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PHOTOCHEMICAL ACTIVITY AND COMPONENTS OF MEMBRANE PREPARATIONS FROM BLUE-GREEN ALGAE
II. LOW-TEMPERATURE PHOTOOXIDATION OF CYTOCHROME b_{559}

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SUMMARY

In view of some conflicting reports we investigated the properties of cytochrome b_{559} present in membrane fragments of *Nostoc muscorum* (Strain 7119) cells capable of high rates of photooxidation of water. We found that (i) the membrane-bound cytochrome b_{559} exists in more than one form, the high-potential form being the predominant one, (ii) the high-potential form of cytochrome b_{559} was photooxidized at cryogenic temperatures (−196 °C), and (iii) the low-temperature photooxidation was accomplished by short-wavelength light characteristic of Photosystem II but not by long-wavelength light characteristic of Photosystem I.

Since similar properties of cytochrome b_{559} have also been found in chloroplasts, they appear to be general characteristics of the O_2-evolving apparatus in photosynthesis.

INTRODUCTION

Of the three cytochromes (*f*, b_6, and b_{559}) embedded in the membrane structure of chloroplasts, cytochrome b_{559} has recently been found to have several unusual properties. Despite the uncertainty and divergence of views about its role in photosynthetic electron transport [1–5], there is now wide agreement [6–11] that cytochrome b_{559} is the only chloroplast cytochrome that undergoes photooxidation at cryogenic (liquid N_2) temperature [12]. Moreover, it has also been confirmed [9–11] that the low-temperature photooxidation of cytochrome b_{559} proceeds effectively only in Photosystem II (short-wavelength) light [12], a fact that is consistent with an interpretation that cytochrome b_{559} is situated in close proximity to the reaction center chlorophyll of Photosystem II. The association of cytochrome b_{559} with Photosystem II was also demonstrated when spinach chloroplasts were fractionated with detergents [13–15] and when Tris-treated spinach chloroplasts gave (at room

Abbreviations: $DCIPH_2$, reduced 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

temperature) contrasting action spectra for the photooxidation of cytochromes b_{559} and *f*: that for cytochrome b_{559} was characteristic of Photosystem II and that for cytochrome *f*, of Photosystem I [16]. However, a photooxidation of cytochrome b_{559} by Photosystem I light has also been observed in chloroplasts isolated from *Chlamydomonas reinhardii* [17], lettuce [18], and under special experimental conditions, spinach [19].

Another distinctive feature of cytochrome b_{559} in chloroplasts is its existence in forms that differ from one another in their oxidation–reduction potentials [10, 20, 21]. The high-potential form (E_m about 330–350 mV) is reducible by hydroquinone, the middle-potential form (E_m about 50–80 mV) is reducible by ascorbate but not by hydroquinone, and the low-potential form is reducible by dithionite but not by ascorbate [20]. The high-potential form was the predominant one in freshly prepared chloroplasts and was strongly correlated with Photosystem II activity [20]. Several treatments, singly or jointly, converted the high-potential form to the middle-potential form (a conversion that was usually accompanied by a decrease in Photosystem II activity). The association of the low-potential form of cytochrome b_{559} with Photosystem II is not well established; this form has recently been observed in spinach chloroplast fragments having Photosystem I activity but lacking Photosystem II activity [22, 23].

The work with chloroplasts suggested that the predominance of the high-potential form of cytochrome b_{559} and its preferential photooxidation at low temperature by short-wavelength light may be general characteristics of the O_2-evolving system in photosynthesis (regardless of how the significance of these two special features of cytochrome b_{559} may be finally evaluated). Two recent reports, however, have cast doubt on the applicability of this generalization to the most primitive (in an evolutionary sense) photosynthetic cells that produce O_2 and are devoid of chloroplasts, i.e. the blue-green algae. Bendall and Sofrova [10] noted that a cell-free preparation from the blue-green alga *Plectonema boryanum* did not contain the high-potential form of cytochrome b_{559}. Knaff [24], using membrane fragments of another blue-green alga, *Nostoc muscorum*, identified in them a high-potential form of cytochrome b_{558} (synonymous with cytochrome b_{559}) but observed that (at room temperature) it was photooxidized preferentially not by Photosystem II (664 nm) but by Photosystem I (715 nm) light.

The aim of this study was to determine whether two of the special features of cytochrome b_{559} in chloroplasts are also found in O_2-evolving membrane fragments of blue-green algae, i.e. whether their cytochrome b_{559} exists in several forms, of which the high-potential one is the dominant one, and whether, at low temperature, the high-potential form of cytochrome b_{559} is preferentially photooxidized by Photosystem II light. The results showed that photosynthetic membranes from *N. muscorum* (Strain 7119) cells are similar to chloroplasts in both respects. They contain different forms of cytochrome b_{559}, of which the high-potential form is the most abundant. At 77 °K, a temperature at which thermochemical reactions are minimal, the high-potential form was photooxidized by Photosystem II light (664 nm); Photosystem I light (720 nm) was ineffective.

METHODS

The membrane fragments used here were digitonin-treated preparations (Fraction C) from *N. muscorum* Strain 7119 cells grown and fractionated as described by Arnon et al. [25]. The membrane fragments showed good electron transport activity, either from water to $NADP^+$ or (in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)) from reduced 2,6-dichlorophenolindophenol ($DCIPH_2$) to $NADP^+$, giving in either case a rate of the order of 200 μmoles of NADPH per mg of chlorophyll *a* per h. These and other properties of the membrane fractions and the estimations of chlorophyll *a* content are described in the companion paper [25].

Absorbance changes were measured with a dual wavelength spectrophotometer (Phoenix Precision Instrument Co.) as described previously [12]. The 664-nm and 720-nm actinic illumination were provided by monochromatic light beams isolated with Baird–Atomic interference filters with half-band widths of 10 nm. The light intensities of the 664-nm and 720-nm beams were, respectively, $1.4 \cdot 10^4$ and $1.0 \cdot 10^4$ ergs/cm^2 per s. The light path at 77 °K was 2 mm and at room temperature, 10 mm. When absorption measurements were made at 77 °K, the membrane fragments were reduced or oxidized prior to the addition of glycerol and freezing at the low temperature. The hydroquinone, ascorbate, dithionite, and ferricyanide used as the reductants and the oxidant were prepared freshly before each experiment.

RESULTS AND DISCUSSION

Evidence for the existence of different forms of cytochrome b_{559} in *Nostoc* membrane fragments was sought at room temperature by reducing the cytochromes in two stages (Fig. 1) and measuring the increase in absorbance at 558 nm. The cytochromes, oxidized initially by ferricyanide, were titrated first with hydroquinone which reduced the high-potential form of cytochrome b_{559} (and cytochrome *f*). Upon the completion of this reduction, dithionite was added to reduce the cytochromes with the more negative potentials.

Several conclusions may be drawn from the two spectra in Fig. 1, one with absorbance peak at 557 nm (hydroquinone minus ferricyanide), and the other at 559 nm (dithionite minus hydroquinone). The predominant form of cytochrome b_{559} in the membrane fragments, accounting for about three-quarters of the total cytochrome b_{559}, was the high-potential form, reduced by hydroquinone. The dithionite minus hydroquinone spectrum provides evidence for the presence of cytochrome b_{559} of a lower potential, not reducible by hydroquinone but reducible by dithionite (no distinction was made here between the middle- and low-potential forms of cytochrome b_{559} described in [20]. The same spectrum also shows the reduction of cytochrome b_6 (in the 563-nm region). The shoulders in the 550-nm region suggest the presence of membrane-bound *c*-type cytochromes. The possibility is being investigated that one of these may be similar to the low-potential *c*-type cytochrome found by Holton and Myers [26] or to one of the two *c*-type cytochromes found by Ogawa and Vernon [17] in *Anabaena variabilis*.

Turning to photooxidation at low temperature (−196 °C), we observed that it was the high-potential form of cytochrome b_{559} that was being photooxidized by illumination with a 664-nm light beam (Fig. 2). The experiment was carried out as

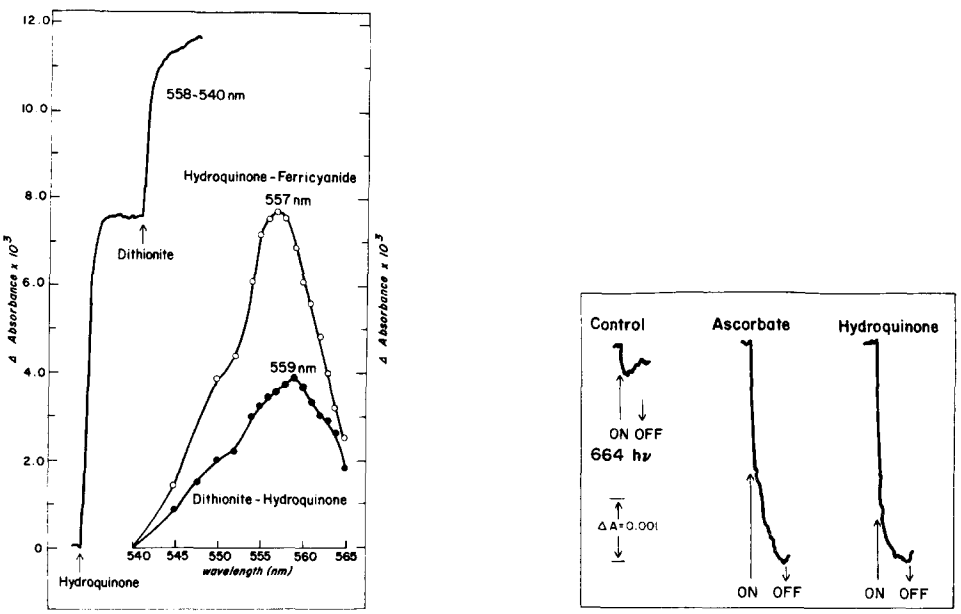

Fig. 1. Titration of cytochromes in *Nostoc* membrane fragments by hydroquinone and dithionite. Temperature, 20 °C. Left: Increase in absorbance at 558 nm due to chemical reduction of cytochromes in two stages. First, hydroquinone (final concn, 2 mM) was added. Next, when reduction by hydroquinone was complete, dithionite (0.184 mg/ml) was added. Right: Absorption spectra resulting from reduction of cytochromes by hydroquinone (hydroquinone minus ferricyanide spectrum) and subsequent reduction by dithionite (dithionite minus hydroquinone spectrum). Each point in the spectra was obtained on a different aliquot of the reaction mixture by the same procedure at each measured wavelength. Reference wavelength in all cases was 540 nm. The initial reaction mixture contained (per 1.0 ml) *Nostoc* membrane fragments (60 μg chlorophyll *a*) and the following: 50 μmoles Tricine (*N*-tris(hydroxymethyl)methylglycine) buffer (pH 7.7), 500 μmoles sucrose, 10 μmoles $MgCl_2$, and 0.5 μmole ferricyanide.

Fig. 2. Photooxidation at −196 °C of cytochrome b_{559} in *Nostoc* membrane fragments prereduced with hydroquinone or ascorbate. The *Nostoc* membrane fragments were pretreated with 2 mM ferricyanide. The photooxidation of cytochrome b_{559} was measured (at 556–537 nm) without subsequent reduction (left) or after reduction by hydroquinone (2 mM) (right) or ascorbate (10 mM) (centre). The reaction mixture contained (per 1.0 ml) *Nostoc* membrane fragments (100 μg chlorophyll *a*), 0.6 ml glycerol and the following: 20 μmoles Tricine buffer (pH 7.7), 200 μmoles sucrose and 4 μmoles $MgCl_2$.

follows: prior to the addition of glycerol and freezing by immersion in liquid N_2, ferricyanide was added to three samples of the membrane fragments to oxidize all cytochromes. Next, the cytochromes were reduced in one sample by the addition of excess hydroquinone and in the second sample by the addition of an excess of ascorbate; no reductant was added to the third sample which served as the control. Fig. 2 shows that in the sample produced by hydroquinone subsequent illumination at 77 °K by a 664-nm actinic light gave a marked decrease in absorption at 556 nm, a wavelength indicative of the α peak of cytochrome b_{559} at low temperature [12]. The extent of photooxidation was about the same as in the sample prereduced by ascorbate, suggesting that here only the high-potential form of cytochrome b_{559} was photooxidized.

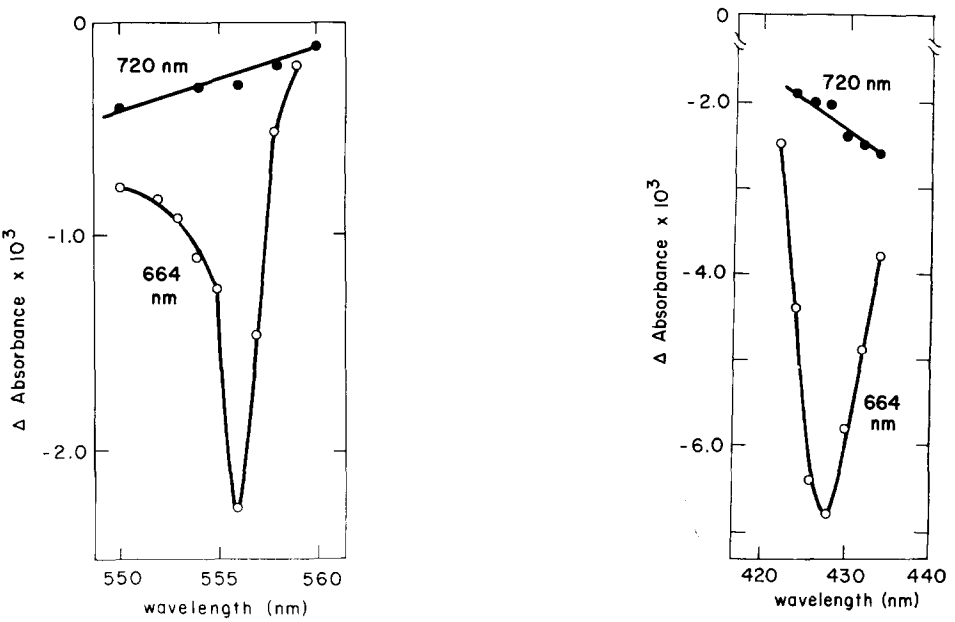

Fig. 3. Photooxidation in the α-band region of cytochrome b_{559} in *Nostoc* membrane fragments at −196 °C. Reaction mixture as in Fig. 2 except that ascorbate (10 μmoles/ml) was added. Reference beam, 562 nm.

Fig. 4. Photooxidation in the Soret region of cytochrome b_{559} in *Nostoc* membrane fragments at −196 °C. The reaction mixture contained (per 1.0 ml) *Nostoc* membrane fragments (50 μg chlorophyll *a*) 0.6 ml glycerol, and the following: 20 μmoles Tricine buffer (pH 7.7), 200 μmoles sucrose, 4 μmoles $MgCl_2$, 10 μmoles sodium ascorbate and 0.001 μmole DCIP. Reference beam, 515 nm.

Very little change in absorption occurred in the control sample which was not prereduced.

Other experiments were undertaken to document, first, that the low-temperature photooxidation was produced by Photosystem II and not by Photosystem I actinic light and, secondly, that the photooxidation was reflected in spectral changes characteristic of the α and γ (Soret) regions of the absorption spectrum of cytochrome b_{559}. Fig. 3 shows that illumination of the *Nostoc* membrane fragments at −196 °C by a Photosystem II (664-nm) light beam produced in the α region decreases in absorbance that had a maximum at 556 nm which coincides with the (low temperature) maximum α absorption peak of cytochrome b_{559}, whether measured in situ in chloroplasts [12] or in an isolated and purified preparation [28].

When changes in absorbance in the *Nostoc* membrane fragments were measured under similar conditions in the Soret region, a maximum decrease in absorbance was observed at 428 nm (Fig. 4) a wavelength very close to the γ absorption peak at 429 nm obtained (at low temperature) for the purified cytochrome b_{559} isolated from chloroplasts by Wasserman and associates [28].

The absorbance changes of cytochrome b_{559} produced by Photosystem II illumination (664 nm), in both the Soret and α regions, did not occur upon illumination with Photosystem I light (720 nm). The absorbance elicited in this region by

720-nm illumination is probably due to P700 [29, 30]. We conclude, therefore, that in *Nostoc* membrane fragments, as in chloroplasts, cytochrome b_{559} is photooxidized at cryogenic temperatures only by Photosystem II light.

As already mentioned, the *Nostoc* membrane fragments used in these experiments (Fraction C) were treated with digitonin [25] a detergent that might create some artifacts. Similar results were obtained, however, with membrane fragments (Fraction A) that were prepared without a detergent treatment [25].

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REFERENCES

1 Knaff, D. B. and Arnon, D. I. (1969) Proc. Natl. Acad. Sci. U.S. 64, 715–722
2 Hiller, R. G., Anderson, J. M. and Boardman, N. K. (1971) Biochim. Biophys. Acta 245, 439–452
3 Cox, R. P. and Bendall, D. S. (1972) Biochim. Biophys. Acta 283, 124–135
4 Butler, W. L. (1973) Acc. Chem. Res. 6, 177–184
5 Vermeglio, A. and Mathis, P. (1973) Biochim. Biophys. Acta 292, 763–771
6 Erixon, K. and Butler, W. L. (1971) Photochem. Photobiol. 14, 427–433
7 Erixon, K. and Butler, W. L. (1971) Biochim. Biophys. Acta 234, 381–389
8 Floyd, R. A., Chance, B. and DeVault, D. (1971) Biochim. Biophys. Acta 226, 103–112
9 Boardman, N. K., Anderson, J. M. and Hiller, R. G. (1971) Biochim. Biophys. Acta 234, 126–136
10 Bendall, D. S. and Sofrova, D. (1971) Biochim. Biophys. Acta 234, 371–380
11 Lozier, R. H. and Butler, W. L. (1974) Biochim. Biophys. Acta, 333, 460–464
12 Knaff, D. B. and Arnon, D. I. (1969) Proc. Natl. Acad. Sci. U.S. 63, 956–962
13 Boardman, N. K. and Anderson, J. M. (1967) Biochim. Biophys. Acta 143, 187–203
14 Arnon, D. I., Tsujimoto, H. Y., McSwain, B. D. and Chain, R. K. (1968) in Comparative Biochemistry and Biophysics of Photosynthesis (Shibata, K., Takamiya, A., Jagendorf, A. T. and Fuller, R. C., eds), pp. 113–132, Univ. Park Press, St. College
15 Ke, B., Vernon, L. P. and Chaney, T. H. (1972) Biochim. Biophys. Acta 256, 345–357
16 Knaff, D. B. and McSwain, B. D. (1971) Biochim. Biophys. Acta 245, 105–108
17 Levine, R. P. and Gorman, D. S. (1966) Plant Physiol. 41, 1293–1300
18 Ben-Hayyim, G. and Avron, M. (1970) Eur. J. Biochem. 14, 205–213
19 Cramer, W. A., Fan, H. N. and Böhme, H. (1971) Bioenergetics 2, 289–303
20 Wada, K. and Arnon, D. I. (1971) Proc. Natl. Acad. Sci. U.S. 68, 3064–3068
21 Erixon, K., Lozier, R. and Butler, W. L. (1972) Biochim. Biophys. Acta 267, 375–382
22 Anderson, J. M. and Boardman, N. K. (1973) FEBS Lett. 32, 157–160
23 Knaff, D. B. and Malkin, R. (1973) Arch. Biochem. Biophys. 159, 555–562
24 Knaff, D. B. (1973) Biochim. Biophys. Acta 325, 284–296
25 Arnon, D. I., McSwain, B. D., Tsujimoto, H. Y. and Wada, K. (1974) Biochim. Biophys. Acta 357, 231–245
26 Holton, R. W. and Myers, J. (1967) Biochim. Biophys. Acta 131, 375–384
27 Ogawa, T. and Vernon, L. P. (1971) Biochim. Biophys. Acta 226, 88–97
28 Garewal, H. S., Singh, J. and Wasserman, A. R. (1971) Biochem. Biophys. Res. Commun. 44, 1300–1305
29 Ke, B., Ogawa, T., Hiyama, T. and Vernon, L. P. (1971) Biochim. Biophys. Acta 226, 53–62
30 Hiyama, T. and Ke, B. (1971) Biochim. Biophys. Acta 226, 320–327