

Bioenergetics Studies of the Cyanobacterium *Anabaena variabilis*

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A cell-free system exhibiting both photophosphorylation ($P/2e = 1$) and oxidative phosphorylation (P/O up to 0.8) is described for the cyanobacterium *Anabaena variabilis*. NADH and NADPH were found to be equally effective as electron donors for oxidative phosphorylation. Low concentrations of UHDBT, an inhibitor of the cytochrome b/c complex of mitochondria and chloroplasts, were found to inhibit photosystem-II electron transport reactions, but did not affect the cytochrome b_6/f -complex of *Anabaena*. The inhibition by myxothiazol, antimycin and heptylhydroxyquinoline corroborates the hypothesis that both respiration and photosynthesis share the cytochrome b_6/f -complex.

Introduction

Cyanobacterial photosynthetic electron transport is similar to that of higher plants and proceeds with a rate 10 to 20 times as fast as respiratory electron transport [1–3]. This may explain the limited attention given to respiration studies in cell-free cyanobacterial systems (for review see ref. [4]). Oxygen-dependent ATP synthesis with *intact* cyanobacterial cells has been demonstrated for several species with a P/O ratio of up to 3 in *Anacystis* (*Synechococcus*) and *Mastigocladus* [5–8] which is close to the figure expected from the established knowledge of mitochondrial bioenergetics. The few reports on oxidative phosphorylation of a cyanobacterial cell-free system document very low activities [9–12]. Respiratory electron transport in cyanobacteria seems to be localized predominantly on the thylakoid membrane, although a transmembrane electron transport in the cytoplasmic membrane was reported for some species [4]. The interaction of respiratory and photo-

synthetic electron transport has been investigated by several groups, resulting in the hypothesis that plastoquinone, the cytochrome b_6/f -complex and cytochrome $c-553$ or plastocyanin are used for both electron transport systems (for review see ref. [13]).

In this paper a membrane preparation of *Anabaena variabilis* is described as being active in oxidative phosphorylation having both a comparatively high rate and P/O ratio. This preparation is used to examine the activity of some inhibitors directed to the cytochrome b_6/f -complex with respect to the interaction of photosynthetic and respiratory electron transport.

Materials and Methods

Anabaena variabilis ATCC 29413 was grown with air nitrogen enriched with 1.5% CO_2 (v/v) at 30 °C as reported [14] and harvested after 40 to 50 h of growth in the late logarithmic phase. Spheroplasts were prepared according to [15] with 5–8 mg lysozyme per mg chlorophyll for 1 h at 34 °C. After washing the spheroplasts (centrifugation for 1 min at $3000 \times g$), photosynthetic and respiratory electron transport were measured after an osmotic shock of the spheroplasts in the medium described below for phosphorylation, using a Clark-type oxygen electrode. The protein: chlorophyll ratio of the spheroplasts was 31:1 (w/w). Electron-transport measurements given in Fig. 1 were performed with membranes prepared by a French-press treatment in a buffer system described by Stürzl *et al.* [16]. Phosphorylation was measured under continuous shaking of the reaction vessels either in white light ($140 \mu E/m^2 \times s$) or in the dark at 25 °C. The 1-ml final re-

Abbreviations: ATCC, American Type Culture Collection; BSA, bovine serum albumin; CCCP, carbonylcyanide-*m*-chlorophenylhydrazine; Chl, chlorophyll *a*; DAD, 3,6-diaminodurene; DHQ, duroquinol (tetramethyl-*p*-hydroquinone); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ, 2,5-dimethyl-*p*-benzoquinone; FeCy, potassium ferricyanide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-N-oxide; MBq, megabequerel; MV, methylviologen (1,1'-dimethyl-4,4'-bipyridilium dichloride); PMS, N-methylphenazonium methosulfate; SF-6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole.

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action medium contained 2 mM ADP; ^{32}P equivalent to approximately 0.1 MBq; 10 mM MgCl_2 ; 3 mM KH_2PO_4 – K_2HPO_4 /3 mM NaH_2PO_4 – Na_2HPO_4 , pH 7.8; 10 mM tricine/KOH, pH 7.8 and 0.1% (w/v) BSA. The concentration of inorganic phosphate varied between 8–18 mM; this variation had no influence on the rates. Further additions are given in tables and legends. The reaction was started by adding the spheroplasts yielding a final concentration of 30 μg Chl/ml and stopped with 50 μl of 60% (v/v) HClO_4 after a 3-min reaction time.

Labeled ATP was extracted according to Avron [17] and Almon and Böhme [18], quantified by liquid-scintillation counting (Rackbeta II 1215, LKB, Sweden) using Cerenkov radiation.

SF-6847 was purchased from Wako Chemicals, Japan, others from Serva, Heidelberg, FRG (NADH, NADPH, methylviologen); Sigma, Deisenhofen, FRG (nigericin, CCCP) and Amersham Buchler, Braunschweig, FRG (^{32}P).

Results and Discussion

ATP-synthesis in the light was found linear over 3 min for the systems $\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$ and PMS/ascorbate. To characterize the preparation the effect of inhibitors on photophosphorylation are shown in Table I. The results are in accordance with those from cell-free higher plant photophosphorylation. Parallel measurements of linear electron transport

Table I. Influence of electron-transport inhibitors (lines 2 and 6) and uncouplers (lines 3–5) on linear and cyclic photophosphorylation exhibited by shocked spheroplasts of *Anabaena variabilis*.

Additions	Photophosphorylation — electron flow —	
	linear	cyclic
	$\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$	PMS/ascorbate
1 Control	143–170	380–394
2 DCMU, 10 μM	11	371
3 CCCP, 50 μM	0	14
4 Nigericin, 10 μM	14	47
5 SF-6847, 5 μM	24	8
6 UHDBT, 10 μM	50	342

Data are from three different preparations, the variance is given as the range in the control values. Figures represent $\mu\text{mol ATP}/\text{mg Chl} \times \text{h}$. For uncoupling properties of SF 6847 see [31]. For concentrations of the components of the electron-flow reaction systems see Table IV, PMS was 50 μM .

($\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$) under the same conditions yielded P/2e ratios around 1 (data not shown). Thus the results are in good agreement with data reported previously for other cyanobacterial membrane preparations [2, 11, 15]. Evidently, the photophosphorylative capacity of the membranes used has not been substantially affected by preparation of the reaction system.

Oxidative phosphorylation of this membrane preparation was assayed with NADH and NADPH as (physiological) electron donors [9, 16, 19, 20]. A certain rate of endogenous ATP-synthesis was found, which could be stimulated by NAD(P)H by 50 to 100%. Washing the membranes decreased the endogenous rate considerably enhancing the stimulatory effect of the reduced pyridine nucleotides (data not shown). Washing decreased the overall activity by depleting the membranes of either cytochrome *c* [21] or the NADPH-oxidizing enzyme, which is thought to be the ferredoxin-NADP⁺ oxidoreductase [22]. Therefore, no washing step was included after shocking the spheroplasts. In Table II, the influence of CCCP and KCN on oxidative phosphorylation is shown. CCCP inhibited both NADH- and NADPH-dependent activity to approx. 60 to 70%. The inhibition by cyanide was found in the same range. The formation of labeled adenine nucleotides in the presence of CCCP presumably was due to an ADP/P_i-exchange independent of electron transport, as has been shown with a membrane preparation of the cyanobacterium *Mastigocladus* [11]. Rates of oxidative phosphorylation obtained with NAD(P)H using membranes of *Anabaena* [9] or *Plectonema* [12] have been reported significantly lower than the figures in Table II. In our preparation, NADPH was not

Table II. Oxidative phosphorylation with NADH and NADPH as electron donors using a membrane preparation of *Anabaena variabilis*.

Additions	Oxidative phosphorylation	
	NADH	NADPH
Control	8.5 ± 1.3	8.1 ± 2.0
CCCP, 50 μM	3.2 ± 0.2	2.7 ± 0.9
KCN, 1 mM	3.1 ± 0.7	3.8 ± 0.2
UHDBT, 10 μM	8.5 ± 1.8	7.4 ± 2.1

Average values are given, the standard deviation based on 4 to 6 different preparations. Figures represent $\mu\text{mol ATP}/\text{mg Chl} \times \text{h}$. NADH and NADPH concentration was approximately 0.5 mM, controlled by absorbance at 340 nm.

superior to NADH in contrast to several data on electron transport in the literature [18–21]. We assume that this is due to the membrane-bound NADPH-oxidizing enzyme [22], which is partially soluble and results in preparations of different NADPH-driven phosphorylation activity.

P/O ratios are important data to characterize the membrane preparation. For this purpose, NAD(P)H-dependent oxygen uptake was measured parallel to oxidative phosphorylation (Table III). The highest P/O ratios were about 0.8 to 0.9, which is up to four-fold higher than the previously reported ratios [9, 11, 12]. These figures, however, are far below the P/O ratios of 1.5 to 3 determined in the cellular system [6–8], although the photophosphorylative capacity of the membrane preparation is intact as being judged by the P/2e ratios of 1 obtained in the light. Higher instability of oxidative phosphorylation as compared to photophosphorylation has been reported for *Plectonema* [12] and *Mastigocladus* [11]. This different activity may be taken as evidence that both energy-conserving systems are localized on different membranes (the thylakoid membrane exhibiting photosynthetic, the cytoplasmic membrane respiratory electron transport). The data can also be explained by a small, but unavoidable proton leakage of the isolated membrane vesicles, which can be overcome by the high proton-pumping capacity of the photosynthetic electron transport but not by the small proton fluxes induced by the low rates of respiratory electron transport. This situation would lead to a substantial decrease of P/O ratios in the dark but not in the light.

In our experiments no significant difference was found between the P/O ratios of NADH- and

NADPH-supported respiration of shocked spheroplasts (Table III), which was also observed in control experiments using washed membranes (data not shown). Accordingly, no evidence emerges from these data that electron transport from NADPH and NADH to oxygen includes different energy-conserving sites, although different dehydrogenases are involved (ref. 16, 22; I. Alpes, this laboratory, unpublished results).

It has been suggested that photosynthetic and respiratory electron transport share a common segment including the cytochrome *b₆/f*-complex (for review see refs. [4, 13, 23]). Antimycin A, myxothiazol and heptylhydroxyquinoline, known as strong inhibitors of the cytochrome *b/c* or cytochrome *b₆/f*-complex [24, 25], exhibited no differential influence on photosynthetic and respiratory electron transport (Fig. 1, A–C), which is in accordance with a dual role of the *b₆/f*-complex. UHDBT, however, reported to be active in both chloroplasts and mitochondria [24], revealed a strong impact on linear photosynthetic electron flow with little effect on NADH-dependent dark oxygen uptake (Fig. 1D). To check whether this tolerance of respiration is due to an autooxidation of UHDBT in the dark (indeed, at higher concentrations oxygen uptake is stimulated), the inhibition of oxidative- and photophosphorylation by UHDBT was determined (Tables I and II). Respiratory activities were practically not inhibited, no inhibition was found for cyclic PMS-mediated photophosphorylation either. Linear photophosphorylation was inhibited substantially ($\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$) as was found for electron transport (comp. Fig. 1D). These results show that the cytochrome complex responsible for respiration in *Anabaena* is not affected by UHDBT. This can be explained either by two different cytochrome complexes used for photosynthetic and respiratory electron transport, or by the action of UHDBT directed to another target than the cytochrome *b₆/f*-complex in linear photosynthetic electron transport.

To obtain some insight photosynthetic electron transport was measured with different donor → acceptor systems (Table IV), which represent several partial redox reactions. Photosystem I activity, with the cytochrome *b₆/f*-complex either excluded ($\text{DAD}/\text{ascorbate} \rightarrow \text{MV}/\text{O}_2$), or included ($\text{DHQ} \rightarrow \text{MV}/\text{O}_2$; compare ref. [26]) was only slightly inhibited by 10 μM UHDBT, while linear electron transport ($\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$) was inhibited to 58% of the control, and the

Table III. P/O ratios of NADH- and NADPH-supported respiration in *Anabaena variabilis*.

Activities	Electron donor	
	NADH	NADPH
Oxygen uptake	3.1 – 5.1	2.6 – 5.2
Oxidative phosphorylation	2.5 – 5.6	3.5 – 6.3
P/O ratio (means \pm σ)	0.52 \pm 0.19	0.55 \pm 0.16

Only cyanide-sensitive activities are given. Data are means from 8 preparations and 31 determinations. Oxygen uptake is expressed as $\mu\text{mol O}_2/\text{mg Chl} \times \text{h}$, oxidative phosphorylation as $\mu\text{mol ATP}/\text{mg Chl} \times \text{h}$. For concentrations of reduced pyridine nucleotides see legend of Table II. σ = standard deviation.

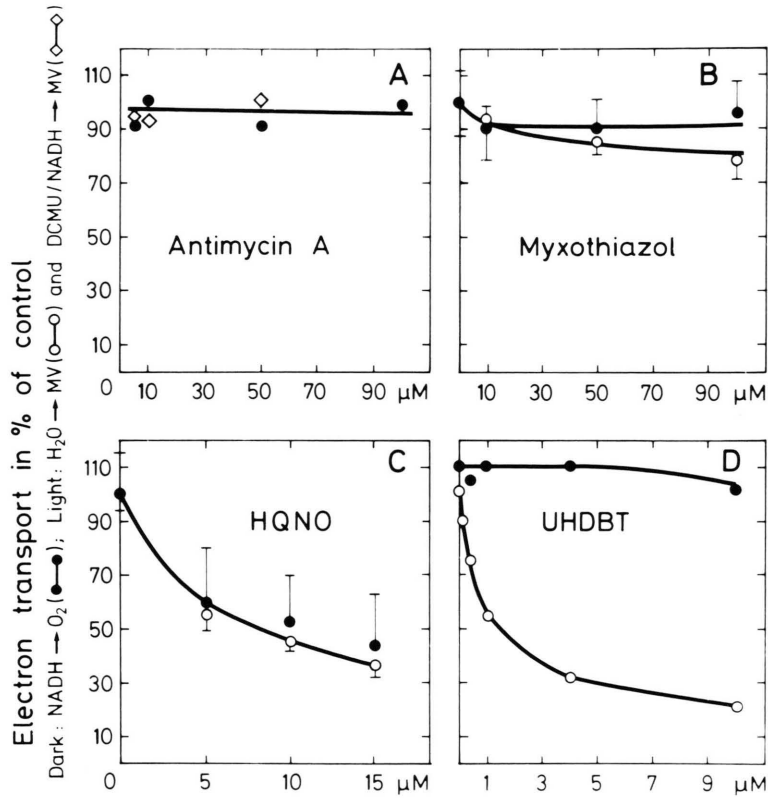


Fig. 1. Effect of four inhibitors of the membrane-bound cytochrome complex assaying linear photosynthetic and respiratory electron transport of membranes from *Anabaena variabilis*, prepared by French-press treatment. 100% corresponds to a rate of 40–60 ($\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$, $\circ-\circ$), 6–13 ($\text{NADH} \rightarrow \text{MV}/\text{O}_2$, $\bullet-\bullet$), and 2–3 ($\text{NADH} \rightarrow \text{O}_2$, in the dark, corrected for cyanide-insensitive oxygen uptake, $\bullet-\bullet$), expressed as $\mu\text{mol O}_2$ uptake/mg chlorophyll \times hour. MV, 0.1 mM, for other components see Table IV, concentrations of reduced pyridine nucleotides as in Table II. The light-induced NADH oxidation in the presence of DCMU (Fig. 1A) was in some, but not all, preparations stimulated by methylviologen, which was, therefore, added routinely. The antimycin insensitivity was found with and without methylviologen.

photosystem II reaction ($\text{H}_2\text{O} \rightarrow \text{DMBQ}/\text{FeCy}$) was blocked completely by this inhibitor concentration. By uncoupling (with 5 mM NH_4Cl present), the electron transport from either $\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$ or $\text{DHQ} \rightarrow$

MV/O_2 was stimulated two-fold; the action of UHDBT in the uncoupled system was similar to the coupled system. Conclusively, UHDBT inhibits photosystem II, as has been suggested previously for

Table IV. Shocked spheroplasts from *Anabaena variabilis*: Inhibition by UHDBT of light-induced electron transport and partial reactions.

Donor \rightarrow acceptor, redox system	Rates in $\mu\text{eq}/\text{mg Chl} \times \text{h}$		
	Control	(+) UHDBT	(+) UHDBT, rate in % of control
$\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$	323	136	42
$\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$; NH_4Cl	665	102	31
$\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$; DCMU	11	n.d.	n.d.
$\text{H}_2\text{O} \rightarrow \text{DMBQ}/\text{FeCy}$	1004	12	1
$\text{DHQ} \rightarrow \text{MV}/\text{O}_2$; DCMU	298	278	93
$\text{DHQ} \rightarrow \text{MV}/\text{O}_2$; DCMU, NH_4Cl	647	634	98
$\text{DAD}/\text{asc} \rightarrow \text{MV}/\text{O}_2$; DCMU	2732	2518	92

Concentrations used: UHDBT, 10 μM ; DAD, 0.5 mM; ascorbate, 5 mM; MV, 0.1 mM; DHQ, 0.25 mM; DMBQ, 0.5 mM; FeCy, 0.5 mM; DCMU, 10 μM ; NH_4Cl , 5 mM; n.d., not determined.

spinach [27]. In contrast to the findings with spinach [23, 27], the cytochrome *b₆/f*-complex of *Anabaena* is not inhibited (see also ref. [28]).

The mode of action of inhibitors, as assayed in this study with respect to electron transport as well as to oxidative- and photophosphorylation, is in accordance with the hypothesis that photosynthetic and respiratory electron transport share a common segment, which includes the cytochrome *b₆/f*-complex (comp. ref. [13, 29, 30]).

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