 1969 Crystalline propionyl-CoA carboxylase from pig heart. Methods Enzymol 13: 181–190
12. LAMED R, Y CEVIN, M WITETEK 1973 Covalent coupling of nucleotides to agarose for affinity chromatography. Biochim Biophys Acta 304: 231–235
13. LAVAL-MARTIN D, J FARINEAU, B PINEAU, R CALVAYRAC 1981 Evolution of enzymes involved in carbon metabolism (phosphoenolpyruvate and ribulosebisphosphate carboxylases, phosphoenolpyruvate carboxykinase) during the light-induced greening of *Euglena gracilis* strains Z and ZR. Planta 151: 157–167
14. LEVEDAHL BH 1968 Heterotrophic CO_2 fixation in Euglena. In DE Buetow, ed, The Biology of Euglena, Ed 1, Vol II. Academic Press, New York, pp 85–96
15. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
16. MARCH SC, I PARIHL, P CUATREASAS 1974 A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal Biochem 60: 149–152
17. MIYATAKE K, T ITO, S KITAOKA 1984 Subcellular location and some properties of phosphoenolpyruvate carboxykinase (PEPCK) in *Euglena gracilis*. Agric Biol Chem 48: 2139–2141
18. OHMAN E, F PLHÁK 1969 Reinigung und Eigenschaften von PEPCK aus *Euglena gracilis*. Eur J Biochem 10: 43–55
19. PEAK JG, MJ PEAK 1981 Heterotrophic carbon dioxide fixation by Euglena. Function of Phosphoenolpyruvate Carboxykinase. Biochim Biophys Acta 677: 390–396
20. PIETRZAK ROHRER S, HJ SAZ, T NOWAK 1986 Purification and characterization of phosphoenolpyruvate carboxykinase from the parasitic helminth *Ascaris suum*. J Biol Chem 261: 13049–13055
21. PÖNSGEN E, A BETZ 1986 Isocitrate lyase of the alga *Chlorogonium elongatum*: immunological quantification in relation to growth conditions. Planta 167: 569–574
22. ROSENBERG A 1963 A comparison of lipid patterns in photosynthesizing cells of *Euglena gracilis*. Biochemistry 2: 1148–1154
23. SATOH Y 1986 Effects of Mn^{2+} on the exchange reaction of phosphoenolpyruvate carboxykinase in the presence of high concentrations of Mg^{2+}. Biochim Biophys Acta 872: 177–182
24. SCHNEIDER T, C BORKOWSKI, A BETZ 1984 Wax ester formation in Euglena gracilis during anaerobiosis and photoheterotrophic growth. *In* C Sybesma, ed, Advances in Photosynthesis Research, Vo. III. Martinus Nijhoff, The Hague, pp 445–448
25. SCHNEIDER T, C BORKOWSKI, A BETZ 1984b Rotenone sensitive waxmonoester synthesis in anaerobic Euglena gracilis: Evidence for operation of methylmalonyl-CoA pathway. *In* PA Siegenthaler, W Eichenberger, eds, Structure, Function and Metabolism of Plant Lipids. Elsevier, Amsterdam, pp 577–580
26. SCHNEIDER T, A BETZ 1985 Waxmonoester fermentation in *Euglena gracilis* T. Factors favouring the synthesis of odd-numbered fatty acids and alcohols. Planta 166: 67–73
27. STUDIER FW 1973 Analysis of bacteriophage T7 early RNAs and proteins on slabs gels. J Mol Biol 79: 237–248
28. TORTORA P, GM HANOZET, A GUERRITORE 1985 Purification of PEPCK from *Saccharomyces cerevisiae* and its use for bicarbonate assay. Anal Biochem 144: 179–185
29. WICHEANVONAGOON S, IJ ARINZE 1984 Phosphoenolpyruvate carboxykinase from guinea pig liver mitochondria. Biochem J 221: 105–111