

# Herbicide-Binding Protein, Binding Sites and Electron-Transport Activity: Quantitative Relations

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Z. Naturforsch. **39 c**, 430–433 (1984); received February 24, 1984

32–35 kDa Protein, Q<sub>B</sub>-Protein, *Bumilleriopsis*, [<sup>35</sup>S]Met Labeling, Light-Induced Turnover

In *Bumilleriopsis filiformis*, the thylakoid-membrane protein showing the highest turnover in the light has an apparent molecular weight of 35 kDa. Loss of this protein after chloramphenicol treatment leads to a corresponding reduction of herbicide-binding sites as well as photosystem-II activity (measured by e.g. H<sub>2</sub>O → silicomolybdate) and cellular photosynthesis. This correlation is quantitated by comparison of the amount of herbicide-binding protein (autoradiography), electron transport, and herbicide binding. An increase of the binding constant after chloramphenicol treatment is evident.

## Introduction

The rapidly synthesized 32–35 kDa thylakoid peptide (Q<sub>B</sub>-protein) has been identified as the receptor or binding protein for many herbicides acting as inhibitors of photosynthetic electron transport [1]. It has been shown that the 32–35 kDa protein is encoded by a chloroplast gene [2], the transcription of which is light-dependent in developing plastids [3], and it is known to have the shortest half-life of all rapidly labeled membrane proteins [4–6] in the mature chloroplast.

However, no agreement exists as to whether this protein is functional in electron transport [5], assuming this binding protein to be an integral (redox) compound, or whether it is not directly involved in electron transport [4, 7], merely assigning it the role of auxiliary functions, its loss not necessarily impairing photosynthesis. It appears that investigations ought to contribute some clarifying data. Reliable quantitative correlations have not yet been published between the amount of binding protein, binding sites as well as electron-transport activity (and photosynthesis). Furthermore, it is still unclear whether chloramphenicol treatment does not only decrease the amount of the 32–35 kDa peptide, but also change its binding affinity. It was reported [7] that the inhibition constant ( $k_i$  = approximately the  $I_{50}$ -value) does not change.

Due to the rapid turnover in the light the 32–35 kDa peptide can be decreased by administering

D-threo-chloramphenicol which is known to inhibit translation on 70-S plastidic ribosomes.

The unicellular eukaryotic alga *Bumilleriopsis filiformis* was chosen for the experiments, since it can easily be labeled, exhibits good response to chloramphenicol, and yields active isolated thylakoids.

## Materials and Methods

*Bumilleriopsis filiformis* (Xanthophyceae, Konstanz stock) was cultivated autotrophically under sterile conditions in liquid medium for 6 days under continuous light, as described previously [8]. Cell density after this period was 18 µl packed cell volume per ml culture medium (pcv) equivalent to  $4.5 \times 10^6$  cells per ml. Cells were harvested and washed once with a medium containing 150 mM NaCl and 10 mM Tris-buffer, pH 8.0, then resuspended in growth medium and adjusted to a final density of 4 µl pcv/ml. This suspension, supplemented with D-threo-chloramphenicol (400 µg/ml), was incubated in 1.5 l Fernbach flasks either in the light or in the dark, as indicated, for 43 h. Light intensity was 165 µEinstein/m<sup>2</sup> × s (measured with a quantum sensor LI-COR 190 SB).

Isolation of chloroplasts and measuring of electron transport was carried out as described [8, 9]. For binding studies of herbicides, or electrophoresis of membrane peptides, thylakoid isolation was modified according to [11].

*In-vivo* labeling of proteins was performed with washed cells (see above) which were resuspended in growth medium and the density adjusted to 80 µl pcv/ml. After preincubation for 1 h at 23 °C in a

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0341-0382/84/0500-0430 \$ 01.30/0



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Warburg apparatus in the light ( $140 \mu\text{Einsteins}/\text{m}^2 \times \text{s}$ ), [ $^{35}\text{S}$ ]methionine (from Amersham-Buchler, Braunschweig) was added yielding 18 nM (specific activity 1360 Ci/mmol). Uptake and incorporation of the radiolabel into protein was allowed to proceed for 2.5 h in the light. The cells were then washed twice as indicated above, before either isolation of thylakoids was carried out (for the control) or chloramphenicol treatment performed as described above. Additional methods are given in the legends.

## Results and Discussion

With microalgae, *in-vivo* synthesis of membrane proteins can be easily monitored by incorporation of [ $^{35}\text{S}$ ]methionine. Autoradiograms of solubilized thylakoids showed a broad pattern of labeled proteins after a 2.5-h incubation time (Fig. 1). A strong label is seen in a protein with an apparent molecular weight of 35 kDa. This matches with reports of Weinbaum *et al.* [4] and Steinback [1] who found the major label in the region of 32 kDa for *Spirodela* and 34 kDa for higher-plant leaves. Further incubation of this sample without radiolabel for 43 h in the presence of chloramphenicol preserved the low-molecular weight labeling pattern as shown by the control, except for this 35-kDa protein which disappeared substantially (about –70%) in the light, but only to a minor extent in the dark. The 35-kDa peptide had the highest turnover in the light.

If this protein were a functional and essential member of the photosynthetic electron-transport chain, a 70% loss should be correlated with an equivalent decrease of photosynthetic activity as well as with a loss of herbicide-binding sites.

Data of photosynthetic electron transport are shown in Table I as  $\text{O}_2$ -evolution of intact cells, which is compared with partial electron-transport activities of isolated thylakoids. It is obvious that the rates of the three cell-free assay systems give almost the same values for the light control and for the dark incubation samples with or without chloramphenicol treatment. After incubation with chloramphenicol in the light, however, a dramatic reduction (about –70%) was observed of cellular photosynthesis as well as of photosystem-II activity of isolated thylakoids (as measured with silicomolybdate and DAD/ferricyanide as electron acceptors).

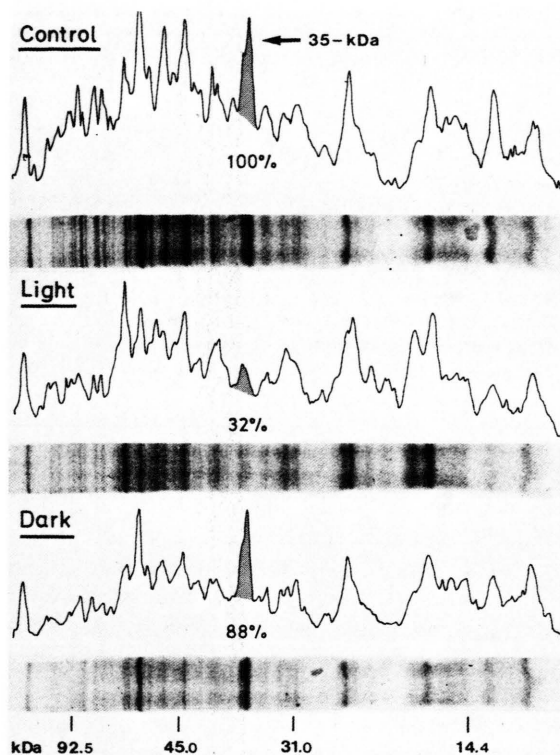


Fig. 1. Autoradiograms of peptide patterns of thylakoid membranes obtained from *Bumilleriopsis*: Gel bands and their scanning.

**Control:** Cells were harvested immediately after radiolabeling with [ $^{35}\text{S}$ ]methionine (25  $\mu\text{Ci}/\text{ml}$ , 2.5 h in the light).

**Light:** Aliquots of washed control cells were resuspended in fresh growth medium containing 400  $\mu\text{g}$  of chloramphenicol (CAP) per ml and then illuminated for 43 h.

**Dark:** Same procedure as in the light, but keeping the cells in the dark for 43 h in the presence of CAP.

After harvesting, isolated thylakoid membranes equivalent to 20–30  $\mu\text{g}$  of chlorophyll were solubilized in a medium containing 0.08 M Tris-buffer, pH 6.8, 20% (v/v) glycerol, 2% dithiothreitol, 4% SDS (sodium dodecyl sulfate); chlorophyll/SDS ratio was adjusted to 1/20–40 (w/w). After 1 h at  $60^\circ\text{C}$ , the thylakoids were dissolved. The mixture was centrifuged (5 min at  $9,000 \times g$ , Eppendorf centrifuge, mod. 5412), and aliquots of the clear green supernatant were electrophoresed on an SDS-polyacrylamide gel according to Laemmli [10] (12 cm resolving gel with 17.5% polyacrylamide, 0.375 M Tris-buffer, pH 8.7, 0.1% SDS; 1 cm stacking gel: 5% polyacrylamide, 0.125 M Tris buffer, pH 6.8, 0.1% SDS; running buffer: 0.19 M glycine, 0.025 M Tris-base leading to pH 8.4, 0.1% SDS. Electrophoresis was performed at 12 mA overnight at  $10^\circ\text{C}$ . All lanes had been loaded with the same amount of chlorophyll. Autoradiograms were scanned densitometrically using a laser densitometer (LKB Ultrosan, mod. 2202). Percent data indicated refer to the 35-kDa peptide as [ $^{35}\text{S}$ ]methionine label remaining in the 35-kDa band after the 2.5-h pulse chase-labeling (= control).

Table I. Rates of cells and isolated chloroplast material under the influence of chloramphenicol.

| No. | Culture conditions | Cells<br>( $10^{-12}$ mol O <sub>2</sub> /cell × h) | Isolated thylakoids, rates in $\mu$ mol O <sub>2</sub> per mg chlorophyll and h |                         |                    |
|-----|--------------------|---|---|-------------------------|--------------------|
|     |                    |   | H <sub>2</sub> O → DAD/FeCy   | H <sub>2</sub> O → SiMo | DAD/ascorbate → MV |
| 1   | Dark, (–CAP)       | 1.22  | 93  | 135                     | 430                |
| 2   | Dark, (+CAP)       | 1.53  | 102   | 146                     | 470                |
| 3   | Light, (–CAP)      | 1.16  | 107   | 141                     | 409                |
| 4   | Light, (+CAP)      | 0.39 (–66%)   | 31 (–71%)   | 42 (–70%)               | 400                |

*Bumilleriopsis* was grown for 6 days, then adjusted to a density of  $10^6$  cells/ml (for chlorophyll content see Table II), chloramphenicol (CAP) was added, and the culture kept in the light or in the dark in nutrient medium for 43 h, as indicated (comp. Methods). Data in parentheses indicate inhibition vs. light control. SiMo was 0.3 mg/ml, DAD 25  $\mu$ M.

Abbreviations: FeCy, potassium ferricyanide; DAD, diaminodurene; SiMo, silicomolybdate (*cf.* [12]); MV, methylviologen (1,4-dimethyl-N,N'-bipyridylum dichloride).

This decrease is clearly determined by a change within the photosystem-II region, because photosystem-I activity is not affected. Since there is no obvious difference between the rate decrease of thylakoids and whole cells (see line 4), it is concluded that the chloramphenicol-affected component is the only rate-limiting step for photosynthesis under these conditions. This finding does not match with data published for *Spirodela* [4], where CO<sub>2</sub>-fixation was barely affected after chloramphenicol treatment while electron-transport rates with isolated chloroplasts were lowered using the assay systems H<sub>2</sub>O → ferricyanide or H<sub>2</sub>O → methylviologen [5]. The H<sub>2</sub>O → silicomolybdate reaction, on the other hand, was reported not to be influenced by chloramphenicol treatment in the light, in contrast to our data. Using spinach chloroplasts, our laboratory had shown previously that the herbicide-binding protein is involved in the silicomolybdate-mediated Hill reaction [12].

Binding data of chlortoluron [3-(3-chloro-4-methylphenyl)-1,1-dimethylurea], an electron-transport inhibitor very similar to diuron (*cf.* [11]), corroborated our finding on the amount of the 35-kDa peptide and the electron-transport activities. Figure 2 exhibits the same  $k_b$  for the light and dark controls and the chloramphenicol-treated chloroplasts in the dark (column a of Table II), whereas the number of binding sites per mg chlorophyll is but slightly different. Chloramphenicol treatment in the light, however, led to a marked reduction of herbicide-binding sites as well as to an increase of the  $k_b$ . Correlation of the reduction of herbicide-binding capacity and increasing  $k_b$  was also ob-

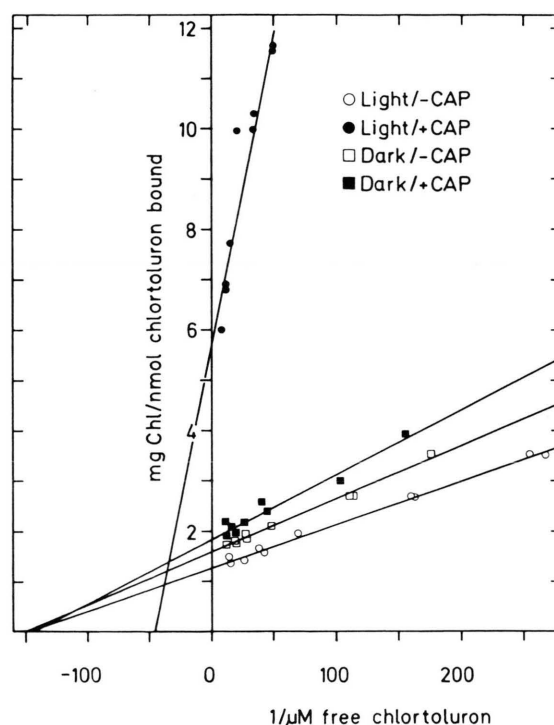


Fig. 2. Double-reciprocal plots of specific binding of [<sup>14</sup>C]chlortoluron to thylakoid membranes of *Bumilleriopsis*. Cells were cultured with or without chloramphenicol present (CAP, 400  $\mu$ g/ml) for 43 h in the light and in the dark. Isolated thylakoids with a final concentration of 90  $\mu$ g chlorophyll/ml were incubated in 1-ml volumes for 10 min, suspended in a medium containing 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 25 mM Tris-buffer, pH 8.0, and with concentrations of herbicide ranging from  $3 \times 10^{-8}$  M to  $1.3 \times 10^{-7}$  M. Incubation was for 10 min in the cold room. After centrifugation in an Eppendorf centrifuge (5 min, at  $9,000 \times g$ ) bound radiolabel was estimated by counting aliquots of the supernatant in a liquid-scintillation counter (comp. [11]). Specific activity of chlortoluron was 6.58 mCi/mmol.

Table II. Binding parameters of isolated thylakoids for the urea herbicide chlortoluron.

| No. | Culture conditions with/without chloramphenicol | (a)<br>Binding constant $k_b$<br>[nM] | (b)<br>Number of Chl molecules per molecule of chlortoluron bound | (c)<br>Number of binding sites (nmol/mg Chl) |
|-----|---|---------------------------------------|---|--|
| 1   | □—□ Dark, (–CAP)                                | 6.6                                   | 1590  | 0.63   |
| 2   | ■—■ Dark, (+CAP)                                | 6.9                                   | 1860  | 0.54 (–14%)                                  |
| 3   | ○—○ Light, (–CAP)                               | 6.8                                   | 1280  | 0.78   |
| 4   | ●—● Light, (+CAP)                               | 21.9                                  | 5690  | 0.18 (–77%)                                  |

Calculation of parameters was carried out with the data points of the double-reciprocal plots of Fig. 2. Symbols refer to the figure. Data in parentheses indicate percent decrease vs. controls. Chl = chlorophyll.

Chloramphenicol does not degrade chlorophyll under the conditions used.

The decrease of the Chl/herbicide ratio (or increase of binding sites/Chl) in the light control (line 3, columns b, c) is due to decrease of chlorophyll content per cell and per thylakoids, as compared to the dark control (line 1), which is generally observed when growth is turned on by increased light gain of cells due to dilution of the culture, as done here at start of this expt. Cells of the dark control had about  $1.7\text{--}2 \times 10^{-5} \mu\text{g}$  chlorophyll/cell, while the light control was decreased to  $1.2\text{--}1.6 \times 10^{-5} \mu\text{g}/\text{cell}$ . The slight decrease of the ratio of line 2, column c, is due to some loss of the 35-kDa peptide (see Fig. 1, last row).

served after trypsin digestion or growth at higher temperatures as shown with a mutant of *Chlamydomonas* [6].

The  $k_b$  and amount of binding sites (column c) are listed in Table II. The strong decrease (–77%) of binding sites in the chloramphenicol-treated light sample correlates well with the reduction of photosystem-II activity (–71%, Table I). In the dark, loss of binding sites in the presence of chloramphenicol was 14% only (vs. dark control). For the increase of binding sites per chlorophyll in the light control (line 3, column c) see legend of Table II.

Comparison of the three experimental approaches reported here gives a good correlation between the

amount of a 35-kDa protein, the number of herbicide-binding sites, and electron transport activity. These results demonstrate, by quantitative data, the functional relevance of the 35-kDa protein as an integral component of the photosystem-II part of the electron-transport chain.

#### Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft (grant no. Bo 310/13-1). The authors are grateful to Ciba-Geigy, Basle, Switzerland, for a gift of [ $^{14}\text{C}$ ]chlortoluron.

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