Oligomeric Forms of Pyruvate Kinase from *Chlorella* with Different Kinetic Properties

G. Ruyters, N. Grotjohann, and W. Kowallik

Fakultät für Biologie, Universität Bielefeld, Postfach 8640, D-4800 Bielefeld 1, Bundesrepublik Deutschland

Fast protein liquid chromatography on Superose 6 of crude extracts from the chlorophyll-free mutant no. 20 of the unicellular green alga *Chlorella kessleri* reveals two possibly oligomeric forms of pyruvate kinase (2.7.1.40). Their occurrence is markedly altered in the course of heterotrophic growth with changing levels of exogenous glucose as carbon source with only one enzyme species with a MW of 400 kDa existing in growing cells, two forms of 400 and 580 kDa in resting cells. Substrate affinity towards PEP of the 400 kDa form is better than that of the 580 kDa species; responses to the effector AMP are different as well. *In vitro*, addition of PEP or of AMP leads to the formation of higher MW enzyme species with MW of 730, 1050 and 1400 kDA without affecting the total activity. *In vivo* alterations in the levels of several metabolites including PEP upon addition of glucose have been shown to occur. Therefore, it is discussed, whether changes in the concentration of intermediates and effectors may provide the mechanism for the increased rate of carbohydrate degradation by affecting the occurrence and/or ratio of the various PK forms with different kinetic and regulatory properties. Upon blue light irradiation, which also stimulates carbohydrate breakdown of the *Chlorella* mutant cells, the distribution of PK is shifted towards the species with higher substrate affinity, a result being in accordance with the above conception.

Introduction

Regulation of metabolic pathways is mainly achieved by two different mechanisms, coarse and fine control. In the coarse control the amount of an enzyme is regulated mostly by changing the rate of protein synthesis or degradation; this occurs rather slowly and often during developmental processes. In contrast, fine control is provoked by fast changes in the activity of existing enzyme molecules, due mainly to rapid alterations in the concentrations of cofactors and effectors. Both principles could be shown to be involved in the control of starch breakdown of *Chlorella* and its chlorophyll-free mutants: On the one hand, synthesis of several enzymes, especially that of the regulatory important phosphofructokinase (PFK) [1, 2] and pyruvate kinase (PK) [3] appeared to be influenced. On the other hand, rapid and pronounced changes in the level of various intermediates and effectors particularly of the glycolytic pathway could be shown to occur when the flux through the glycolytic pathway was altered [4, 5].

Very recently, aggregation of enzymes to oligomeric forms or dissociation of the latter resulting in enzyme forms with different kinetic and regulatory properties have been described as another control mechanism in plants [6–10]. This phenomenon appears also to be present in the green alga *Chlorella kessleri* and its chlorophyll-free mutant no. 20. Among all glycolytic enzymes investigated PFK and PK were the only ones found to exist in several oligomeric forms with MW between 360 and 1500 kDa for PFK and between 305 and 830 kDa for PK [11]. These forms were captured when crude cell extracts rather than purified enzymes were quickly separated by fast protein liquid chromatography (FPLC) on a Superose 6 column. In the case of PFK the observed aggregates were proven to be no artificial products of the separation procedure, since subsequent FPLC of the smallest PFK species of 360 kDa does not yield any larger form [12]. The existence of aggregates *in*

vivo is further supported by observations of Wong *et al.* [8], who found PFK aggregates after FPLC separation of crude carrot extracts which corresponded to oligomeric forms artificially produced from the purified enzyme by addition of certain metabolites. From these results the conclusion seems justified that the various forms of PFK found after FPLC separation of crude extracts also from *Chlorella* cells represent *in vivo* states of the enzyme.

In agreement with the above considerations the PFK oligomers of *Chlorella* possess markedly different kinetic and regulatory properties. Since the degree of oligomerization could be influenced *in vivo* by the light quality used for autotrophic growth and *in vitro* by the addition of specific effectors to the enzyme extract, dissociation or association of this enzyme – mediated by wavelength-dependent alterations in the concentrations of regulatory metabolites – have been considered to be involved in the regulation of carbohydrate breakdown [12]. In continuing our studies on the control of this process in *Chlorella* it appeared consequent to investigate also PK in more detail. First it is asked, whether the occurrence and/or the ratio of the various PK forms as well can be influenced by alterations of the physiological situation and *in vitro* by specific effectors. Second, kinetic and regulatory properties of the different PK species are to be measured to elucidate their possible contribution to the regulation of carbohydrate degradation. The Chlorella mutant no. 20 was chosen for this investigation, since the rate of its carbohydrate degradation varies markedly with changing physiological conditions.

Materials and Methods

The experiments were performed with the chlorophyll-free mutant no. 20 of the unicellular green alga *Chlorella kessleri* Fott et Novakova, formerly *Chlorella vulgaris* (211-11 h/20), obtained from the Culture Collection of Algae of the Institute of Plant Physiology at Göttingen University, F.R.G. The algae were grown in the dark in an inorganic medium [23] with iron supplied as EDTA-complex and 1% glucose added as carbon source.

For experiments, the algae were separated from their nutrient solution and washed twice with distilled water by centrifugation ($2000 \times g$). The re-

sulting cell sediment was resuspended in 100 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, containing 100 mM NaCl and 0.02% Na azide to prevent bacterial growth. The final cell density was 100 or 200 µl packed cell volume ml^{-1}, determined with microhematokrit tubes, which were spun in a Varifuge S (Heraeus Christ, Osterode, F.R.G.) at $4000 \times g$ for 15 min. Preparation of crude cell extracts was performed as described recently [1]. 0.2 ml of the crude extract filtered through a 0.2 µm sterile filter (Sartorius GmbH, Göttingen, F.R.G.) was applied to a FPLC Superose 6 column (HR 10/30; Pharmacia, Uppsala, Sweden). The buffer systems used for equilibration of the column and for elution of the proteins are given in the respective figure legends. The flow rate was 0.5 ml/min; 0.25 ml fractions were collected and assayed for enzyme activity. All experiments were performed at room temperature. For native MW determination the column was calibrated with protein standards [11]. The activity of PK was determined as described previously [3] except for raising the PEP concentration to 10 mM.

Results and Discussion

When the chlorophyll-free *Chlorella* mutant no. 20 is grown heterotrophically in static cultures with glucose as the only carbon source, there is rapid growth for about three to four days under our conditions. After this period of time glucose and nitrate have completely been taken up from the medium; increase in dry weight of the culture ceases, cell division stops due to lacking net protein synthesis. The endogenous carbohydrate reserves slowly decrease during the next days [13]. In the following these two different physiological situations will be referred to as growing (2 to 3 day old cultures) and resting, slightly starved cells (8 to 12 day old cultures), respectively.

In crude extracts of growing *Chlorella* mutant cells the capacity of PK is more than twice compared to that of resting cells (159 *vs.* 72 nmol/mg protein × min in the present experiments). FPLC on Superose 6 of 0.2 ml of such crude extracts, equivalent to about 1 mg total protein, reveals only one huge peak of PK activity with an approximate MW of 400 kDa for rapidly growing cells, whereas the much lower capacity of the enzyme of slightly starved cells is distributed into two smaller

peaks of comparable activities with MW of 400 and 580 kDa (Fig. 1). None of these MW agrees with that of 240 kDa, a value reported for various organisms [14, 15] and determined by molecular sieve chromatography on Sephadex also for the partially purified enzyme of the chlorophyll-free mutant [16] used in the present study. From this the question arises whether the PK forms with the MW of 400 and 580 kDa obtained by FPLC of crude extracts from *Chlorella* mutant cells represent oligomers of PK or aggregates of the 240 kDa monomer with other proteins, and in addition, whether these larger PK species exist *in vivo* or originate from artificial aggregation during the FPLC separation procedure. The latter question is especially interesting, since different aggregation states of PK were not observed in *Selenastrum*, in which PK was very recently reported to exist as a probably chloroplastic enzyme with a MW of again 240 kDa, but in addition as a probably cytosolic form with a MW of 590 kDa [17].

Fig. 2. FPLC Superose 6 elution profiles of PK of crude extracts from slightly starved cells of the chlorophyll-free *Chlorella* mutant no. 20. Extract was dialyzed towards 20 mM phosphate buffer, pH 7.5 (closed circles) or kept on ice as control (open circles). For elution buffer see Fig. 1. The activity of the most active fraction of the non-dialyzed crude extract is set 100. Data are mean values of 3 experiments.
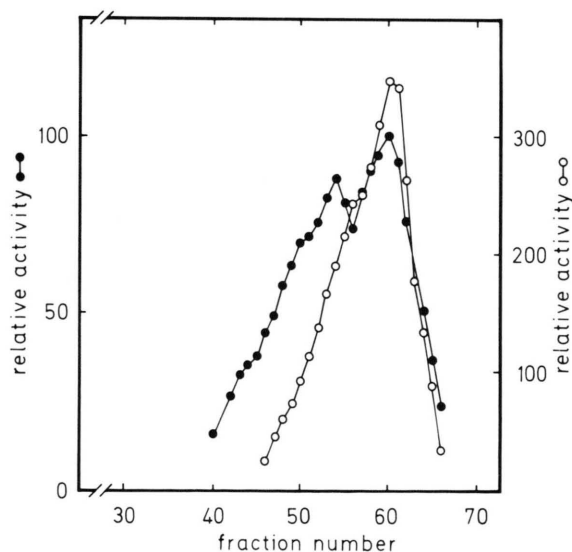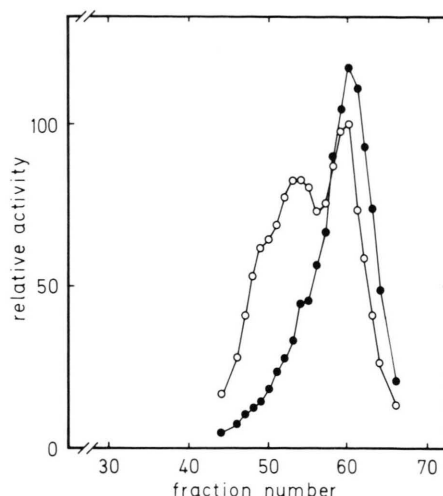

Fig. 1. FPLC Superose 6 elution profiles of PK of crude extracts from the chlorophyll-free *Chlorella* mutant no. 20, grown heterotrophically in the dark with glucose as carbon source. Growing cells = open circles, slightly starved cells = closed circles (elution buffer: 100 mM KH_2PO_4/Na_2HPO_4, 100 mM NaCl, 0.02% NaN_3, pH 7.5). The activity of the most active fraction of crude extracts from resting cells is set 100. Data are mean values of 5 or 8 experiments.

In first attempts to obtain the 240 kDa form the ion concentration was changed. Dialysis of crude extracts from slightly starved cells with PK species of 400 and 580 kDa towards 1 l of 20 mM phosphate buffer pH 7.5 for 3 h with one buffer change in between resulted in only one peak with PK activity with the MW of 400 kDa in the following FPLC (Fig. 2). Compared with the non-dialyzed crude extract the activity of this species increased, although the total activity is decreased by about 15%. From this it is reasonable to assume that dialysis provoked dissociation of the higher MW species of PK into the 400 kDa form. Similar results were obtained with 1 M urea, routinely used to dissociate oligomeric proteins. In contrast, treatment with 20 mM DTT, taken under the assumption that the high MW PK forms might be stabilized *via* sulfur bridges, did not have significant influence on the FPLC Superose 6 elution profile. Likewise, elevation of the pH in the buffer used by one unit from 7.5 to 8.5 was as ineffective as was the lowering of the protein concentration in the crude extract up to 10-fold, both treatments having been described as suitable means for dissociating aggregated forms of enzymes [18]. Finally,

crude extracts were prepared and eluted from the FPLC Superose column in the presence of 2 M NaCl, which had been found to dissociate the oligomeric *Chlorella* PFK into its 360 kDa monomer [12]. For PK, however, this treatment leads to a rather unspecific aggregation, the FPLC separation yielding a variety of badly separated large proteins with PK activity, a main peak being at about 1050 kDa.

If, however, a partially purified PK [16] is applied onto the FPLC Superose 6 column, PK is found as a 240 kDa protein also with this technique. As already demonstrated for PFK [8, 12] this result excludes an artificial self-aggregation during the FPLC separation procedure as cause for the detection of the high MW PK forms. The introductory assumption of the different PK forms representing *in vivo* states of the enzymes is thereby supported. The question whether they are oligomeric forms of PK or aggregates with other proteins cannot be answered unequivocally from these findings. Following the results of Wong *et al.* [8] for PFK and especially those of Lin *et al.* [17] for PK, however, the latter does not seem very probable. Instead, it may be considered that the 400 kDa is the dimer, the 580 kDa PK species the trimer of the 240 kDa monomer, the lower MW compared with the exact double or triple, respectively, arising from an altered quarternary structure with different behaviour in molecular sieve chromatography (compare PFK, [7]). A composition of the two PK species of a different number of 60 kDa subunits as in *Selenastrum* [17] is also feasible, of course. The 240 kDa monomer might not be obtainable by the treatments described above because of different binding forces stabilizing the high MW forms and the dimer, respectively.

Kinetic properties of the different PK forms

The alteration in the occurrence of both the PK forms during the limited heterotrophic growth of our algae would be of regulatory significance, if total activity and/or kinetic and regulatory properties of the various enzyme forms were different. In this case the phenomenon would contribute to the changes in the rates of carbohydrate degradation, the latter being more than 6-fold higher in growing than in resting *Chlorella* mutant cells [13]. Because of the incomplete separation of the 400 and

580 kDa forms only the four most active fractions of each species were pooled after the FPLC separation procedure and taken for these examinations. Still, the results obtained may not give the true values, but valuable indications for the respective properties and thus answers to the questions raised above would be obtained, if the differences were sufficiently pronounced. Table I shows markedly different substrate affinities for PEP with K_m values of 1.4 and 7.8 M, respectively, but similar affinities towards ADP at saturating PEP concentrations for the 400 and 580 kDa enzyme form. Both species follow Michaelis-Menten kinetics with PEP as well as with ADP as variable substrate ($n = 1$). For comparison, Table I also includes the kinetic properties of the 240 kDa monomeric PK. With a $S_{0.5}$ value for PEP of 1.9 its affinity is a little lower than that of the 400 kDa form, the enzyme additionally exhibiting a slightly negative cooperativity. The affinity towards ADP is similar to those given for the 400 and 580 kDa species. From these data it becomes evident, that the alteration in the ratio of the 400 and 580 kDa PK occurring during cell growth (compare Fig. 1) leads to an enrichment of the PK species with lower substrate affinities in resting cells at the expense of the PK species with the better substrate affinities; this occurs in addition to a pronounced decrease in total capacity of PK.

From the generally known effectors of PK, AMP was chosen for investigation, since this compound had previously been shown to influence the kinetic behaviour of partially purified PK of the mutant [16]. At the suboptimal concentration of 1 mM PEP, presence of 0.3 to 20 mM AMP in the

Table I. Kinetic properties of the different MW forms of PK from the chlorophyll-free *Chlorella* mutant no. 20. All data have been determined by Hill plots — in the case of Michaelis-Menten kinetics additionally by Lineweaver-Burk plots — and are mean values of at least 4 measurements.

PK species	PEP dependence		ADP dependence		PEP dependence in presence of AMP	
	$K_m/S_{0.5}$ [mM]	n	K_m [mM]	n	$K_m/S_{0.5}$ [mM]	n
580 kDa	7.8	1.0	0.19	1.0	0.59	1.0
400 kDa	1.4	1.0	0.14	1.0	0.44	1.0
240 kDa	1.9	0.89	0.17	1.0	0.98	0.92

enzyme assay provoked an increase in the activities of the 400 and 580 kDa forms. A maximal rise of 40 and 60%, respectively, was achieved by AMP concentrations from 3 to 10 mM. Detailed analysis of the kinetics reveals a slight decrease of the K_m value for PEP from 1.4 to 0.44 mM for the 400 kDa, but a marked one from 7.8 to 0.59 mM for the 580 kDa species by addition of 6 mM AMP. The affinity towards PEP of the partially purified 240 kDa monomer is increased as well by this treatment (Table I). Altogether these results indicate that the different PK forms of the *Chlorella* mutant cells do not only differ in their affinities towards PEP, but also in their response to the positive effector AMP. To elucidate the regulatory significance of these findings, the next step was to look for specific factors influencing the occurrence of the different forms of PK *in vitro.*

Metabolites affecting the FPLC Superose 6 elution profile of PK

Presence of 10 mM PEP during the preparation of crude extracts from growing cells and during the elution of the proteins from the Superose 6 column leads to an FPLC elution pattern comparable to that of starved cells without PEP; *i.e.,* the 580 kDa species with the comparatively low substrate affinity shows up at the expense of the 400 kDa form (Fig. 3a). In slightly starved cells two larger forms

with MW of approx. 1050 and 730 kDa appear as main peaks in addition to the also present 400 and 580 kDa forms (Fig. 3b). The total enzyme activity, obtained by measuring at saturating substrate concentrations, is not altered by this treatment, thus – in contrast to the results for PFK [12] – rejecting the assumption of the oligomerization property being a means of regulation of PK activity. Instead, the decrease in total capacity shown above to occur during growth has to be due to an altered amount of the enzyme. The regulatory importance of the described changes in the ratio of the PK forms is therefore based on their different substrate affinities. For these to become effective suboptimal PEP concentrations are required, a presupposition indeed given with a PEP level of 0.5 mM determined for instance for resting *Chlorella* mutant cells [4]. The concentration of 10 mM PEP always present during the short period of the enzyme assay itself obviously does not dramatically affect the oligomerization; otherwise various PK forms with different kinetic properties would not be detectable.

From the various effector molecules of PK, AMP and ATP were investigated. 10 mM AMP added to extracts of resting cells as well as to the elution buffer leads to proteins of about 1400 and 1050 kDa as main enzyme forms, whereas the species with 580 and 400 kDa are significantly reduced (Fig. 4). Again the total activity is not sig-
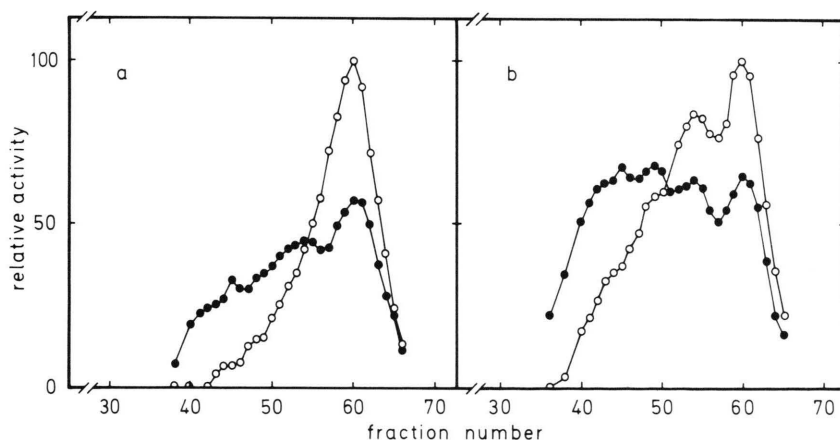

Fig. 3. FPLC Superose 6 elution profiles of PK of crude extracts from growing (a) or slightly starved cells (b) of the chlorophyll-free *Chlorella* mutant no. 20. Extracts were prepared and eluted from the column in the presence of 10 mM PEP (closed circles) or in its absence for control (open circles). For elution buffer see Fig. 1. The activity of the most active fraction of each control is set 100. Data are mean values of 3 experiments.
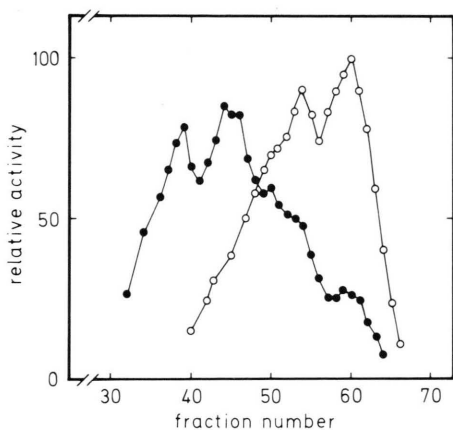
Fig. 4. FPLC Superose 6 elution profiles of PK of crude extracts from slightly starved cells of the chlorophyll-free *Chlorella* mutant no. 20. Extracts were prepared and eluted from the column in the presence of 10 mM AMP (closed circles) or in its absence for control (open circles). For elution buffer see Fig. 1. The activity of the most active fraction of the control is set 100. Data are mean values of 3 experiments.

nificantly changed. MgATP, however, highly effective in dissociating the oligomers of PFK [12], did not provoke significant effects on PK at concentrations of 5 or 10 mM.

Taken together these results demonstrate that the substrate PEP and the effector AMP cause aggregation of PK *in vitro*. Certainly, the metabolite concentration of 10 mM is higher than the *in vivo* overall concentrations estimated. Since these may not necessarily agree, however, with those at the site of the enzyme reaction, we have chosen a concentration which led to pronounced effects *in vitro* thereby demonstrating the ability of the enzyme to respond to these metabolites. From these results changes in the concentration of the above metabolites in the algal cell would probably affect the ratio of the different PK forms. Therefore, knowledge about the *in vivo* concentrations of those compounds and their alterations upon varying physiological conditions would be of high value. Changes in the concentrations of PEP, pyruvate, glucose-6-phosphate, ATP, and ADP have recently been demonstrated occurring immediately after addition of exogenous glucose [5].

Similar results were obtained when *Chlorella* mutant cells were irradiated with low intensity blue light [4]. This treatment is reported to have important regulatory effects on carbon metabolism of many green algae, especially to stimulate carbohydrate degradation and respiration (for review see [19]). Especially the rapid and pronounced decrease in the concentration of PEP deserves attention in view of the results presented above: Since high PEP concentrations lead to aggregation of PK (compare Fig. 4), a decrease in PEP would favour the formation of the low MW forms. In order to check if these considerations become true *in vivo,* the PK elution pattern of crude extracts from blue light-irradiated, slightly starved *Chlorella* mutant cells, in which light responses appear especially pronounced, has been compared to that of dark-kept cells. It is evident from Fig. 5, that 60 min of blue light illumination indeed provoke a shift in the ratio of the two PK species in favour of the 400 kDa form, *i.e.,* towards the form with the higher substrate affinity, whereas 15 min of illumination are not effective yet. In both cases total activity is not changed by the blue light irradiation, the latter treatment leading to an enhanced PK activity *via* protein synthesis only after several hours of illumination [3]. The primary drop in PEP, which has to occur prior to the shift in the different PK forms, may be due to an acceleration of the PEP carboxylase reaction, which is shown to become stimulated by blue light or by addition of glucose [20, 21].

Altogether these results indicate that changes in the concentration of metabolites occurring for instance in dependence on the supply with exogenous glucose or upon irradiation with different light qualities contribute to the alterations of the rate of carbohydrate breakdown, measured under these conditions [22], by affecting the occurrence and/or the ratio of the various PK forms with different kinetic and regulatory properties. Therefore, aggregation/dissociation of (regulatory?) enzymes such as PFK and PK probably have to be taken into account as control mechanisms in addition to the well-known coarse control *via* protein synthesis and fine control *via* changing concentrations of effectors also in *Chlorella*.

For a complete view certainly much more information is needed about the levels of intermediates, their glucose- or light-induced changes and their effects on the occurrence and/or ratio of the different PK forms of the *Chlorella* cells with different
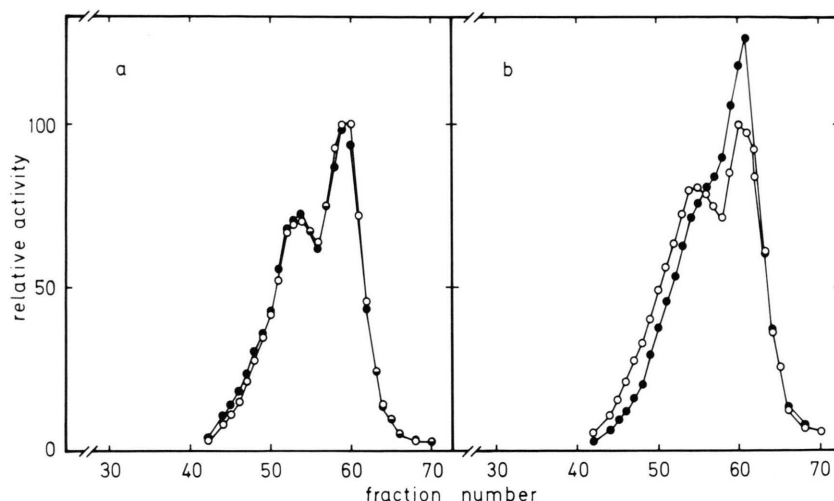
Fig. 5. FPLC Superose 6 elution profiles of PK of crude extracts from slightly starved cells of the chlorophyll-free *Chlorella* mutant no. 20. Cells were irradiated with blue light (< 550 nm, approx. 250 W/cm^2; closed circles) for 15 min (a) or for 60 min (b) or kept in the dark for control (open circles). For elution buffer see Fig. 1. The activities of the most active fraction of the dark controls are set 100. Data are mean values of 3 experiments.

kinetic and regulatory properties. Respective experiments are in progress and will hopefully contribute to the understanding of the regulation of carbohydrate degradation of *Chlorella* in darkness and in blue light.

[1] W. Kowallik and N. Grotjohann, Physiol. Plant. **74**, 101 (1988).
[2] N. Grotjohann and W. Kowallik, Physiol. Plant. **75**, 43 (1989).
[3] W. Kowallik and G. Ruyters, Planta **128**, 11 (1976).
[4] G. Ruyters, Plant Cell Physiol. **29**, 355 (1988).
[5] G. Ruyters, Plant Cell Physiol. **29**, 365 (1988).
[6] F. C. Botha and D. H. Turpin, Plant Physiol. **93**, 871 (1990).
[7] A. R. Goldhammer and H. H. Paradies, Curr. Top. Cell. Reg. **15**, 109 (1979).
[8] I. H. Wong, B. C. Yee, and B. B. Buchanan, J. Biol. Chem. **262**, 3185 (1987).
[9] M.-X. Wu and R. T. Wedding, Arch. Biochem. Biophys. **240**, 655 (1985).
[10] M.-X. Wu, D. A. Smyth, and C. C. Black, Proc. Natl. Acad. Sci. **81**, 5051 (1984).
[11] W. Kowallik, N. Grotjohann, and G. Ruyters, Bot. Acta **103**, 197 (1990).

[12] N. Grotjohann, W. Kowallik, and G. Ruyters, Bot. Acta **104**, 77 (1991).
[13] M. Georgi, PhD thesis, Cologne (1974).
[14] W. C. Plaxton, Plant Physiol. **86**, 1064 (1988).
[15] D. Vaccaro and M. H. Zeldin, Plant Physiol. **54**, 617 (1974).
[16] G. Ruyters, Z. Pflanzenphysiol. **108**, 207 (1982).
[17] M. Lin, D. H. Turpin, and W. C. Plaxton, Arch. Biochem. Biophys. **269**, 219 (1989).
[18] C. Frieden, Annu. Rev. Biochem. **40**, 653 (1971).
[19] W. Kowallik, Annu. Rev. Plant Physiol. **33**, 51 (1982).
[20] A. Kamiya and S. Miyachi, Plant Cell Physiol. **16**, 729 (1975).
[21] G. Ruyters, Z. Pflanzenphysiol. **100**, 107 (1980).
[22] W. Kowallik and S. Schätzle, in: The Blue Light Syndrome (H. Senger, ed.), p. 344, Springer, Berlin 1980.
[23] H. G. Ruppel, Flora **152**, 113 (1962).