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VARIABILITY IN THE SYNTHESIS OF CYTOCHROMES *f* AND *b*-563 DURING THE CELL CYCLE OF *EUGLENA GRACILIS*

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The study of the successive formation of the photosynthetic apparatus in *Euglena gracilis* (Brandt, P. and Von Kessel, B. (1983) Plant Physiol. 72, 616–619) was extended to the determination of the stage-specific synthesis of cytochrome *b/f* complex during the cell cycle of this alga. Most of the cytochrome *f* (33 kDa) has properties of an intrinsic membrane protein, but part of it is soluble. Cytochrome *b*-563 (18 kDa) is only intrinsic. The intensity of binding the intrinsic cytochromes in the thylakoids depends on the developmental stage of the organism. The light-independent synthesis of cytochrome *f* takes place prior to the assembly of the chlorophyll-protein complex I (CP I). Immediately after this assembly of CP I, cytochrome *b*-563 is synthesized in the light. Hence, the ratio cytochrome *b*-563 / cytochrome *f* changes during the cell cycle of *E. gracilis*. The physiological implication of presumably non-complexed cytochrome *f* and of complex-bound cytochromes *f* and *b*-563 on the stage-specific efficiency of photosynthesis of *E. gracilis* is discussed.

Introduction

In general, the chlorophyll-protein complexes related to Photosystem I (PS I), chlorophyll-protein complex I (CP I) and Photosystem II (PS II), respectively, the chlorophyll-protein complex CPa and the light-harvesting-chlorophyll-protein complex (LHCP) exist within the thylakoids as separate intrinsic protein entities [1–3]. In *Euglena gracilis*, their synthesis takes place at fixed stages during the ontogenetic cell development [4] correlating with stage-specific changes of the photosynthetic efficiency [4] and also with certain modifications of the actual PS II particle population [5]. However, so far no information exists about the time of synthesis of the electron carrier systems of photosynthetic electron transport and a probable connection of their synthesis to the formation of the chlorophyll-protein complexes in the chloroplasts of *E. gracilis*. Of special interest are the two cytochromes *b*-563 and *f*. Both are components of a special thylakoid complex [6] and the independence or connection of their synthesis and assembly into the complex could influence non-cyclic as also PS I cyclic electron transport [7,8] during the cell cycle of *E. gracilis*. It was the aim of this study to investigate such relationships.

Abbreviations: CPa, chlorophyll-protein complex related to Photosystem II; CP I, chlorophyll-protein complex related to Photosystem I; LHCP, light-harvesting chlorophyll-protein complex; PS, photosystem. Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Materials and Methods

Euglena gracilis, strain z (1224-5/25) (Sammlung von Algenkulturen, Pflanzenphysiologisches Institut, Universität Göttingen) was cultivated phototrophically [9] and synchronized by daily light-dark periods of 14 h light and 10 h dark with

dilution of the culture at the end of the dark period.

The following investigations were all done at 4°C. For preparation of chloroplasts, cells were harvested and broken in a YEDA press (Research and Development Co., Ltd., Rehovot, Israel). Chloroplasts were isolated on a Ficoll/saccharose/glycerol gradient [10], resuspended in a medium comprising 0.33 sorbitol/1 mM $MgCl_2$/2 mM EDTA/4 mM mercaptoethanol/50 mM Tricine-KOH (pH 8.4) [10]. The isolated chloroplasts were osmotically shocked in 25 mM Tricine/12.5 mM KCl (pH 6.8). The soluble chloroplast proteins were separated from the membrane proteins by centrifugation for 60 min at 100000 × *g*. The resulting membrane preparation was incubated with 0.1% sodium deoxycholate (0.1% sodium deoxycholate/0.25 mM saccharose/10 mM Tricine-HCl (pH 8.0)/1 M KCl) for 40 min. This membrane suspension was centrifuged for 1 h at 100000 × *g*. The resulting supernatant is the first membrane fraction. The pellet was incubated once more with sodium deoxycholate (0.75% sodium deoxycholate/0.5 M saccharose/34 mM Tris-HCl (pH 8.0)/1 M KCl) for 40 min and subsequently centrifuged for 20 min at 100000 × *g*. The resulting supernatant is the second membrane fraction and the resulting pellet is the third membrane fraction [10]. The proteins of the four fractions, e.g., soluble proteins and the first, second and third membrane fraction, were separated in 15% acrylamide gels [10] without treatment at 100°C preventing proteolytic degradation [11,12]. The cytochrome *b/f*-complex was isolated from the second or third membrane fraction by isoelectrofocusing in acrylamide gels [13]. Protein was stained with Coomassie Blue [10]. Cytochromes were identified by tetramethylbenzidine-staining [14]. Proteolytic degradation during the whole procedure of thylakoid isolation and of sample preparation for SDS-polyacrylamide gel electrophoresis can be excluded, because identical profiles of soluble proteins, first, second and third membrane fraction were established without and with the improved addition [15,16] of proteinase inhibitors (5 mM ε-amino-caproic-acid/1 mM phenylmethylsulfonylfluoride/1 mM benzamidine) to all buffers.

The amount of cytochrome was estimated for cytochrome *f* from the ascorbate-reduced minus ferricyanide-oxidized difference spectrum at 554 nm in 10 mM phosphate buffer (pH 7.2), using an *E* of 18 mM [6] and for cytochrome *b*-563 from the dithionite-reduced minus ascorbate-oxidized difference spectrum at 563 nm in 10 mM phosphate buffer (pH 7.2), using an *E* of 21 mM [6]. The amount of chlorophyll was determined by the method of Arnon [17].

Results and Discussion

Four heme-binding proteins are present in the protein spectra of the chloroplasts from *E. gracilis* identified by tetramethylbenzidine-staining in SDS polyacrylamide gels (Fig. 1). In accordance with their presumed location and published molecular weight values of *Euglena* cytochromes, these four tetramethylbenzidine bands show the following correspondence: the polypeptide with the apparent molecular weight of 33000 corresponds with cytochrome *f* [6,14,18,19]; the polypeptide with the molecular weight of 18000 corresponds with cytochrome *b*-563 [6,14,20]; the polypeptide with the molecular weight of 10000 corresponds with cytochrome *c*-522 [21]; the polypeptide with the molecular weight of 5000–8000 corresponds with cytochrome *b*-559 [14,22].

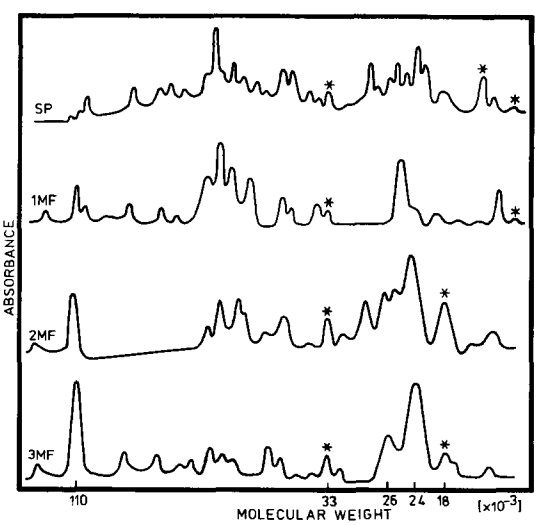

Fig. 1. Protein patterns of the soluble proteins (SP) and three membrane fractions of thylakoid proteins (1MF–3MF) of chloroplasts from *Euglena gracilis*. *, heme-binding proteins.

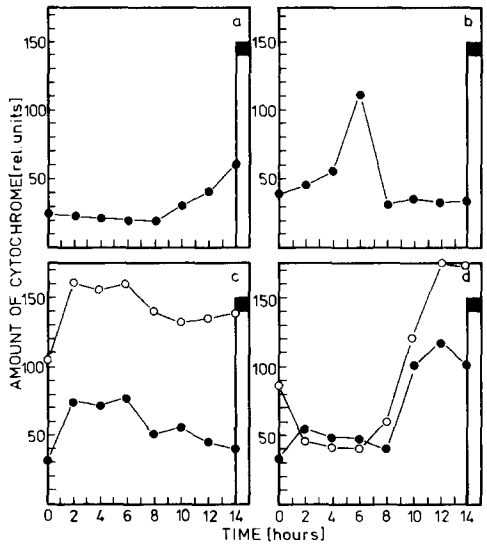

Fig. 2. Thylakoid content of cytochrome *f* (●) and of cytochrome *b*-563 (○) in the soluble protein fraction (a) as well as in the first (b), second (c) and third (d) membrane fraction isolated by chloroplast-fractionation.

During the stage-specific modulation of the thylakoid composition in *E. gracilis* [4,5,10,23] the binding forces within the thylakoids alter as can be judged from the characteristic changes in the amounts of cytochromes *b*-563 and *f* in the four membrane fractions. Their relative amounts are given as relative units in relation to the other proteins of the actual membrane fraction (Fig. 2). During the first six hours of the light-dark cycle the amount of cytochrome *f* increases in the first membrane fraction and less in the second; but in the second part of the light period there is a decrease of the cytochrome *f* amount in these two membrane fractions accompanied by a slight increase in the soluble proteins and a drastic increase in the third membrane fraction. There are comparable alterations of the cytochrome *b*-563 binding within the thylakoids. Its amount increases in the second membrane fraction by about 50% during the first 2 h of light and by about 400% in the third one after the sixth hour of light. The separation of the plastid proteins into four fractions by deoxycholate treatment [10] is only suitable for the estimation of the binding properties of the cytochromes within the thylakoids. Therefore, we also calculated the average cytochrome concentrations of the chloroplasts from measurements of difference spectra of reduced minus oxidized cytochromes (Fig. 3). Relative to the amount of chlorophyll, the concentration of cytochrome *b*-563 stays constant all over the light period, whereas the concentration of cytochrome *f* decreases by about 50%. During the first 10 h of the cell cycle of *E. gracilis* the ratio of cytochrome *b*-563 : cytochrome *f* is about 1.5 as has been reported also for spinach [24]. During the last 4 h of the light period, this ratio increases to about 3.0. It follows that the syntheses of cytochrome *f* and cytochrome *b*-563 are separated in time during the cell cycle of *E. gracilis* and that the donor site of PS I changes in a stage-specific manner. The

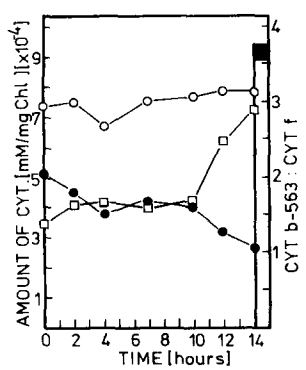

Fig. 3. Chloroplast content of cytochrome *f* (●) and of cytochrome *b*-563 (○) related to the plastid chlorophyll content during the light period of synchronized *E. gracilis*. Ratio of cytochrome *b*-563 : cytochrome *f* (□).

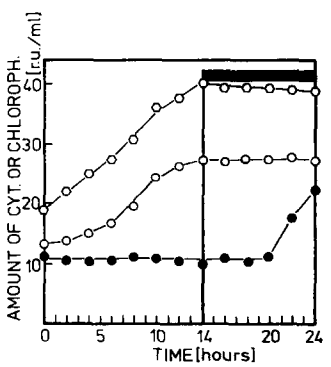

Fig. 4. Chlorophyll content (○), cytochrome *b*-563 content (○) and cytochroem *f* content (●) during the cell cycle of synchronized *E. gracilis*.

amount of the cytochromes *b*-563 and *f* can be calculated for the complete cell cycle of *E. gracilis* (Fig. 4) without relation to the stage-specific chlorophyll content of the cells [4]. The synthesis of cytochrome *b*-563 occurs only together with chlorophyll synthesis during the light period of the light-dark cycle, whereas cytochrome *f* is synthesized in the second part of the dark period just after the division of the cells and chloroplasts [4] and just prior to the beginning of the light period and the assembly of CP I [4].

For further elucidation of the organizational form of cytochrome *b*-563 and cytochrome *f* within the thylakoids of *E. gracilis* the combined second and third membrane fractions were separated by polyacrylamide gel electrofocussing [13]. This procedure revealed one heme-containing band, which is located at pH 3.6 at the beginning of the light period and at pH 4.5 at its end (Fig. 5). Reelectrophoresis of this heme-containing band shows a pattern of seven proteins (Fig. 6) all over the light period. Two of these proteins were identified by tetramethylbenzidine-staining and their molecular weights are identical with cytochrome *f* and cytochrome *b*-563 (Fig. 1). This protein profile corresponds to some extent with the composition of the intrinsic cytochrome *b/f* complex of spinach chloroplasts [6]. The stage-specific pH-shift of the cytochrome complex of *E. gracilis* probably results from a modification of the complex composition during the cell cycle, but it is impossible to detect distinct modulations in the relative amounts of the components within the isolated cytochrome complex.

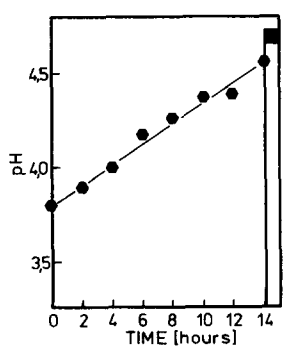

Fig. 5. Change of the isoelectric point of the heme-binding protein complex isolated by polyacrylamide gel electrophoresis from thylakoids of *E. gracilis* during the light period of the cell cycle.

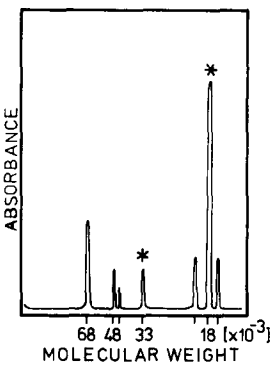

Fig. 6. General protein pattern of the cytochrome *b/f*-complex isolated by isoelectrofocusing and subsequent SDS-polyacrylamide gel electrophoresis of the combined second and third membrane fraction of *Euglena* chloroplasts. *, heme-binding proteins.

The increasing ratio of cytochrome *b*-563 : cytochrome *f* in the chloroplasts of *E. gracilis* in the last 4 h of the light period (Fig. 3) correlates with the main part of the cytochrome *b*-563 synthesis (Fig. 4) as well as with the increasing binding of both cytochromes in the thylakoids (Fig. 2). The relative amount of cytochrome *f*, however, increases at this stage of the cell cycle concomitantly in the soluble proteins (Fig. 2). This indicates presumably the existence of two 'kinds' of cytochrome *f*, e.g., one is bound in the intrinsic complex and the other one as extrinsic protein in the soluble proteins is less bound on the thylakoids. This view of cytochrome *f* location in the thylakoids of *E. gracilis* corresponds with immunological studies, which demonstrate that that part of cytochrome *f*, which is involved in linear electron transport, is located on the outer surface of the membrane, whereas the cytochrome *f* involved in the cyclic phosphorylation seems to reside inside the membrane [25]. In this connection it is noteworthy that the assembly of the LHCP takes place in the middle of the light period of the cell cycle of *E. gracilis* [4]. The resulting increase of the energy supply to PS II as well as to PS I [26] correlates with the cytochrome *b*-563 synthesis (Fig. 4), which has also been involved in the cyclic electron transport [7,8]. At this stage of the cell cycle, the increased energy supply of PS I presumably can be used for an increased cyclic phosphorylation.

Acknowledgement

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