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ENERGY METABOLISM IN THE CYANOBACTERIUM *PLECTONEMA BORYANUM*

PARTICIPATION OF THE THYLAKOID PHOTOSYNTHETIC ELECTRON TRANSFER CHAIN IN THE DARK RESPIRATION OF NADPH AND NADH

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The oxidation of NADPH and NADH was studied in the light and in the dark using sonically derived membrane vesicles and osmotically shocked spheroplasts. These two types of cell-free membrane preparations mostly differ in that the cell and thylakoid membranes are scrambled in the former type and that they are more or less separated in the latter type of preparations. In the light, using both kinds of preparations, each of NADPH and NADH donates electrons via the plastoquinone-cytochrome *b*/*c* redox complex (Qbc redox complex) to the thylakoid membrane-bound cytochrome *c*-553 preoxidized by a light flash and to methylviologen via Photosystem I. NADPH donates electrons to the thylakoid membrane via a weakly rotenone-sensitive dehydrogenase to a site that is situated beyond the 3(3′,4′-dichlorophenyl)-1,1-dimethylurea sensitive site and before plastoquinone. Ferredoxin and easily soluble cytoplasmic proteins are presumably not involved in light-mediated NADPH oxidation. Inhibitors of electron transfer at the Qbc redox complex as the dinitrophenylether of 2-iodo-4-nitrothymol, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone and 2-*n*-heptyl-4-hydroxy-quinone-*N*-oxide are effective, but antimycin A and KCN are not. The oxidation of NADH showed comparable sensitivity to these inhibitors. However, the oxidation of NADH is antimycin-A-sensitive regardless of the kind of membrane preparation used, indicating that in this case electrons are donated to a different site on the thylakoid membrane. In the dark, NADPH and NADH donate electrons at sites that behave similar to those of light-mediated oxidation, indicating that the initial steps of electron transfer are situated at the thylakoid membranes. However, NADPH oxidation is in some cases not sensitive to inhibitors active at the Qbc redox complex. It is concluded that O_2 reduction takes place at two different sites, one partly developed in vitro, situated near the rotenone-sensitive NADPH dehydrogenase, and another, highly KCN-sensitive one, situated beyond the Qbc redox complex and used in vivo. The terminal oxygen-reducing step of NADPH and NADH oxidation in the dark showed a preparation-dependent sensitivity for KCN, more than 80% inhibition in sonically derived membrane vesicles and less than 30% inhibition in osmotically shocked spheroplasts. From this result we tentatively conclude that the highly KCN-sensitive oxidase is not necessarily located at the thylakoid membrane and could be located at the cytoplasmic membrane.

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Abbreviations: Chl a, Chlorophyll *a*; cyt, cytochrome; DBMIB, 2,5 dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DNP-INT, dinitrophenylether of 2-iodo-4 nitrothymol; DQH_2, duroquinol; HOQNO, 2-*n*-heptyl-4-hydroxy-quinoline-*N*-oxide; PS I, Photosystem I; PQ, plastoquinone; Qbc redox complex, the redox complex containing plastoquinone, Cytochromes *b*-563 and *c*-557 and the Rieske non-haem iron and sulfur protein; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]-glycine.

Introduction

In bioenergetic studies of cyanobacteria (blue-green algae) a major and so far only partially resolved question is the localization of the dark respiratory and associated phosphorylation activities. Since, in cyanobacteria, photosynthetic activities are localized in separate organelle-like thylakoid structures, unlike the other photosynthetic prokaryotes that have both their photosynthetic and respiratory machineries situated in one continuous membrane, the question is whether in dark respiration the thylakoid membrane, the cell membrane or perhaps both in cooperation participate [1,2]. Reports on the involvement of the thylakoids in respiration are abundant [2–16], on the other hand the cell membrane was shown to participate in respiration as well [2,6,14,17,18].

NADPH was shown to donate electrons to a rotenone-sensitive dehydrogenase [19,20], the electrons then reducing a plastoquinone containing Qbc redox complex [7,15,16], since ubiquinone is absent in cyanobacteria [21]. The pathway(s) of electrons from the Qbc redox complex to O_2 are still largely unresolved [2,14,17,21,22]. Interestingly, Peschek and coworkers [23,24] have recently detected cyt aa_3 in several species of cyanobacteria.

The aim of the present work is to study the light-induced and dark electron transfer in sonically-derived membrane vesicles and osmotically shocked spheroplasts with NADPH and NADH as electron donors and to compare its sensitivity to inhibitors in the light and in the dark with methylviologen (via PS I), oxidized TMPD and O_2 as terminal electron acceptors.

Our results strongly indicate that in the first steps of NAD(P)H oxidation the thylakoid membrane participates, both in the light and the dark. Thus, the dark substrate oxidation pathway shares redox components with the photosynthetic electron transfer chain. It is shown that more than one O_2-reducing site may exist and that the highly KCN-sensitive terminal oxidase is not necessarily located at the thylakoid membrane and could be located at the cell membrane. A scheme of electron transfer pathway is presented in Fig. 3 of the accompanying paper [32].

Materials and Methods

Culture. Plectonema boryanum 594 Gomont, according to Rippka et al. [25] properly called LPP 73110, was kindly provided by Dr. E. Padan and was grown in axenic batch culture as described previously [26].

Intact cells were collected by centrifugation of 400 ml cells, in the late logarithmic phase of growth, and washing them twice in medium B containing 10 mM Tricine-NaOH/5 mM sodium phosphate-potassium phosphate/10 mM $MgCl_2$ (final pH 7.8) [27], and resuspending them in this medium to a protein concentration of about 10 $mg \cdot ml^{-1}$.

Preparation of membrane vesicles. Intact cells were collected in medium A (equal to medium B + 0.5 M mannitol), lysozyme (1 $mg \cdot ml^{-1}$) was added and the suspension was incubated at 32°C for 30 min in the dark as described in detail previously [26]. Next, the cells were sonicated for 5 times 1 min (1 min intervals) at 4°C with a MSE Soniprep 150 suited with the microtip at an amplitude of 28 μm. Intact cells were centrifuged at $2000 \times g$ for 10 min and the particles were collected by centrifugation of the supernatant for 10 min at $25000 \times g$ and resuspension in medium A. The final protein concentration was 15 $mg \cdot ml^{-1}$, corresponding to about 0.5 mg chl *a* per ml, the preparation was stored on ice until use (within 3 h).

Preparation of spheroplasts. The procedure was essentially as described previously [26]. Lysozyme treatment time was 90 min after which the spheroplasts were stored on ice until use.

Preparation of concentrated soluble proteins. From 4 l cell culture (about 0.5 mg protein per ml) spheroplasts were prepared and washed twice in medium A (no phycocyanin was set free during this procedure) and resuspended in medium B. The suspension was gently mixed for 5 min and the spheroplasts were collected at $2000 \times g$ for 5 min. The faint blue supernatant was stored at 4°C. The pellet was treated with medium B and centrifuged once more. The supernatants were pooled and brought to 70% of saturation with $(NH_4)_2SO_4$. The mixture was left for 1 h at 4°C and the precipitated protein was collected by centrifugation at $40000 \times g$ for 20 min. The blue

pellet was resuspended in a final volume of 2 ml of medium B and dialyzed overnight at 4°C against three changes of medium B. The bluish solution, containing about 40 mg protein per ml, was stored in liquid nitrogen until use.

Flash experiments. Measurements were made in a thermostated multipurpose cuvette [28], accomodating two measuring light paths, and O_2 and other electrodes, the actinic light being provided from the bottom. Light flashes from a G.E. GT-230 xenon flash tube (2 kV), of 5 μs half-amplitude width and tail-depressed, were triggered by a programmable pulse generator and filtered through a Schott filter (above 715 nm). Usually, the signals of 64 flashes fired at a frequency of 0.5 Hz were averaged, digitized and stored in an on-line disc-based mini-computer system as described by Schuurmans et al. [29]. A computer program for experimental data fit to a sum of exponential-decay functions was applied [30]. At the start of the experiments either intact cells or freshly prepared spheroplasts were diluted in 1.8 ml of medium C containing 20 mM NaCl, 20 mM KCl, 7 mM glucose, 10 mM $MgCl_2$, 5 mM sodium-potassium phosphate, 5 mM Tricine-NaOH (final pH 7.8), at a final Chl *a* concentration of 20 $\mu g \cdot ml^{-1}$ and equilibrated at 25°C while stirred. When semi-anaerobic conditions were required a stream of N_2 was passed over the solution; strict anaerobiosis was obtained by the addition of 8 $U \cdot ml^{-1}$ glucose oxidase, 1000 $U \cdot ml^{-1}$ catalase and 2 μl ethanol and passing a stream of Ar over the solution. In order to minimize unspecific absorbance changes caused by scattering of the long intact cell filaments in the experiments of Fig. 1, the reaction mixture was not stirred during measurements (maximal 2.5 min) and 7% (v/v) glycerol was added to decrease the scattering of the solution further. Measurements were made single-beam at the indicated wavelengths (see Results). Aspecific scattering in the case of spheroplast preparations was low, the cuvette was stirred during the entire experiment and no glycerol was added. O_2 concentrations were monitored continuously in this case; methylviologen (50 μM) was added as electron acceptor.

NADPH, NADH and duroquinol oxidation was measured with an Aminco DW-2a dual-wavelength spectrophotometer at 340 nm against 400 nm as reference or, alternatively, through polarographic assay of O_2 uptake [26]. NADPH, NADH, duroquinol and TMPD were added at a concentration of 0.1 mM. Measurements could be performed simultaneously in the same cuvette as used in the flash experiments [28]. Continuous PS I light was provided by a 250 W quartz halogen lamp via a Schott fibre-optic light guide and a Schott filter (715 nm). The photomultiplier was protected against the actinic light by a filter combination absorbing light above 600 nm. In the case that anaerobiosis was required medium B was replaced by medium C and the appropriate additions were made (see above).

Chlorophyll a and protein were determined as described previously [26].

Controls. Appropriate controls in which inhibitors and electron donors or acceptors were omitted, were always run in parallel with each experiment. Unless indicated otherwise, at least three experiments were done; single determinations deviated maximally 25% of the corresponding average, deviations are expressed as S.D. values. Separate control runs showed that ethanol (nor methanol in the case of duroquinol) used at the maximum concentration of 17 mM was without any detectable effect.

Chemicals. DNP-INT and DBMIB were generous gifts of Prof. Dr. A. Trebst. DCMU was obtained from K & K laboratories; rotenone, HOQNO, antimycin A, glucose oxidase, catalase and lysozyme were all from Boehringer; Ferredoxin type III from spinach was from Sigma; BHAM from Koch Light Laboratories; DQ was prereduced with $NaBH_4$ in acidic methanol. DNP-INT, DBMIB, DCMU, HOQNO and antimycin A were dissolved in 96% ethanol.

Results

Reduction of flash-oxidized cyt c-553 by NADPH

Fig. 1 shows the spectrum of the absorbance changes in the 540–570 nm region after flashing intact cells with PS I light (715 nm). The maximal absorbance change is observed around 550 nm, most likely due to oxidation of cyt *c*-553 [7]. The time course of the flash-induced absorbance decrease (shown as positive deflections in Figs. 1 and

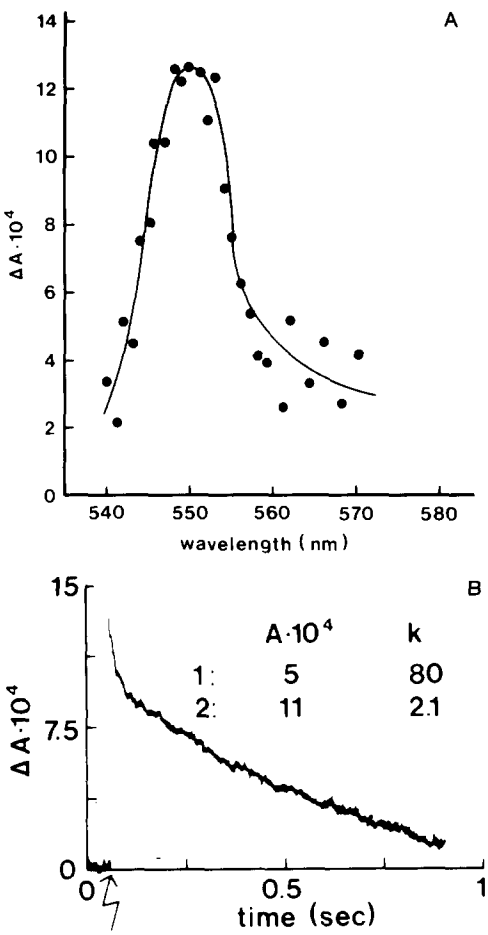


Fig. 1. (A) Light minus dark difference spectrum of 715 nm flash-induced absorbance changes in intact cells. (B) For further details, see also Materials and Methods.

2) at 550 nm and the relaxation (dark reduction by endogeneous substrates) is pictured in Fig. 1B.

Reduction of flash-oxidized cyt c-553 by NADPH and NADH in osmotically shocked spheroplasts

Upon cell disruption by lysozyme treatment and dilution in hypoosmotic medium the endogenous rate of cyt *c*-553 reduction after the flash decreased, but could largely be restored by addition of NADPH as shown in Fig. 2, or NADH (not shown). As it is presumed that the thylakoid and cell membranes are not scrambled in the osmotically shocked spheroplast preparations, it is concluded from this experiment that NAD(P)H directly donates electrons to the thylakoid membrane.

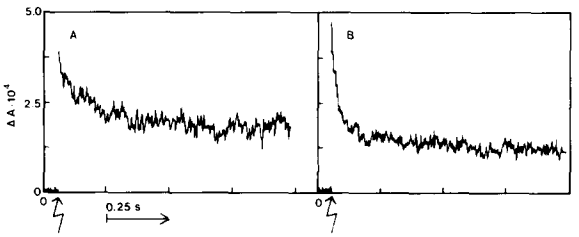
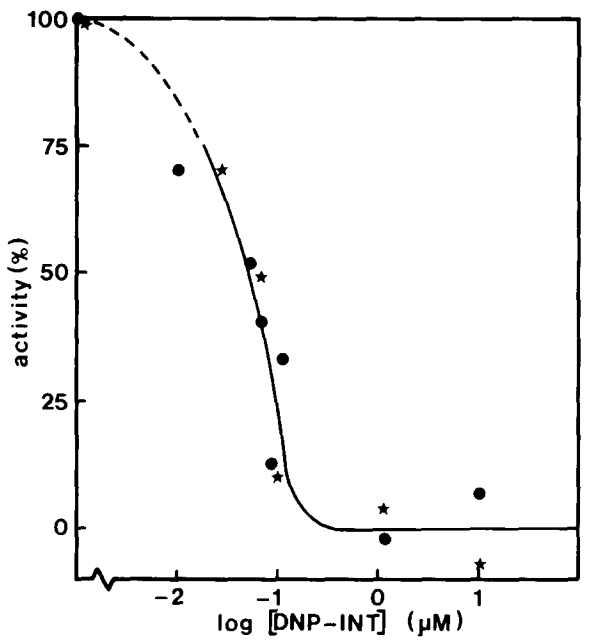


Fig. 2. Flash-induced absorbance changes at 550 nm in the presence (B) and absence (A) of NADPH in osmotically shocked spheroplasts. NADPH was added at a final concentration of 0.1 mM. (A), $k_1 = 8.3$; $A_1 = 1.5 \cdot 10^4$; $k_2 = 0.1$; $A_2 = 2.0 \cdot 10^4$. (B), $k_1 = 40$, $A_1 = 3.5 \cdot 10^4$; $k_2 = 0.4$; $A_2 = 2.0 \cdot 10^4$. Further details in Materials and Methods.

The rates of reduction could be fitted to a sum of two exponential decay functions according to: $\Delta A = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$. Where A_1 and A_2 represent the amplitudes and k_1 and k_2 are the respective rate constants. A_1 and k_1 represent the fast reduction; the slow reduction is represented by A_2 and k_2. The rates of NADPH-induced reduction were compared under different experimental conditions (Fig. 2). A high rate was found under micro-aerophilic conditions (10–25 nmol O_2 per ml) (Fig. 2B), while the rate was about 30% lower under complete anaerobic conditions, presumably due to overreduction (not shown). The fast reduction was almost absent in air-saturated medium or in micro-aerophilic medium when using aged spheroplasts (not shown). These spontaneously develop a capacity for fast NADPH oxidation after storage on ice for about 3 h [26], presumably due to the use of the alternative O_2-reducing sites. We conclude from these experiments that O_2 reduction may take place at the thylakoid membranes, as the cell and thylakoid preparations are more or less separated in the preparations used.

Effect of inhibitors and soluble proteins on NADPH and NADH oxidation in the light with methylviologen as electronacceptor in sonically derived membrane vesicles and osmotically shocked spheroplasts

From the inhibition curve the K_i for DNP-INT was estimated to be 50 ± 20 nM in both types of preparations (Fig. 3). This shows that DNP-INT is a very effective inhibitor of electron transfer in *P. boryanum*; the corresponding K_i-values reported for spinach chloroplasts are about 200 nM for linear and 10 μM for cyclic electron transfer [39].



We conclude from this experiment that electrons from NADPH pass through the DNP-INT sensitive part of the photosynthetic pathway, i.e., the Qbc redox complex.

In Table I the effects are listed of several inhibitors and proteins that might stimulate the PS-I-mediated oxidation of NADPH and NADH. Unless indicated otherwise, similar results were obtained with both kinds of preparations. DCMU does not inhibit NADPH oxidation in the light; rotenone weakly inhibits. DNP-INT (added prior to NADPH), DBMIB and HOQNO strongly inhibit, whereas antimycin A and KCN do not inhibit at all. We found no inhibition by DNP-INT

Fig. 3. DNP-INT inhibition of electron transfer from NADPH to cyt *c*-553. Conditions were anaerobic; DNP-INT was added prior to NADPH in a concentration range of 10 nm–10 μM: ●, sonically derived membrane vesicles; ★, osmotically shocked spheroplasts. Further details in Materials and Methods.

TABLE 1

RELATIVE RATES OF PS-I-MEDIATED OXIDATION OF NADPH AND NADH IN THE LIGHT

Either sonically derived membrane vesicles (A) or osmotically shocked spheroplasts (B) were used. Experiments were done by spectrophotometry under completely anaerobic conditions with 715 nm actinic light and methyl viologen as terminal electron acceptor. The NADPH and NADH concentrations were 0.1 mM. Control rates of NADPH oxidation varied between 60 and 170 nmol/mg Chl *a* per min in membrane preparations from different cultures; NADH was oxidized at a rate of 70 nmol/mg Chl *a* per min. Data represent mean ± S.D. for the indicated number of experiments. More details in Materials and Methods.

Electron donor	Addition	Rate of NAD(P)H oxidation (% of control)	Number of experiments
(A) Sonicated membrane vesicles			
NADPH	none	100 ± 12	5
	DCMU (10 μM)	101 ± 15	4
	rotenone (50 μM)	65, 72	2
	DNP-INT (1 μM)	101 ± 26	3
	DNP-INT (1 μM) [a]	0 ± 20	3
	DNP-INT (1 μM) [a], O_2	85 ± 16	3
	DBMIB (10 μM)	0 ± 15	5
	HOQNO (50 μM)	40	1
	antimycin A (10 μM)	93 ± 7	4
	KCN (1 mM)	100	1
	ferredoxin (10 μM)	102, 106	2
	CSP [b] (10 μl·ml^{-1})	96, 103	2
NADH	none	100 ± 15	4
	antimycin A (10 μM)	53 ± 29	4
(B) Shocked spheroplasts			
NADPH	antimycin A (10 μM)	93 ± 16	3
NADH	antimycin A (10 μM)	32 ± 26	3

[a] DNP-INT added prior to NADPH.

[b] CSP, concentrated soluble proteins.

when it was added after the addition of NADPH or when O_2 was present. Neither added spinach ferredoxin, nor concentrated soluble proteins had any effect in this system, indicating that PS-I-mediated NADPH oxidation is a membrane-bound process, not dependent on cytoplasmic proteins. NADH also serves as an electron donor to PS I. Interestingly, the electron transfer was now sensitive to antimycin A in contrast to the NADPH-induced electron transfer. The difference in antimycin A sensitivity of NADPH and NADH oxidation was most prominent when using osmotically shocked spheroplasts. (Table I (B)). A similar effect of antimycin A was found on the rate of reduction of flash-oxidized cyt *c*-553 (not shown).

Effect of inhibitors on the dark respiration of NADPH, NADH and duroquinol

Table II lists the effect of several electron donors, electron acceptors and inhibitors on dark electron transfer reactions in sonically derived membrane vesicles. Unless indicated otherwise, similar results were obtained when using osmotically shocked spheroplasts.

DCMU does not inhibit NADPH-induced O_2 uptake, while rotenone inhibits to about the same

TABLE II

RELATIVES RATES OF NADPH AND NADH RESPIRATION

Experiments were done in the dark by spectrophotometry (S) or by polarography (O); either sonically derived membrane vesicles (A) or osmotically shocked spheroplasts (B) were used. NADPH oxidation rates (A) in preparations from different cultures (with acceptor O_2) varied between 20 and 60 nmol/mg chl *a* per min, with oxidized TMPD as acceptor the rates were about two times higher. NADH oxidation rates (A) varied between 10 and 25 nmol/mg chl *a* per min. The control rates of NADPH (B) of NADH (B) were 90 and 35 nmol/mg chl *a* per min, respectively. Further details in Materials and Methods.

Electron donor	Electron acceptor	Addition	Rate of NAD(P)H oxidation or O_2 reduction (% of control)	Method	Number of experiments
(A) Sonicated membrane vesicles					
NADPH (0.1 mM)	O_2	none	100 ± 10	S	5
		none	100 ± 12	O	5
		rotenone (50 μM) 58, 66	O	2	
		DNP-INT (1 μM)	98 ± 12	S	3
		DNP-INT [a] (1 μM)	94 ± 6	S	4
		DBMIB (10 μM)	140 ± 35	O	3
		antimycin A (10 μM)	94 ± 8	S, O	5
		KCN (1 mM)	10 ± 10	O	3
		antimycin A + KCN (1 MM)	14	O	1
NADPH (0.1 mM)	TMPD (0.1 mM)	none, anaerobic	200 ± 19	S	3
		anaerobic, DNP-INT (1 μM)	0 ± 10	S	3
		anaerobic, DNP-INT (1 μM) [a]	0 ± 10	S	3
		DNP-INT (1 μMO) [a], O_2	132 ± 12	S	4
NADH (0.1 mM)	O_2	none	100 ± 10	S, O	5
		antimycin A (10 μM)	48 ± 16	O	3
		KCN (1 mM)	12	O	1
		antimycin A (10 μM), KCN (1 mM)	15 ± 10	S, O	5
(B) Shocked spheroplasts					
NADPH (0.1 mM)	O_2	none	100 ± 12	O	5
		antimycin A (10 μM)	96 ± 10	O	4
		KCN (1 mM)	48 (20–70) [b]	S, O	5
NADH (0.1 mM)	O_2	none	100 ± 12	O	4
		antimycin A (10 μM)	26 ± 16	O	5
		KCN (1 mM)	40, 65	O	2

[a] DNP-INT was added prior to NADPH.

[b] Lowest and highest of five separate measurements.

extent as in the oxidation in the light (cf. Table I). DNP-INT does not inhibit in the presence of O_2, independent of its addition prior to or after NADPH. However, electron transfer from NADPH to oxidized TMPD in the dark under anaerobic conditions is completely inhibited by DNP-INT, the inhibition being abolished upon introduction of O_2. Remarkably, the order of addition of NADPH and DNP-INT does not influence the degree of inhibition with TMPD as the electron acceptor in contrast to the results presented in Table I. We therefore conclude that dark reduction of O_2 by NADPH in *P. boryanum* may proceed prior to the DNP-INT-sensitive site and that a relatively oxidized DNP-INT binding site is required for its inhibition. Addition of DBMIB to NADPH-respiring membrane vesicles even results in a stimulation of O_2 uptake, presumably due to autooxidation of reduced DBMIB as was also reported by Houchins and Hind [12]. However, with reduced DQH_2 as electron donor DBMIB completely inhibited O_2 uptake (not shown). Antimycin A hardly inhibits NADPH oxidation; with KCN present in addition, about the same inhibition was found as with KCN only. NADH oxidation is inhibited by antimycin A to about the same extent as in the oxidation in the light (cf. Table I), KCN inhibits O_2 uptake in this case as well. The different effects of antimycin A in NADPH and NADH respiration are again more pronounced in osmotically shocked spheroplasts. Most remarkably the KCN inhibition of NADPH and NADH respiration is far less in the osmotically shocked spheroplasts than in the sonicated preparations. Thus, when the thylakoid and cell membranes are more or less separated KCN inhibition of respiration is less complete.

Discussion

In the work presented we have focused on the pathways of NADPH and NADH oxidation mediated by electron transfer to PS I or to O_2 in cell-free membrane preparations of *P. boryanum*. Evidence that both NADPH and NADH are effective electron donors to flash-oxidized cyt *c*-553 (Fig. 2) supports the results of Hirano et al. [7] who showed that endogenous catabolism is able to supply reducing compounds for reduction of flash-oxidized cyt *c*-553 in intact cells of *Synechococcus lividus*. Furthermore, Peschek and coworkers have recently shown that plastoquinol-cytochrome *f*/*b*-563 reductase is a common electron donor to P-700 and cytochrome *c*:O_2 oxidoreductase in cyanobacteria. The rate of cyt *c*-553 reduction is markedly influenced by the dissolved O_2 concentration; O_2 saturation nearly suppressed this reduction. This is indicative for a close association between electron transfer from NADPH to PS I and to O_2 in the initial redox steps. O_2 seems to regulate the diversion of reducing equivalents from the photosynthetic electron transfer chain; a branching point seems to be situated prior to the DBMIB and DNP-INT sensitive sites. Fig. 3 of the accompanying paper [32] shows a tentative scheme of electron transfer pathways in *P. boryanum*.

Our results also show that in PS-I-mediated NADPH oxidation, electrons are accepted at the thylakoids beyond the DCMU-sensitive site via a weakly rotenone-sensitive dehydrogenase (Table I). Similar observations are evident when O_2 is the terminal electron acceptor (Table II). The results are suggestive for a common NADPH dehydrogenase site for PS-I- and O_2-mediated NADPH oxidation. The NADPH dehydrogenase may be analogous to the NADH dehydrogenase at the thylakoids of the green alga *Chlamydomonas reinhardii* CW-15, described by Godde and Trebst [33]. NADPH electron donation to the thylakoids is not dependent on added ferredoxin. This observation makes it unlikely that NADPH electron donation to the thylakoids is proceeding via the ferredoxin-dependent cyclic pathway as is present in chloroplasts [42,43]. The involvement of possibly retained ferredoxin should not be excluded, however (Böhme, H., personal communication). Because NADPH oxidation in the light via PS I is highly sensitive to DNP-INT (K_i of about 50 nM, Fig. 3) and DBMIB (Table I), it is likely that electrons are donated to the photosynthetic electron transfer chain prior to PQ [31,36]. In marked contrast, dark respiratory electron transfer from NADPH to O_2 is not inhibited. However, in spinach chloroplasts [31] or in *Aphanocapsa* [37], O_2 does not affect the inhibition of electron transfer by DNP-INT. Yet, DNP-INT inhibits electron transfer in the dark as is demonstrated when

oxidized TMPD serves as electron acceptor instead of O_2. Moreoever, electron donation from duroquinol to O_2 is inhibited by DBMIB, demonstrating participation of the Qbc redox complex. We conclude from these results that with NADPH as electron donor, two pathways of O_2 reduction may be used. One is situated near the NADPH dehydrogenase, and prior to the DNP-INT-sensitive site, and is presumably (partly) inducible in vitro. The other one is situated beyond the DBMIB-sensitive site and is highly KCN-sensitive and may be used in in vivo respiration. The site of O_2 reduction near the NADPH dehydrogenase may be analogous to the alternative oxidase present in higher plant mitochondria [38] and also found in *Anacystis nidulans* [20]. The use of the highly KCN-sensitive oxidase in NAD(P)H respiration could clearly be detected in sonically derived membrane vesicles, but its use in osmotically shocked spheroplasts was very much reduced. Thus, the presumed scrambling of the thylakoid and cell membranes in the former preparations also enhances respiratory electron transfer via the highly KCN-sensitive oxidase. In the osmotically shocked spheroplast preparations the initial steps of respiratory electron transfer and the highly KCN-sensitive oxidase seem to be physically separated, suggesting that these activities are localized at the thylakoid and cell membranes, respectively.

NADH also serves as a respiratory substrate in cell-free membrane preparations of several cyanobacteria [19,20,37] and in addition, is able to reduce flash-oxidized cyt *c*-553 and PS I (Tables I, II). NADH oxidation by O_2 in cell-free membrane preparations was studied by Leach and Carr [19] who found antimycin A to inhibit this reaction for about 60% in *Anabaena variabilis* and by Peschek [20] and recently by Sandmann and Malkin [37] who did not observe any inhibition in *Anacystis nidulans* and *Aphanocapsa*, respectively. In contrast to the almost antimycin-A insensitive NADPH oxidation, we found antimycin A to inhibit NADH oxidation for about 50% and even up to 80% in osmotically shocked spheroplast preparations both in the light and in the dark. The decreased inhibition in the sonicated preparations may be caused by a decreased specificity of the dehydrogenases for NADPH and NADH after sonication.

NADH seems to donate its reducing equivalents at a site different from NADPH. Although the antimycin A effects on photosynthetic electron transfer are not well-defined so far, it seems likely that cyt *b*-563 involved in cyclic electron flow is the primary inhibition target [39]. The NADH dehydrogenase, also shown to be rotenone-sensitive [19], may then donate to the Qbc redox complex from the 'cyclic' rather than the 'linear' end.

The terminal steps of NADPH respiration involving the highly KCN-sensitive oxidase may be localized at the thylakoid membrane according to Binder [10], at a membrane region different from that bearing the photosynthetic chain as described by Scherer et al. [13,18], or also at the cell membrane, as suggested by Peschek et al. [2,6,14,17]. At this point, we want to emphasize that cell disrupture will easily lead to vesicles consisting of partially separated, associated or scrambled thylakoid and cell membrane fragments. Thus, in order to arrive at conclusions concerning the localization of the highly KCN-sensitive terminal oxidase, the method of cell-free membrane preparation is of crucial importance.

In the present study we have shown that the first steps of NADPH and NADH respiration are restricted to the thylakoid membranes and that the highly KCN-sensitive oxidase may be physically separated from the thylakoid membranes. In the accompanying paper, we will enlarge on this aspect; a scheme of electron transfer pathways will be presented.

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References

1 Stanier, R.Y. (1977) Carlsberg Res. Commun. 42, 77–98
2 Peschek, G.A. (1984) in Subcellular Biochemistry, Vol. 10 (Roodyn, B.D., ed.), pp. 83–189, Plenum Press, New York
3 Brown, A.H. and Webster, G.C. (1953) Am. J. Bot. 40, 753–757
4 Biggins, J. (1969) J. Bacteriol. 99, 570–575
5 Bisalputra, T., Brown, D.L. and Weier, T.E. (1969) J. Ultrastruct. Res. 27, 182–197
6 Peschek, G.A., Schmetterer, G., Lockau, W. and Sleytr, U.B. (1981) In Photosynthesis. V. Chloroplasts Develop-

ment (Akoyunoglou, G., ed.), pp. 707–719, Balaban International Science Services, Philadelphia, PA
7 Hirano, M., Satoh, K. and Katoh, S. (1980) Photosynth. Res. 1, 149–162
8 Metzler, H. (1981) Arch. Microbiol. 129, 123–126
9 Eisbrenner, G. and Bothe, H. (1979) Arch. Microbiol. 123, 37–45
10 Binder, A., Bohler, M.C., Wolf, M. and Muster, P. (1981) in Energy Coupling in Photosynthesis. Developments in Biochemistry, Vol. 20, (Selman, B.R. and Selman-Reimer, S. eds.), pp. 313–322, Elsevier/North-Holland, Amsterdam
11 Peschek, G.A., Muchl, R., Kienzl, P.F. and Schmetterer, G. (1982) Biochim. Biophus. Acta 679, 35–43
12 Houchins, J.P. and Hind, G. (1982) Biochim. Biophys. Acta 682, 86–96
13 Scherer, S., Stürzl, E. and Böger, P. (1982) Arch. Microbiol. 132, 333–337
14 Peschek, G.A., Schmetterer, G. and Sleytr, U.B. (1981) FEMS Microbiol. Lett. 11, 121–124
15 Peschek, G.A. and Schmetterer, G. (1982) Bioch. Biophys. Res. Commun. 108, 1188–1195
16 Peschek, G.A. (1983) Biochem. J. 210, 269–272
17 Peschek, G.A., Schmetterer, G., Lauritsch, G., Muchl, R., Kienzl, P.F. and Nitschmann, W.H. (1983) in Photosynthetic Prokaryotes. Cell Differentiation and Function (Papageorgiou, G.C. and Packer, L., eds.), pp. 147–162, Elsevier Science Publishers, Amsterdam
18 Scherer, S., Stürzl, E. and Böger, P. (1981) Z. Naturforsch. 36c, 1036–1040
19 Leach, C.K. and Carr, N.G. (1970) J. Gen. Microbiol. 64, 55–70
20 Peschek, G.A. (1980) Arch. Microbiol. 125, 123–131
21 Peschek, G.A. (1980) Biochem. J. 186, 515–523
22 Lockau, W. (1981) Arch. Microbiol. 128, 336–340
23 Peschek, G.A. (1981) Biochim. Biophys. Acta 635, 470–475
24 Peschek, G.A., Schmetterer, G., Lauritsch, G., Nitschmann, W.H., Kienzl, P.F. and Muchl, R. (1982) Arch. Microbiol. 131, 261–265
25 Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y. (1979) J. Gen. Microbiol. 111, 1–61
26 Matthijs, H.C.P., Scholts, M.J.C. and Schreurs, H. (1981) in Photosynthesis II. Photosynthetic Electron Transport and Phosphorylation (Akoyunoglou, G., ed.), pp. 269–278, Balaban International Science Services, Philadelphia, PA
27 Binder, A., Tel-Or, E. and Avron, M. (1976) Eur. J. Biochem. 67, 187–196
28 Kraayenhof, R., Schuurmans, J.J., Valkier, L.J., Veen, J.P.C., Van Marum, D. and Jasper, C.G.G. (1982) Anal. Biochem. 127, 93–99
29 Schuurmans, J.J., Peters, A.L.J., Leeuwerik, F.J. and Kraayenhof, R. (1981) in Vestorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F., Quagliariello, E., Siliprandi, N. and Slater, E.C., eds.), pp. 359–369, Elsevier/North-Holland, Amsterdam
30 Schuurmans, J.J., Leeuwerik, F.J., Siu Oen, B. and Kraayenhof, R. (1981) in Photosynthesis I. Photophysical Processes Membrane Energization (Akoyunoglou, G. ed.), pp. 543–552, Balaban International Science Services, Philadelphia, PA
31 Binder, R.G. and Selman, B.R. (1980) Biochim. Biophys. Acta 592, 314–322
32 Matthijs, H.C.P., Ludérus, E.M.E., Scholts, M.J.C. and Kraayenhof, R. (1984) Biochim. Biophys. Acta 766, 38–44
33 Godde, D. and Trebst, A. (1980) Arch. Microbiol. 127, 245–252
34 Mills, J.D., Crowther, D., Slovacek, R.E., Hind, G. and McCarty, R.E. (1979) Biochim. Biophys. Acta 547, 127–137
35 Peters, F.A.L.J., Van Spanning, R. and Kraayenhof, R. (1983) Biochim. Biophys. Acta 724, 159–165
36 Böhme, H., Reimer, S. and Trebst, A. (1971) Z. Naturforsch. 26b, 341–352
37 Sandmann, G. and Malkin, R. (1983) Biochim. Biophys. Acta 725, 221–224
38 Laties, G.G. (1982) Annu. Rev. Plant Physiol. 33, 519–555
39 Gimmler, H. (1977) in Encyclopedia of Plant Physiol. New Series, Vol. 5, Photosynthesis I. Electron Transport and Photophosphorylation (Trebst, A. and Avron, M. eds.), pp. 456–458, Springer-Verlag, Berlin