CHARACTERIZATION AND FUNCTION OF A 33 000 M_r POLYPEPTIDE IN DCMU-SENSITIVE AND RESISTANT STRAINS OF A CYANOBACTERIUM, *APHANOCAPSA* 6714

C. ASTIER and F. JOSET-ESPARDELLIER
Laboratoire de Photosynthèse, CNRS, 91190 Gif-sur-Yvette, France

Received 30 March 1981

1. Introduction

The thylakoids contain numerous polypeptides, most of which have not yet been associated with a photosynthetic function. In the case of chloroplast membranes, only the components of photosystem I reaction centers [1], the chlorophyll *a*/*b* light harvesting complex [2,3] and the coupling factor [4] have been clearly identified. Studies performed on cyanobacterial thylakoids have shown a number of similarities between their components and those of chloroplasts from algae or higher plants, and led to the identification of some of their polypeptides [5,6]. Photosynthetic functions have yet to be assigned to the remaining polypeptides. One approach to relate individual polypeptides to functions has been to use selective trypsin digestion: the alteration of specific polypeptides can thus be correlated to the modifications of photosynthetic parameters [7]. A more direct approach is to select and study mutants of the photosynthetic functions.

The herbicide 3-(3,4-chlorophenyl)-1,1-dimethylurea (DCMU) blocks electron transport on the reducing side of photosystem II, between the primary acceptor Q [8] and the secondary acceptor B [9]. Different authors have attempted, using various approaches, to determine the molecular site of action of several photosystem II inhibitors having the structural group –CO–N= in common with DCMU [10,11]. A protein, ranging from 25 000–40 000 M_r depending on the authors and the organisms, has been put forward as being the attachment site of this class of inhibitors.

We have isolated and characterized several DCMU-resistant mutants of the cyanobacterium, *Aphanocapsa* 6714 [12]. Here, we report the comparison of the polypeptide composition of thylakoids isolated from the wild type and the mutants DCMU^r-II in [12]. We show a good correlation between DCMU sensitivity and the presence of a 33 000 M_r polypeptide. The role of this polypeptide is discussed.

2. Materials and methods

The strain *Aphanocapsa* 6714 (ATCC 27178) was kindly given by Dr R. Y. Stanier [13]. Growth conditions, pigment concentration measurements and photosystem II activity assays (Hill reaction) were done as in [12].

Thylakoids were prepared from exponentially growing cells (~2×10^{10} cells) harvested by centrifugation at 3000 × *g* for 5 min and washed in tricine buffer (10 mM tricine, 10 mM NaCl, 10 mM $MgCl_2$, pH 7.7). Cells which had been cultured in the presence of DCMU were washed twice. After resuspension in ~20 ml of the same buffer the cells were broken with a French press at 56 kg/cm^2. The suspension was then immediately diluted with an equal volume of cold tricine buffer, containing 0.4 M sorbitol, with or without 0.5% bovine serum albumin. Cell disruption and subsequent centrifugation procedures were performed at 4°C. Unbroken cells were removed by centrifugation at 500 × *g* for 5 min. The cell-free supernatant was spun at 17 000 × *g* for 1 h to sediment the thylakoid membranes. The pellet was gently resuspended in tricine–sorbitol buffer. The preparation, which contained ~1 mg chl/ml, was kept frozen at –20°C.

Thylakoid fragmentation was obtained by thawing 0.5 ml samples and diluting them in 4.5 ml tricine buffer. The samples were stirred for 1 h at room tem-

perature and spun at 17 000 X *g* for 1 h to sediment the remaining thylakoid membranes. The pellets were resuspended in 0.5 ml tricine–sorbitol buffer. The supernatants were dialysed against distilled water for 4 h and concentrated with a dehydrating reagent (aquacide; Calbiochem) to 0.5 ml final vol.

Trypsin digestion of isolated thylakoids was carried out on samples adjusted to ~50 μg chl/ml, in tricine buffer; 10 μg/ml trypsin (Sigma T 8253) was added to the sample. After 20 min incubation at room temperature, under slow agitation, 20 μg/ml trypsin inhibitor (Sigma T 9128) was added to the reaction system. The samples were stirred for 2 min, diluted 3-fold with cold tricine buffer and tested for DCMU sensitivity of the Hill reaction.

Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis of the isolated thylakoids was done as in [14]. The samples were mixed with an equal volume of a solution of 2% SDS, 1.5% Cleland's reagent, 10% glycerol and 0.2% bromophenol blue in phosphate buffer (pH 6.8); ~1 mg chl/ml; 10–30 μl aliquots of the mixture were layered onto 16 cm slab gels of 18% polyacrylamide. Electrophoresis was performed using 0.1% SDS, 0.2 M glycine, 25 mM Tris buffer at 30 mA. The gel was fixed with a methanol–water–acetic acid (5:5:1, by vol.) solution and stained with 0.2% Coomassie blue. A M_r calibration curve was obtained by running cytochrome *c*, γ-globulin, β-lactoglobulin, ovalbumin and bovine serum albumin as standards.

Measurement of the decay of chlorophyll fluorescence yield following a saturating dye laser flash (1 μs) was done as in [15]. The photomultiplier was gated during 5 ms when the flash was fired. A weak modulated beam from a He-Cd laser allowed a continuous, synchronous detection of the fluorescence yield.

3. Results

The thylakoids of DCMU^r-II mutant cells showed the same high level of resistance to DCMU as those of whole cells [12]. The sensitivity to DCMU of the Hill reaction, measured on thylakoids prepared from wild-type cells and mutants grown in the absence (non-adapted, denoted na) or in the presence (adapted, denoted a) of DCMU, 10^{-5} M is shown in fig.1a. The half-inhibitory DCMU concentration for wild-type thylakoids was 10^{-7} M, while [DCMU^r-II]^a thylakoids needed 10^{-5} M to reach half inhibition. In the case of [DCMU^r-II]^{na} thylakoids, we observed a somewhat variable level of resistance, with half-inhibition concentrations ranging from 10^{-6}–10^{-5} M DCMU, depending upon the preparation. The addition of 0.5 μg/ml bovine serum albumin, as well as the fewest possible freeze–thaw cycles of the extracts were found to be important to maintain the highest level of resistance.

It was anticipated that the mutation could have produced a modification in a protein implicated in the electron transfer between Q and B, that is at the step inhibited by DCMU. This might result in changes of the efficiency of electron transfer at this step. Such

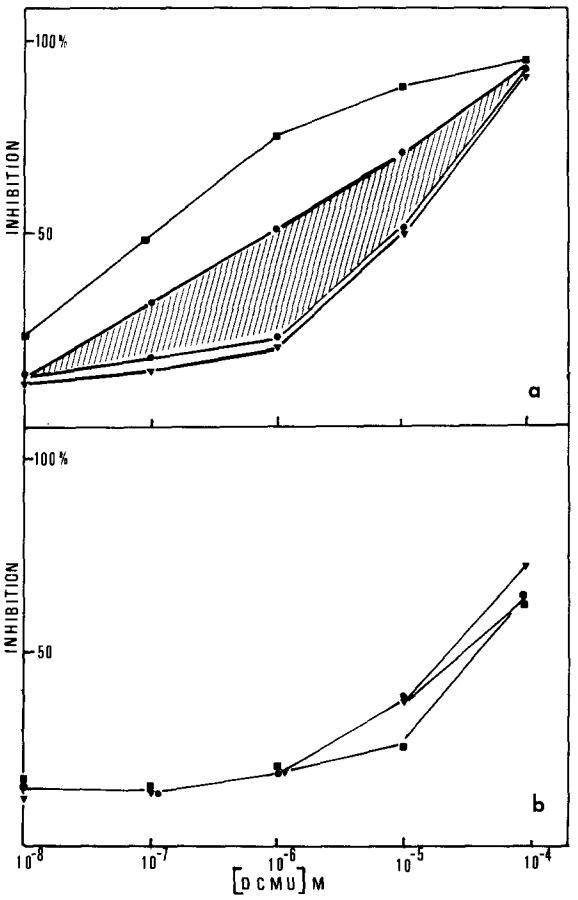

Fig.1. Inhibition of the rate of DCIP photoreduction *vs* DCMU concentration (log scale) in (a) untreated and (b) trypsin-treated thylakoids isolated from (■) wild type, (●) [DCMU^r-II]^{na} and (▲) [DCMU^r-II]^a cells. Thylakoids were incubated with or without trypsin as in section 2. Assays were performed at ~10 μg chl/ml with 4 X 10^{-4} M DPC and 10^{-4} M DCIP. The photoreduction rates of the non-inhibited cells are in μg DCIP . mg chl^{-1} . h^{-1}: (a) WT = 22, [DCMU^r-II]^{na} = 15, [DCMU^r-II]^a = 25; (b) WT = 17, [DCMU^r-II]^{na} = 12, [DCMU^r-II]^a = 27.

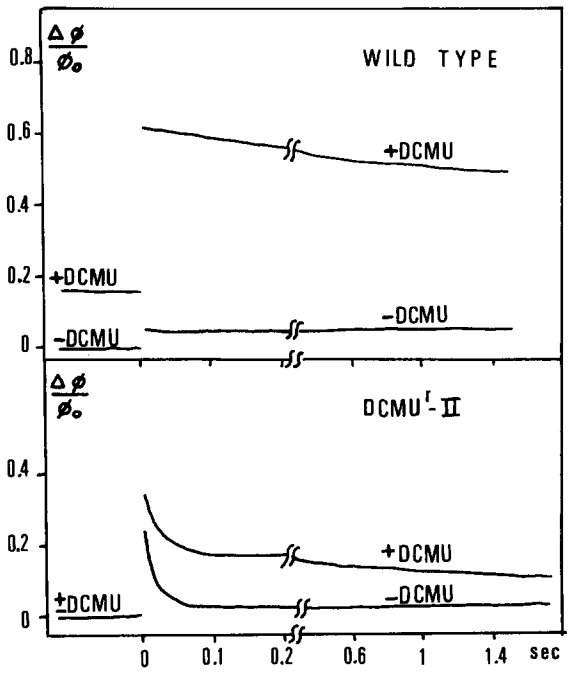

Fig.2. Fluorescence yield decay following a short saturating laser flash in wild-type and DCMU^r-II strains. Where indicated, the algae were incubated for 5 min with 10^{-5} M DCMU. The flash was fired at zero time. Ordinate scale: variable fluorescence $\Delta\Phi$ in Φ_o units (dark-adapted level of Φ in the absence of DCMU). Notice the much smaller $\Delta\Phi_{max}/\Phi_o$ ratio (0.6) observed compared to the usual results in green algae or chloroplasts [3,4].

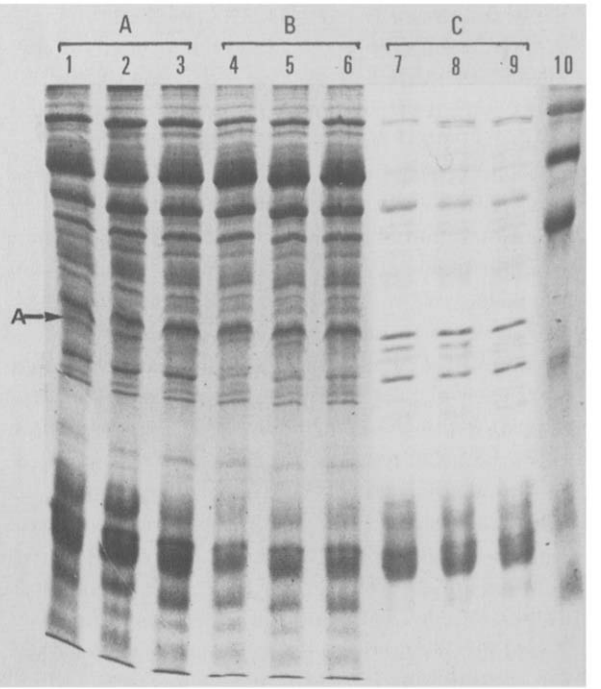

Fig.3. SDS–polyacrylamide gel separation of the polypeptides from: (A) complete thylakoids from WT ①, [DCMU^r-II]^{na} ②, [DCMU^r-II]^a ③; (B) pellet fraction of fractionated thylakoids from WT ④, [DCMU^r-II]^{na} ⑤, [DCMU^r-II]^a ⑥; (C) concentrated supernatant of fractionated thylakoids from WT ⑦ [DCMU^r-II]^{na} ⑧, [DCMU^r-II]^a ⑨ and molecular mass standards ⑩.

changes can be estimated from measurement of the decay of the fluorescence yield, Φ, after a saturating laser flash, in wild type and [DCMU^r-II]^{na} whole cells (fig.2). In the absence of DCMU, the rapid phases of fluorescence decay were completed during the 5 ms blanck period (photomultiplier gate) in the wild-type sample. In the mutant strain a slow phase of Φ decay was observed during the first 100 ms. Addition of DCMU caused an upward shift of the dark-adapted Φ level (back-transfer $QB^- \xrightarrow{DCMU} Q^-B$ [16,17]) in the wild strain but not in the mutant. The Φ decay was drastically slowed down in the wild strain, whereas only a part of the variable fluorescence of the mutant was similarly affected. A fast phase of Φ decay in the 100 ms range was still observed in the mutant, with DCMU present.

The effect of trypsin digestion on the DCMU sensitivity of the various thylakoid preparations was studied. The thylakoids were isolated and treated with trypsin as in section 2. As shown in fig.1b, the enzymatic treatment increased the resistance to DCMU of the wild type thylakoids, to a level identical to that of the [DCMU^r-II]^a extract. The sensitivity of the latter remained unaltered by the trypsin digestion. The membrane extracts from [DCMU^r-II]^{na} cells, which initially showed variable half-inhibitory DCMU concentrations, were now fully resistant, to the level of the [DCMU^r-II]^a thylakoids.

These results suggested that the integrity or the presence of a (or several) protein(s) were implicated in the electron transfer from Q to B and were responsible for the sensitivity to DCMU. We thus analysed the polypeptide composition of various extracts (fig.3). An important difference was observed in the case of the [DCMU^r-II]^a thylakoids: a single band (A) corresponding to a 33 000 M_r polypeptide, was absent from that preparation.

In order to confirm the probable relationship

between the sensitivity to DCMU and the presence of the 33 000 M_r polypeptide, we determined the conditions which allow a selective separation of this polypeptide from the membranes and have measured the resulting DCMU sensitivity. Thylakoids from the 3 different types of cells, (wild-type, $[DCMU^r\text{-}II]^a$ and $[DCMU^r\text{-}II]^{na}$), were fractionated as in section 2 (dilution in buffer and mild stirring at room temperature). From each thylakoid preparation, two fractions, the pellet containing the large membrane fragments, and the concentrated supernatant, were obtained. The polypeptide composition of these different fractions was analysed by electrophoresis. A few bands present in the whole membranes (fig.3A) were absent from the pellet fraction (fig.3B) and were recovered in the supernatants (fig.3C). In particular, the 33 000 M_r polypeptide, band A, from the two preparations containing it (wild type and $[DCMU^r\text{-}II]^{na}$) has thus been solubilized. The DCMU sensitivity of the Hill reaction measured on the corresponding pellet fractions now showed complete resistance to DCMU, the half-inhibitory concentration being $\geqslant 10^{-5}$ M.

4. Discussion

A number of arguments prove the proteic nature of the DCMU binding site in the photosynthetic membranes. In several cases, the polypeptide has been clearly or with little ambiguity, determined. While it has long been established that mild trypsin digestion could selectively result in the loss or modification of several reactions linked to PS II activity [7,11,18] an increased resistance towards DCMU has been observed simultaneously with the degradation of either 1 or 2 polypeptide(s) from PS II particles [7], of 33 000 and 27 000 M_r, respectively.

Similar results have been obtained using DCMU analogs having a similar inhibitory step on the electron-transport chain. Phenolic herbicides bind selectively and competitively to a 30 000–40 000 M_r protein from spinach chloroplasts [10]. An azido derivative of atrazine can be covalently linked to a 32 000 M_r protein from a susceptible weed biotype and not to the spontaneous resistant one [11].

The isolation of DCMU resistant mutants from several algae, both eucaryotic, *Euglena gracilis* [19] and *Chlamydomonas reinhardtii* [20] and procaryotic, *Aphanocapsa* 6714 [12], indicates that the protein nature of the molecular site of action of this herbicide is a general phenomenon. The simultaneous appearance, after a single mutational event, of resistance towards both DCMU and atrazine [12] suggests that the sites of action for both inhibitors are on the same protein.

These data show that only 1 polypeptide needs to be modified to cause resistance. Its molecular mass identifies it with those polypeptides described from other organisms. The interesting result obtained here concerns the process through which the resistant cells express this character: they are still capable of synthesizing a 33 000 M_r polypeptide (non-adapted mutant), which the present analysis does not distinguish from that of the wild type. However the mutation results in a higher lability of this polypeptide from the photosynthetic membrane. The expression of the resistance (adapted mutant cells) would coincide with its complete release, or very rapid turnover. The presence of DCMU in the growth medium is necessary to induce the appearance of the resistant phenotype. However the present results do not allow to choose between the two possible effects:

(i) Either the release of the protein from the membrane, through a structural modification brought about by the herbicide;

(ii) Or a repression of the synthesis of the protein.

Mechanism (ii) seems less probable in view of the absence of transcriptional regulation of several biosynthetic pathways in cyanobacteria [21]; the kinetics of appearance of the resistant phenotype, much faster than the kinetics of growth, implies that at least the first mechanism must be active.

The similar characteristics (resistance towards DCMU and loss of the 33 000 M_r polypeptide from the membrane) observed in the adapted mutant, and after trypsin treatment of both the wild-type and the non-adapted mutant, are in agreement with the topological model proposed in [7,18]: located on the outer side of the membrane, the 33 000 M_r polypeptide would be easily reached by trypsin, and easily detached Its solubilization, facilitated in the mutant, would ipso facto prevent the inhibitory effect of the herbicide on the electron transfer.

Our results both in vitro on wild-type thylakoids treated with trypsin or stirred after dilution, and in vivo on adapted mutants, however, disagree with Renger's hypothesis [18] that this polypeptide would be neccessary to promote the electron transfer between Q and B, its absence leading to complete loss of PS II activity. The only function that can now be proposed

for this polypeptide is to facilitate this step of the electron transfer. This is confirmed by the fluorescence decay data which can be interpreted as showing a marked reduction in the rate of reoxidation of Q^- by B in the mutant, compared to the wild-type. The intermediate sensitivity to DCMU observed with the mutant would reflect a heterogeneity of the PS II centers, in this strain. This result agrees with an alteration of the structure implicated, at the level of the acceptor B, leading to an increased lability of the DCMU-binding protein. Similar findings have been reported [22,23] in chloroplasts isolated from atrazine resistant weed strains. In *Aphanocapsa* 6714, the absence of the polypeptide, however, does not decrease the rate of electron transfer below that of another, not defined, limiting step, since the overall efficiency of the photosynthetic process, measured as the growth rate of the cells, is not decreased in the mutant grown in the presence of the inhibitor.

The existence of this mutant, and the possibility of isolating the DCMU-binding polypeptide, will most probably be of a great importance to promote a better knowledge of the molecular mode of action of the inhibitor and of the structure of the photosynthetic membrane.

Acknowledgements

We wish to thank Dr J. Lavergne for the fluorescence experiment, and Dr M. Herdman for critically reading the manuscript. This work was supported in part by grant 125 (1979–80) from the French Solar Energy Commission, Pirdes. Part of this work has been presented at the 5th International Congress on Photosynthesis, Halkidi (Greece), September 1980.

References

[1] Bengis, C. and Nelson, N. (1977) J. Biol. Chem. 252, 4564–4569.
[2] Thornber, J. P. (1975) Annu. Rev. Plant Physiol. 26, 127–158.
[3] Burke, J. J., Ditto, C. L. and Arntzen, C. J. (1978) Arch. Biochem. Biophys. 187, 252–263.
[4] Nelson, N. (1976) Biochim. Biophys. Acta 456, 314–338.
[5] Reinman, S. and Thornber, J. P. (1979) Biochim. Biophys. Acta 547, 188–197.
[6] Newman, P. J. and Sherman, L. A. (1978) Biochim. Biophys. Acta 503, 343–361.
[7] Croze, E., Kelly, M. and Horton, P. (1979) FEBS Lett. 103, 22–26.
[8] Duysens, L. N. M. and Sweers, H. E. (1963) in: Studies on Microalgae and Photosynthetic Bacteria (Jap. Soc. Plant Physiol. ed) pp. 353–372, Univ. of Tokyo Press.
[9] Velthuys, B. R. (1976) PhD Thesis, Rijksuniversiteit Leiden, The Netherlands.
[10] Oettmeier, W., Masson, K. and Johanningmeier, U. (1980) FEBS Lett. 118, 267–270.
[11] Pfister, K., Steinback, K. E., Gardner, G. and Arntzen, C. J. (1980) Plant Physiol. suppl. 65–70.
[12] Astier, C., Vernotte, C., Der-Vartanian, M. and Joset-Espardellier, F. (1979) Plant Cell Physiol. 20, 1501–1510.
[13] Rippka, R., Deruelles, J., Waterbuty, J. B., Herdman, M. and Stanier, R. Y. (1979) Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria, J. Gen. Microbiol. 111, 1–61.
[14] Thomas, J. O., Kornberg, R. D. (1975) Proc. Natl. Acad. Sci. USA 72, 2626–2630.
[15] Lavergne, J. (1980) in: Proc. 5th Congr. Photosynthesis, Halkidiki.
[16] Velthuys, B. R. and Amesz, J. (1974) Biochim. Biophys. Acta 333, 85–94.
[17] Wollman, F. A. (1978) Biochim. Biophys. Acta 503, 263–273.
[18] Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300.
[19] Laval-Martin, D., Dubertret, G. and Calvayrac, R. (1977) Plant Sci. Lett. 10, 185–195.
[20] McBride, J. C., McBride, A. C. and Togasaki, R. K. (1977) Plant Cell Physiol. spec. iss. 239–241.
[21] Joset-Espardellier, F., Astier, C., Evans, E. H. and Carr, N. G. (1978) FEMS Microbiol. Lett. 4, 261–264.
[22] Bowes, J., Crofts, A. R. and Arntzen, C. J. (1980) Arch. Biochem. Biophys. 200, 303–308.
[23] Darr, S., Souza Machado, V. and Arntzen, C. J. (1981) Biochim. Biophys. Acta 634, 219–228.