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OSCILLATIONS OF THE NAD(P)H POOL SIZE AND OF THE REDOX STATE OF A CYTOCHROME *b* DURING DARK RESPIRATION OF THE BLUE-GREEN ALGA, *ANACYSTIS NIDULANS*

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Summary

Oscillations of the oxygen uptake rate of the blue-green alga (cyanobacterium) *Anacystis nidulans* were induced by light pulses. The pool size of NAD(P)H and the redox state of a cytochrome *b* showed oscillations of similar shape and frequency. Phase diagrams revealed that these three oscillations were presumably linked. The cytochrome *b* should be a part of the respiratory chain of this blue-green alga. The oscillations were inducible only in a limited physiological state of the alga.

Introduction

The rate of respiration of phototrophic blue-green alga is rather low [1] and it is an open question whether the respiratory chain is situated on the photosynthetic membranes as postulated [2]. The electron donor for the respiratory chain appears to be NADPH in vivo [1] though NADH can be oxidized also at least in vitro [3]. Flexibacteria, considered as colourless cyanophyta, contain *b*-type cytochromes in their respiratory chain [4,5]. The *b*-type cytochromes found in phototrophic blue-green algae appear to belong to the photosynthetic electron transport chain [6—9].

The light-triggered oscillation of the oxygen uptake rate of *Anacystis nidulans* [10] offered the possibility to search for other oscillations in the respiratory system of this blue-green alga. Oscillations and their phase relation-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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ship have been frequently used to study the components and the regulation of glycolysis in yeast (e.g. Refs. 11 and 12). Similarily the oscillation of the oxygen uptake rate should be used to identify components of the respiratory system in *A. nidulans*. Some of the results have been reported earlier [13].

Material and Methods

Anacystis nidulans (Kratz and Allen strain, University of Texas culture collection No. 625) was received from Dr. Šetlík, Laboratory of Algology, Třeboň, Č.S.S.R. The alga was grown autotrophically (40°C, 16 h 7000 lumen/m^2 white light, 8 h dark, 1.5% CO_2 in air). 10 ml of the culture were transferred every day before the light period in 310 ml fresh medium (composition [14]) to get roughly synchronized algae. Samples for experiments were harvested after a complete culture cycle and a short preillumination period (up to 1 h, culture conditions) by centrifugation (10 min, $3000 \times g$, about 35°C). The pellet was resuspended and the cell content was adjusted to about 0.5 mg organic nitrogen per ml (measured by the Kjeldahl method) for the absorption and fluorescence measurements.

In vivo changes of the pool size of NAD(P)H were recorded either by fluorescence [15] or absorption measurements. For fluorescence measurements the Eppendorf fluorometer was equipped with a Hg 366 nm filter and with a filter of 2 mm plexiglas No. 701 (Röhm and Haas, Darmstadt, F.R.G.) in front of the photomultiplier. The fluorescence measured was mainly emitted by NAD(P)H as judged by comparison of the emission spectra of algae and NADH. A 0.5-cm path length cuvette with the algal suspension was exposed in the fluorometer to the red actinic light for phosphosynthesis. As some stray light superimposed the fluorescence signal during the light pulse, the pool size changes of NAD(P)H were measured only in the dark. The exciting light for fluorescence (366 nm) did not alter the rate of oxygen exchange.

Absorption changes were measured in an Aminco DW-2 spectrophotometer operated in the dual wavelength mode (bandwidth 10 nm). The photomultiplier was protected against stray actinic light by glass filters (340—400 nm: UG 1 + BG 12; 400—450 nm; BG 7 + BG 12 + BG 28; 500—580 nm: BG 7 + BG 18 + BG 28) from Schott and Gen, Mainz, F.R.G.

The oxygen exchange rate was measured polarographically with a teflon-covered stationary platinum electrode [16,17]. The algae spread on the electrode were covered by a dialysis membrane which separated the algae from the fluid (growth medium or phosphate buffer) streaming over the electrode with constant velocity, temperature and oxygen content. In such a system the polarographic current is a direct measure of the rate of oxygen exchange or in the dark of oxygen uptake.

All measurements were performed at 40°C in the growth medium or in a 2.9 mM potassium phosphate buffer (pH 7.8). The phosphate concentration and the pH of the buffer were identical to those of the growth medium. Measurements which were used for analysis of the phase relationship etc. were made at about the same time with samples of the same suspension of algae under identical conditions.

Results

In *Anacystis nidulans*, an oscillation of the oxygen uptake rate induced by light pulses [10] was always accompanied by an oscillation of the NAD(P)H pool size (Fig. 1a). Both oscillations could only be induced simultaneously, suggesting that they are linked. When the oxygen uptake rate showed only a single minimum after the light pulse, appearing as the second maximum in the oxygen exchange rate trace (Fig. 1b; cf. Ref. 17), the pool size of NAD(P)H reached a minimum and returened to the dark steady state without further transients (Fig. 1b). All light-triggered transients of the NAD(P)H pool size in the dark were abolished by 10 μM CCCP as was already reported for the transients of the oxygen uptake rate [10]. These results point to a common mechanism of both oscillations and of their induction.

Absorption measurements showed that beside the NAD(P)H pool size (Figs. 1a, 2a) the redox state of at least one cytochrome was oscillating with a similar frequency (Fig. 2b). The spectra of the absorption changes forming these oscillations are compared with the mirror image of an absorption spectrum of NADH (X) and with the spectrum of the absorption change during the light (O) (Fig. 3a). The similarity between the absorption spectrum of NADH and the

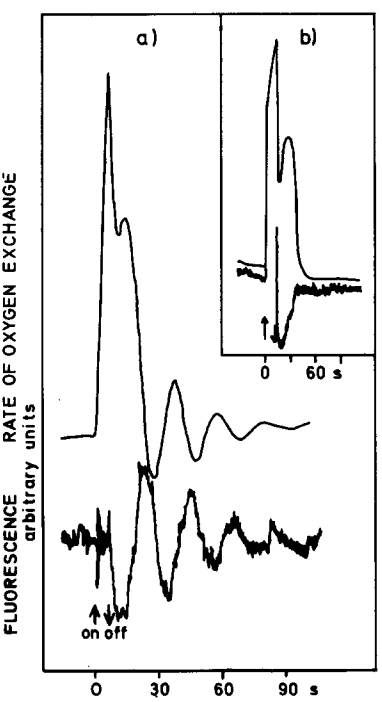

Fig. 1. Comparison of the transients of the oxygen exchange rate and of the NAD(P)H specific fluorescence after light pulses (↑ on, ↓ off; 674 nm, 0.77 nanoeinstein · cm^{-2} · s^{-1} for the oxygen measurements, upper traces; 671 nm, 0.85 nanoeinstein · cm^{-2} · s^{-1} for the fluorescence measurements, lower traces). (a) Samples of a suspension of algae inducible to oscillations of the oxygen exchange rate, harvested after a complete culture cycle + 0.5 h preillumination. Light pulse: 6 s. (b) Samples of a suspension of algae inducible only to a single minimum of the oxygen uptake rate (second maximum of the oxygen exchange trace) after the light pulse (12 s) harvested after a complete culture cycle + 1 h preillumination.

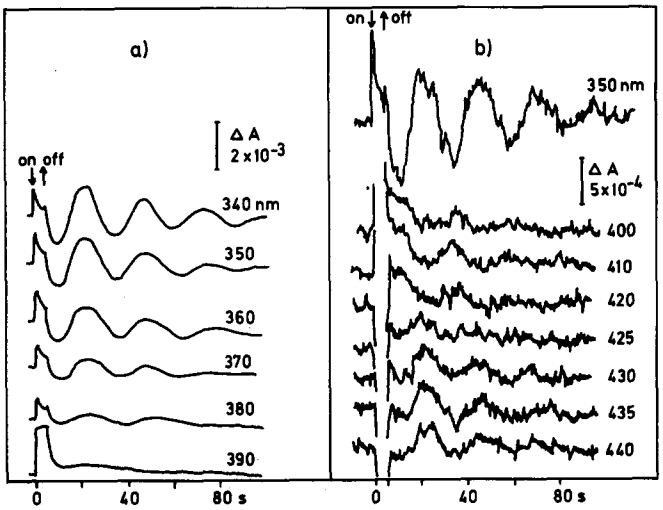

Fig. 2. (a and b) Absorption changes during and after light pulses (↑ on, ↓ off, 5 s, 671 nm, 1.0 nanoeinstein · cm^{-2} · s^{-1}) measured in a dual wavelength spectrophotometer. Reference wavelength 455 nm, measuring wavelength as indicated in the figure.

spectrum of the absorption changes between 340 and 390 nm is obvious and confirms the data obtained by fluorescence measurements. The spectra of the absorption changes between 400 and 450 nm during the light and during the oscillation show distinct minima and maxima supporting that different cytochromes evoked it. During the light a strong oxidation of cytochrome *f* caused

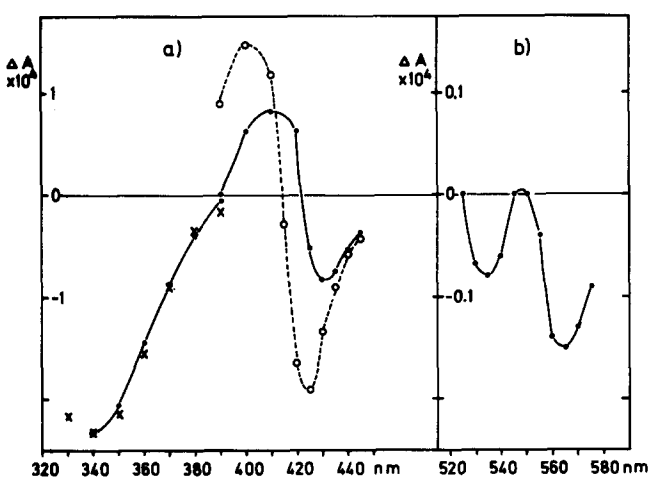

Fig. 3. Spectra (●———●) of the observed absorption changes oscillating in the dark after a light pulse. The differences between the first minimum (resp. maximum) and the first maximum (resp. minimum) of the oscillations were plotted as negative (resp. positive) values against the wavelength of the measuring beam (dual wavelength spectrophotometry). For comparison the absorption spectrum of NADH (X) (inversely plotted, equalized at 340 nm) and the spectrum of the observed absorption changes during the light (○- - - - -○, reduced scale: X0.5) are shown. (a) Reference wavelength 455 nm (change attributable to NAD(P)H absorption and to the Soret-bands of cytochromes. (b) Reference wavelength 500 nm. Spectrum of the α- and β-bands of cytochromes. Note the enlarged scale (X10).

the minimum at 425 nm (cf. Refs. 18 and 19). The small shoulder of the spectrum at about 435 nm may be due to oxidation of *P*-700 (cf. Ref. 19) but it cannot be excluded that the absorption change observed at about the same wavelength after the light pulse occurred already during the light. However, it was assured that *P*-700 did not show transient absorption changes after the light pulse (measuring wavelength 700 nm, reference wavelength 730 nm). Measurements of the very weak absorption changes in the region of the α- and β-bands of the cytochromes showed that apparently the *c*-type cytochromes described in *A. nidulans* [20] could not be induced to oscillatory changes by light pulses. The spectral characteristics of the absorption changes during the oscillation support a light-induced oscillation of the redox state of a *b*-type cytochrome which had in the reduced state an α-band at about 565 nm, a β-band at about 535 nm and a Soret-band at about 430 nm. The oscillations of the NAD(P)H pool size and of the reduced state of the cytochrome were obviously in phase, the oxidized state (Soret-band at about 410 nm) being 180° out of phase.

The phase relationship between the rate of the oxygen uptake and of the NAD(P)H pool size was analysed in a phase plane plot [11,12] (Fig. 4a). From the spiral formed the phase angle could not be determined since a rate (oxygen uptake) was compared with a concentration (NAD(P)H pool). However, the spiral indicates that both oscillations were not firmly linked. The rate of NAD(P)H oxidation (which is equal to the first time derivative of the NAD(P)H concentration) was plotted versus the rate of oxygen uptake (Fig. 4b). This plot shows a spiral which has a longer axis which forms an angle of about 45° with the *x*-axis (Fig. 4b). This angle indicates that these two oscillations were roughly in phase too [12]. An exact determination of the phase angle was impossible as only relative values for both rates could be determined. The varia-

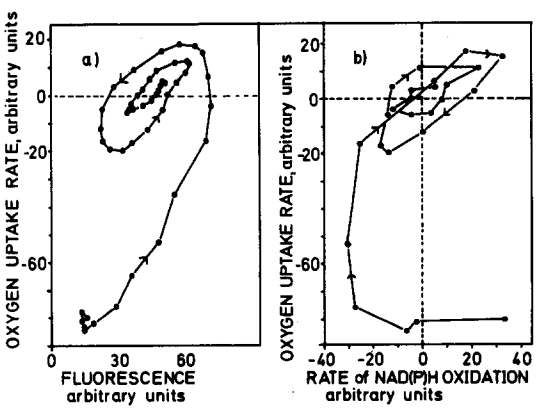

Fig. 4. Phase plane plots of the oscillations of the oxygen exchange rate against the NAD(P)H specific fluorescence. The plots were started 3.6 s after the end of the light pulse to avoid an influence of the photosynthetic oxygen evolution. (a) Rate of oxygen uptake (*y*-axis) against the intensity of NAD(P)H fluorescence (*x*-axis) both in arbitrary units. Distance between the points: 1.2 s. Zero of the oxygen uptake rate was defined as the rate in the dark before the light pulse. (b) Rate of oxygen uptake (*y*-axis) agianst the rate of NAD(P)H oxidation (*x*-axis) both in arbitrary units. The rate of NAD(P)H oxidation was graphically determined from the oscillation of the fluorescence intensity. Distance between the points: 2.4 s.

tions of the phase angle during the oscillations of the NAD(P)H oxidation and of the oxygen uptake (Figs. 1a, 4a, 4b) suggest that the oscillation of the NAD(P)H pool size may not only reflect an oscillatory oxidation by the respiration but also an oscillating reduction of $NAD(P)^+$. However, it is difficult at present to decide whether the pool size oscillations of NAD(P)H evoked or followed the oscillation of the oxygen uptake.

Discussion

The phase relationship between the oscillations and the observed similarities of the frequencies support the notion that all oscillations are linked. The effect of CCCP, however, may be restricted on the induction and does not support a control of the oscillation by the oxidative phosphorylation.

The NAD(P)H oscillation could be expected as Biggins [1] demonstrated the oxidation of NADPH in vivo by the respiration of *Anacystis nidulans*. As fluorescence and absorption measurements do not distinguish between NADH and NADPH the observed variations of the phase angle of the oscillations (Fig. 4) may have been caused by NADH pool size changes. As the respiration rate of *A. nidulans* does not appear to be limited by the oxygen concentration within wide margins [1] it is suggested that an oscillation of the NAD(P)H pool size evoked the oscillation of the oxygen uptake and not vice versa.

The role of the cytochrome *b* with the oscillating redox state could not be clarified definitively. The obvious direct connection to the redox state of the NAD(P)H pool suggests a position in the respiratory chain. While the cytochrome *b*-562 found in flexibacteria presumably belongs to their respiratory chain [5], in phototrophic blue-green algae previous data did not indicate a participation of a cytochrome *b* in the respiratory chain [21]. The photosynthetic electron transport chain of blue-green algae contains at least two *b*-type cytochromes [6—9]. The oscillating cytochrome differed in its spectral characteristics from cytochrome *b*-559(557, 558) [6—9]. The similarity to cytochrome *b*-562 [6] suggests an identity and it is tempting to assume a connection between the photosynthetic and the respiratory electron transport chains by this cytochrome as was found in photosynthetic bacteria. Such a direct interaction between photosynthesis and respiration has been proposed on the basis of electron microscopic and photosynthesis studies [2,22]. However, the induction of the oscillation depended on the inhibition of respiration [10,23] presumably mediated by the adenylate system [23]. The inhibition of respiration (Fig. 1b) was a prerequisite but not the only condition for the oscillations [10].

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References

1 Biggins, J. (1969) J. Bacteriol. 99, 570—575
2 Bisalputra, T., Brown, D.L. and Weier, T.E. (1969) J. Ultrastruct. Res. 27, 182—197

3 Leach, C.K. and Carr, N.G. (1970) J. Gen. Microbiol. 64, 55—70
4 Webster, D.A. and Hackett, D.P. (1966) Plant Physiol. 41, 599—605
5 Biggins, J. and Dietrich, W.E., Jr. (1968) Arch. Biochem. Biophys. 128, 40—50
6 Ogawa, T. and Vernon, L.P. (1971) Biochim. Biophys. Acta 226, 88—97
7 Knaff, D.B. (1973) Biochim. Biophys. Acta 325, 284—296
8 Fujita, Y. (1974) Plant Cell Physiol. 15, 861—874
9 Aparicio, P.J., Ando, K. and Arnon, D.I. (1974) Biochim. Biophys. Acta 357, 246—251
10 Ried, A. and Šetlík, I. (1971) in Proc. 2nd Int. Congr. Photosynthesis Research (Forti, G., Avron, M. and Melandri, A., eds.), Vol. 3, pp. 2077—2087, Junk, The Hague
11 Ghosh, A and Chance, B. (1964) Biochem. Biophys. Res. Commun. 16, 174—181
12 Betz, A. and Chance, B. (1965) Arch. Biochem. Biophys. 109, 585—594
13 Metzler, H. (1979) in Kinetics of Physicochemical Oscillatios, Discussion Meeting, Preprints of Submitted Papers (Franck, U.F. and Wicke, E., eds.), Vol. II, pp. 404—413, Aachen
14 Moyse, A., Couderc, D. and Garnier, J. (1957) Rev. Cytol. Biol. Végét. 18, 293—304
15 Olson, J.M. and Amesz, J. (1960) Biochim. Biophys. Acta 37, 14—24
16 Fork, D.C. (1963) Plant Physiol. 38, 323—332
17 Ried, A. (1968) Biochim. Biophys. Acta 153, 653—663
18 Amesz, J. and Duysens, L.N.M. (1962) Biochim. Biophys. Acta 64, 261—278
19 Murata, N., Throughton, J.H. and Fork, D.C. (1975) Plant Physiol. 56, 508—517
20 Holton, R.W. and Myers, J. (1967) Biochim. Biophys. Acta 131, 362—374
21 Carr, N.G. (1973) in The Biology of Blue-green Algae (Carr, N.G. and Whitton, B.A. eds.), pp. 44—50, Blackwell, Oxford
22 Jones, L.W. and Myers, J. (1963) Nature 199, 670—672
23 Ried, A., Šetlík, I., Bossert, U. and Berková, E. (1973) Photosynthetica 7, 161—176