Two Forms of Phosphoenolpyruvate Carboxylase in *Chlorella kessleri*

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FPLC of crude cell extracts of *Chlorella kessleri* reveals two protein fractions with phosphoenolpyruvate carboxylase activity. Their mole masses, 955 kDa and 543 kDa, suggest that they are dimer and monomer of the same enzyme. Further data, however, indicate that they are more likely two different iso-forms of phosphoenolpyruvate carboxylase: Attempts to interconvert both proteins *in vitro,* such as through treatment with NaCl, metabolites and thiol- or histidine-group effecting reagents, were unsuccessful. The substrate affinity of the large protein was slightly higher than that of the small protein (K_m = 1.13 and 0.76 mM, respectively); and the sensitivity to enhanced temperature was more pronounced in the large than in the smaller protein (half lifes = 23 min and 55–60 min, respectively). Some properties of both fractions, however, proved identical: 1. pH optima at pH 8.5–9, 2. Hill coefficients approx. 1, 3. no significant regulatory effect of glutamine, glutamate, aspartate and malate, 4. increase in K_m and in Hill coefficient by citrate, and 5. identical behaviour in ion exchange chromatography. Functions and localization of the assumed two iso-forms of phosphoenolpyruvate carboxylase remain to be clarified.

No specific effects of red or blue light during autotrophic growth on either protein with PEP Co activity could be found.

Introduction

Phosphoenolpyruvate carboxylase (PEP Co) catalyzes the incorporation of CO_2 in PEP resulting in the production of oxaloacetate. Operating also in the dark, it increases the amount of organically bound carbon even in non-photosynthetic organisms. In studying the regulation of the basic metabolism of plant cells, two observations in literature arouse our interest in this enzyme. Firstly, there are reports of two PEP Co forms in C4 and CAM plants, which exhibit not only different mole masses, but also different catalytic activities. They appear to be interconvertible. The larger form consists of 4 identical subunits of about 100 kDa each. It is thought to be a dimer of the smaller form [1–3]. Total *in vitro* activity of the larger – tetrameric – form is by 70% higher than that of the smaller – dimeric – form [3]. Secondly, an enhancing influence of blue light on the activity of PEP Co in non-green *Chlorella kessleri* mutants has been described. This effect supposedly results from the biosynthesis of an additional enzyme form with different catalytic properties [4, 5].

Analysis of the regulatory effectiveness of blue light on the basic metabolism of *Chlorella kessleri* cells has presented the possibility that aggregation-dissoziation processes of specific enzyme proteins might prove to be a means of regulating their activities [6]. From these data, analysis of PEP Co in *Chlorella* appeared to be rewarding. In the following, we report the existence of two species of PEP Co in wild type cells of *Chlorella kessleri,* for which however, no specific influence of blue light could be detected.

Abbreviations: FPLC, fast protein liquid chromatography.

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Materials and Methods

Organism and growth conditions

The experiments were performed with the unicellular green alga *Chlorella kessleri* Fott et Nováková no. 211–11 h, formerly *Chlorella vulgaris* of the Culture Collection of Algae of the Inst. for Plant Physiology at Göttingen University, Germany. The algae were grown in an inorganic medium according to [7] with iron being supplied as EDTA-complex. Cultur tubes (4 cm diameter, 45 cm length), aeration with air +2% CO_2 and the light thermostat used (30 ± 1 °C) have been described by [8].

Light regimes

White, red and blue light leading to equal dry matter production were produced, either by Osram-L 36 W/30-1 warm white fluorescent tubes, or by passing the radiation of Osram-L 36 W/36 natura fluorescent tubes through a 3 mm red plexiglass or that of Osram-L 115 W/20 cool white fluorescent tubes through a 3 mm blue plexiglass (Rhöm GmbH, Darmstadt, Germany).

Preparation of crude cell extracts

Crude cell extracts were prepared as described in [9], except for the use of Tris glycine buffer consisting of 30 mM Tris(hydroxymethyl)aminomethane, 250 mM glycine and 100 mM NaCl pH 8.5 for extraction.

Ion exchange chromatography

Crude extracts of *Chlorella kessleri* were separated by ion exchange chromatography on DEAE cellulose 23 SN (column 2.2 × 8 cm) using 50 mM Tris buffer pH 7.5 for equilibration and elution. After washing with two bed volumes of this buffer the column was developed with a linear KCl gradient (0–0.6 M). Two ml fractions were collected at a flow rate of 60 ml/h. KCl density in the fractions was calculated from the index of refraction using an Abbe universal refractometer (Schmid and Haensch, Berlin).

Fast protein liquid chromatography

For fast protein liquid chromatography (FPLC) superose 6 has been used as described recently [9]. Extraction buffer was used for equilibration of the column and for elution of the proteins.

Enzyme assay

PEP Co activity was measured in a coupled test by following the oxidation of NADH by malate dehydrogenase (MDH) resulting from the reduction of oxaloacetate produced by PEP Co. In order to detect pyruvate deriving from decarboxylated oxaloacetate, due to the conditions in the test-system lactate dehydrogenase (LDH) was added.

The assay mixture concentrations were: Tris(hydroxymethyl)aminomethane (Tris) buffer pH 8.5 (100 mM) or 2-(N-morpholino)ethane sulfonic acid (MES) buffer pH 5.5 (100 mM), as indicated in the text, $MgCl_2$ 12 mM, $NaHCO_3$ 30 mM, MDH 12 U, LDH 5 U, NADH 0.64 mM, crude extract 50 µl or separated fractions 200 µl (FPLC) or 500 µl (DEAE chromatography) and phosphoenolpyruvate 10 mM (start).

Determination of half life

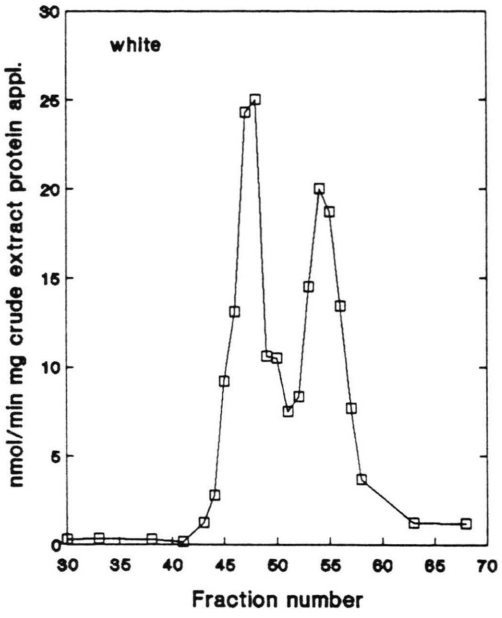
The semilogarithmic plot of PEP Co activity against the time of incubation at 45 °C results in a straight line indicating first order kinetic for the temperature dependent inactivation. Therefore inactivation can be described by the equation $N_t = N_0 \cdot e^{-kt}$ (N_t and N_0: activities at time t and $t = 0$; t: time; k: first order rate constant. Half life $t_{1/2}$ is calculated by the equation $t_{1/2} = \ln 2/k$.

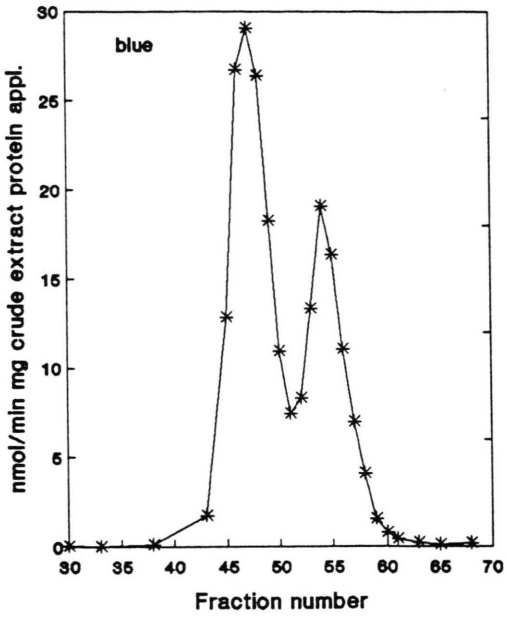
Results and Discussion

Two different protein fractions with PEP Co activity

Separation of the proteins of crude cell extracts from *Chlorella kessleri* by FPLC on superose 6 yields PEP Co activity in the fractions of 955 ± 64 kDa and of 543 ± 34 kDa. This is identical for cells under blue or red light conditions (Fig. 1).

Considering the possibility of different geometrical arrangements, these data might indicate the existence of a dimeric and a monomeric form of the same enzyme. This idea is supported by the occurrence of only one peak with PEP Co activity after protein separation by DEAE column chromatography (Fig. 2). However, the larger aggregate would have to be rather tightly bound since also subsequent separation on superose 6 of the most active fractions previously isolated by DEAE chromatography yields the two activity peaks at the known mole masses. All attempts to transfer the larger form into the smaller form failed. There was the same separation pattern as for untreated extracts, *i.e.* PEP Co activity at about 950 kDa and 540 kDa, using 1 M NaCl, 10 mM PEP, 10 mM citrate, 10 mM malate, 50 µM 5,5′-dithiobis(2-nitrobenzoic acid), 1.25 mM diethylpyro-carbonate, 5 mM dithiothreitol, 1% cystein, 15 µM $CuSO_4$, and 10 mM $MgSO_4$; these are all known to aggregate or split PEP Co proteins [1, 2, 10, 11].





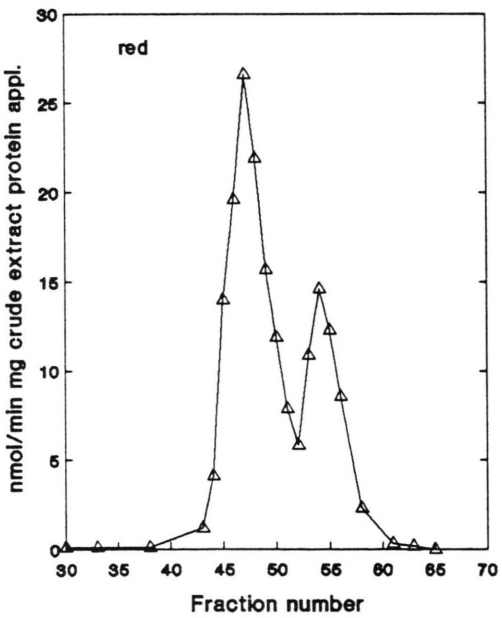


Fig. 1. Distribution of PEP Co activity after FPLC on superose 6 of crude extracts of *Chlorella kessleri* grown autotrophically in white, blue or red light of equal fluence rates of 33.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$. PEP Co activity tested at pH 8.5. Activity given in nmol $min^{-1} \times$ mg crude extract $protein^{-1}$ applied to the column.

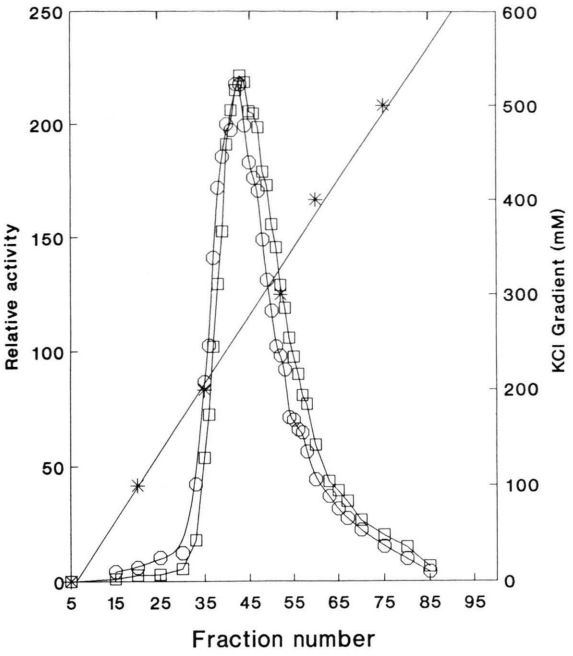


Fig. 2. Distribution of PEP Co activity after protein separation of crude cell extracts of *Chlorella kessleri* grown in blue light (○−○) or red light (□−□) by DEAE chromatography. PEP Co activity tested at pH 8.5

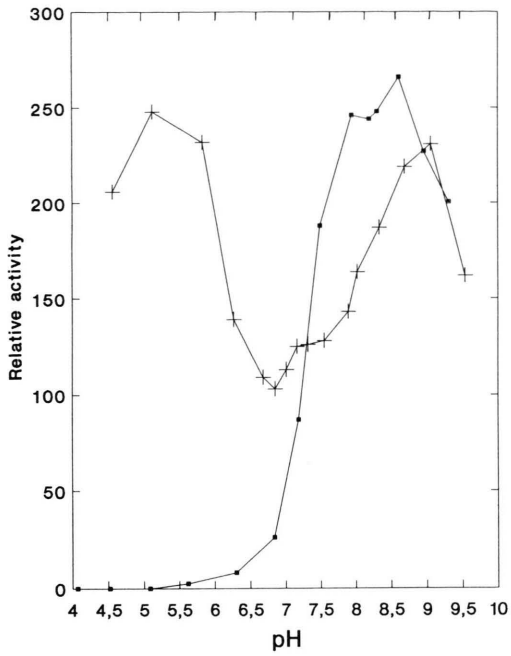


Fig. 3. pH dependence of PEP consumption of the 955 kDa (■−■) and the 543 kDa fractions (+−+) in the PEP Co test separated by FPLC on superose 6 from crude cell extracts of *Chlorella kessleri*.

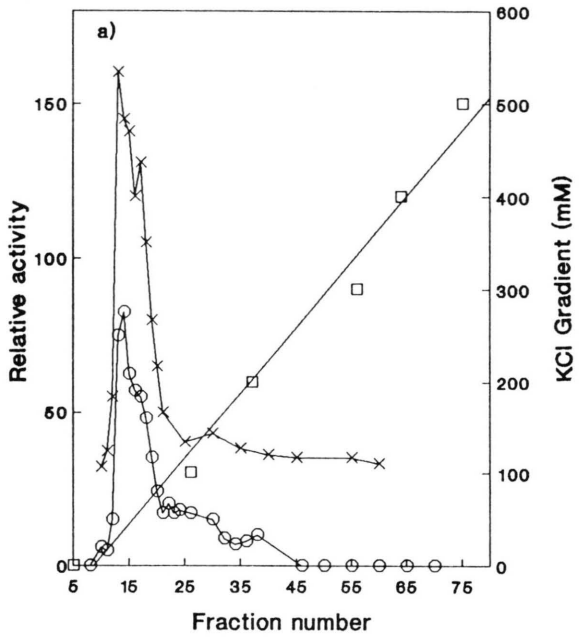
Properties of the two proteins with PEP Co activity

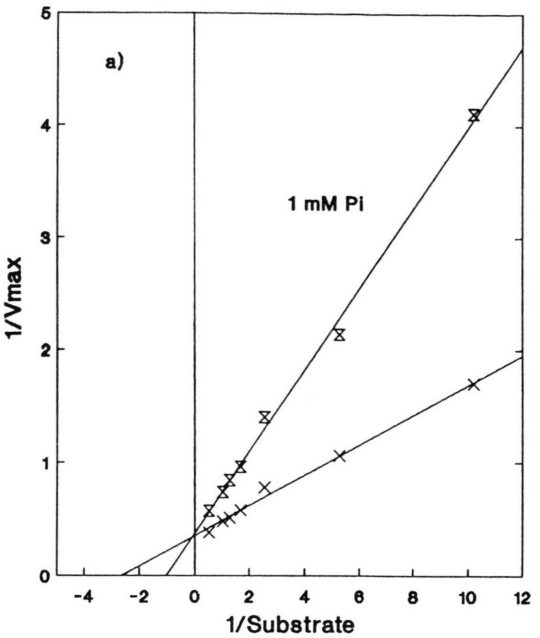
I. pH-dependence

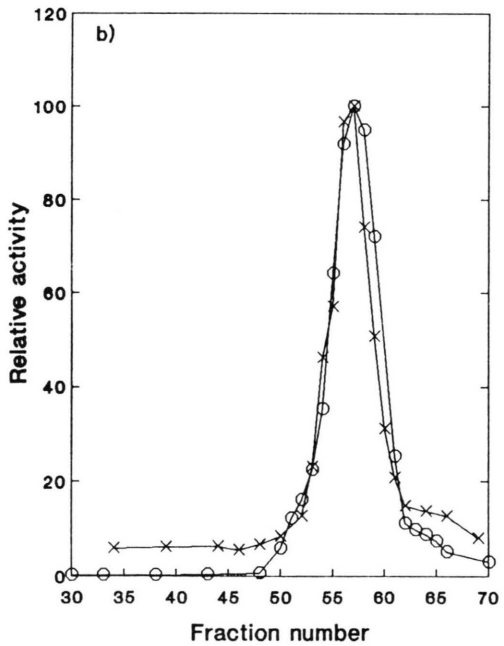
The isolated 955 kDa protein with PEP Co activity exhibits a pronounced pH-optimum at 8.5 (Fig. 3). This corresponds to literature data for lower and higher plants [12]. The isolated 543 kDa-form, however, shows maximum activity at pH 8.5 and 5.5 (Fig. 3). The conclusion that this fraction contains two different types of PEP Co proved to be wrong. The high activity at pH 5.5 depends on phosphatase activity, measured with the test system chosen also. The test system contains LDH besides MDH, in order to detect that portion of the product of PEP Co activity, oxaloacetate, which is disintegrated into CO_2 and pyruvate by test conditions [13]. Pyruvate however, is also produced by a phosphorylase splitting off inorganic phosphate from the substrate added, PEP. Fig. 4 indicates that when measured at pH 5.5 the activity peak of PEP consumption coincides with that of *p*-nitrophenylphosphate dephosphorylation after protein separation by DEAE as well as by FPLC. Fig. 5 shows the well-known inhibiting effect of inorganic phosphate on *p*-nitrophenylphosphate phosphatase (5a), but also for the PEP consuming reaction at pH 5.5 (b). Inhibition of PEP Co activity at standard test conditions, *i.e.* pH 8.5, could not be shown, because at this pH phosphate ions are complexed by Mg^{2+} present. If phosphatase is separated from PEP Co by DEAE chromatography, subsequent FPLC of the latter again yields two activity peaks at 955 and 543 kDa. The lighter form now shows also maximum activity around pH 8.5 only.

II. Substrate dependence

The affinity of the two proteins with PEP Co activity towards their substrate PEP, differs. The 955 kDa-form shows a K_m-value of $1.13 \times 10^{-3} \pm 0.035$ mol·l^{-1}, the 543 kDa-form of $0.76 \pm 0.029 \times 10^{-3}$ mol·l^{-1}. The Hill coefficients are around unity in both cases, indicating no cooperativity. Culturing of *Chlorella kessleri* in red or blue light did not change the kinetic properties of







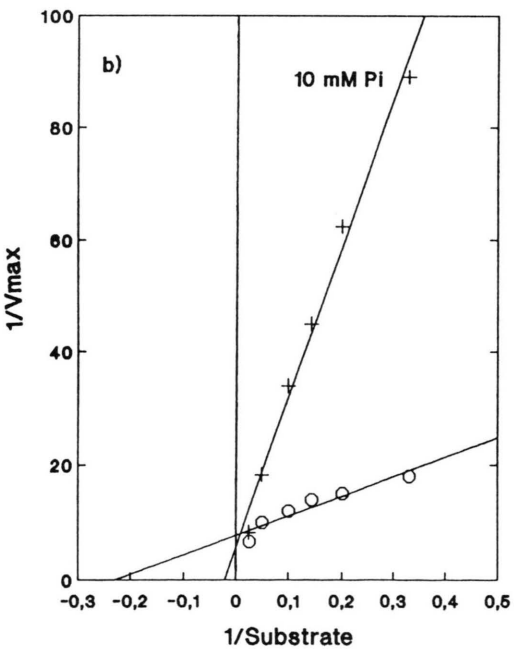


Fig. 4. Distribution of PEP consuming (○−○) and *p*-nitrophenylphosphate dephosphorylating (×−×) activity after protein separation of crude cell extracts of *Chlorella kessleri* by DEAE a) or FPLC b) chromatography. Both tests at pH 5.5.

Fig. 5. Influence of inorganic phosphate on a) *p*-nitrophenylphosphate dephosphorylating and b) PEP consuming activity in the 543 kDa fraction of crude extracts from *Chlorella kessleri*. Test at pH 5.5, in the presence (⧖−⧖) and (+−+) or absence (×−×) and (○−○) of inorganic phosphate.

the proteins with PEP Co activity. This is differs from Ruyters' observations [5]. He reported almost the same K_m-values as above for PEP Co activity found in crude extracts of chlorophyll-free mutant cells of *Chlorella vulgaris (= kessleri)* from dark or blue light conditions leading to the conclusion that there is a specific PEP Co iso-form in short wavelength light.

III. Influence of effectors

There is a pronounced substrate inhibition (Fig. 6), which is identical for both forms. No significant effects on K_m-value or Hill-coefficient for PEP can be found. Glutamine, glutamate, aspartate and malate, all known to effect PEP Co activity in the alga *Selenastrum,* higher plants and bacteria [3, 14–17], exhibit no effect on K_m of either PEP Co activity fraction at test pH 8.5 (Table I). There is, however, a strong inhibiting influence of citrate. The K_m-value increases to about 2×10^{-3} mol · l^{-1}. The results also are identical for enzyme preparations of cells from blue or red light conditions.

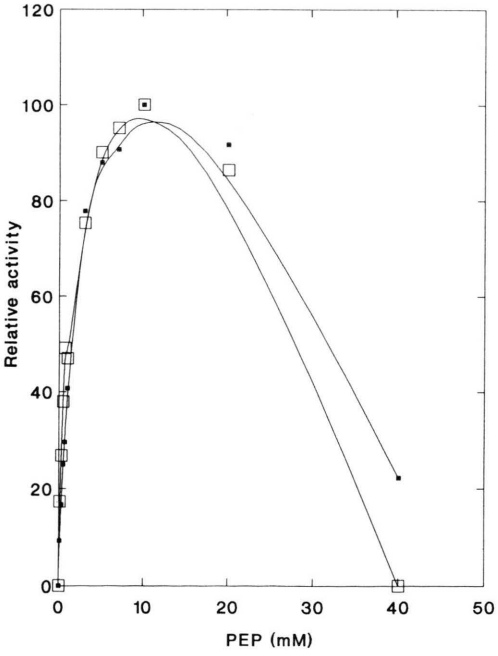


Fig. 6. Dependence on PEP concentration of the 955 (■–■) and the 543 kDa (□–□) proteins with PEP Co activity separated by FPLC on superose 6. PEP Co activity tested at pH 8.5.

Table I. K_m values and Hill coefficients for PEP [mM] of the 955 kDa (peak I) and 543 kDa (peak II) PEP Co activity containing protein, separated by FPLC on superose 6 in the presence of different effectors (10 mM). A: cells from blue light, B: cells from red light. Test at pH 8.5.

Table I A: blue light

Effector:	peak I		peak II	
	K_m	n	K_m	n
Glutamine	1.10	1.03	0.61	0.80
Glutamate	1.09	1.05	0.77	0.87
Aspartate	1.20	1.12	0.80	1.01
Citrate	2.01	1.95	1.59	1.23
Malate	1.16	1.07	0.95	0.96
Control	1.14	1.04	0.78	0.90

Table I B: red light

Effector:	peak I		peak II	
	K_m	n	K_m	n
Glutamine	1.15	1.08	0.87	0.88
Glutamate	1.07	1.19	0.73	0.86
Aspartate	1.04	1.02	0.70	0.88
Citrate	2.00	1.36	1.68	1.29
Malate	0.90	0.91	1.06	1.00
Control	1.12	1.03	0.74	0.87

IV. Temperature dependence

The larger protein with PEP Co activity is more sensitive to enhanced temperatures than the smaller one. While for the 955 kDa-form the halftime for the decay in activity is only 23 min, it is 55–60 min for the 543 kDa-form. These data are also identical for the preparation from blue or from red light conditions.

Conclusion

At the present time we interpret the data obtained of the two protein fractions with PEP Co activity as showing the existence of two different species of this enzyme. Either of the treatments applied should have separated just aggregated units. Consequently, the data lend no support to the hypothesis that aggregation/dissociation processes are a means of activity regulation for PEP Co as suggested to exist for other enzymes of the same organism. In higher plants, regulation of PEP Co activity seems to be accomplished by phosphorylation/dephosphorylation [18, 19]. We have not yet been able to show this for *Chlorella* enzymes, too. However, the possibility that the larger enzyme is a

tight complex of PEP Co and a specific kinase/phosphatase for regulation is certainly an interesting speculation. Continuing on this idea, there might be two types of PEP Co with different tasks and localization at different sites in *Chlorella kessleri*. Only one of them might be regulated by phosphorylation/dephosphorylation. Another possible way to modify PEP Co protein would be a complexation with chaperonins resulting in an altered mole mass of the enzyme.

Considering the known basic cell metabolism the necessity for two such enzymes cannot easily be seen. In literature PEP Co is mostly considered as a cytosolic enzyme [20] but there are also reports of PEP Co in the chloroplast [21, 22]. We have begun, preferably with immunological studies, to clarify this problem for the autotrophic wild type *Chlorella kessleri* used thus far.

In contrast to Ruyters' results [5] with chlorophyll-free *Chlorella* mutant cells, modifying influences on the two PEP Co forms of the wavelengths present at autotrophic cell growth could not be detected.

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