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PYRIDINE NUCLEOTIDES AND H_2 AS ELECTRON DONORS TO THE RESPIRATORY AND PHOTOSYNTHETIC ELECTRON-TRANSFER CHAINS AND TO NITROGENASE IN *ANABAENA* HETEROCYSTS

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The pathways through which NADPH, NADH and H_2 provide electrons to nitrogenase were examined in anaerobically isolated heterocysts. Electron donation in freeze-thawed heterocysts and in heterocyst fractions was studied by measuring O_2 uptake, acetylene reduction and reduction of horse heart cytochrome *c*. In freeze-thawed heterocysts and membrane fractions, NADH and H_2 supported cyanide-sensitive, respiratory O_2 uptake and light-enhanced, cyanide-insensitive uptake of O_2 resulting from electron donation to O_2 at the reducing side of Photosystem I. Membrane fractions also catalyzed NADH-dependent reduction of cytochrome *c*. In freeze-thawed heterocysts and soluble fractions from heterocysts, NADPH donated electrons in dark reactions to O_2 or cytochrome *c* through a pathway involving ferredoxin:NADP reductase; these reactions were only slightly influenced by cyanide or illumination. In freeze-thawed heterocysts provided with an ATP-generating system, NADH or H_2 supported slow acetylene reduction in the dark through uncoupler-sensitive reverse electron flow. Upon illumination, enhanced rates of acetylene reduction requiring the participation of Photosystem I were observed with NADH and H_2 as electron donors. Rapid NADPH-dependent acetylene reduction occurred in the dark and this activity was not influenced by illumination or uncoupler. A scheme summarizing electron-transfer pathways between soluble and membrane components is presented.

Introduction

In many filamentous cyanobacteria, vegetative cells can differentiate into heterocysts; the latter are specialized for their role as the site of N_2 fixation by these organisms during aerobic growth [1]. Photosystem II is eliminated during heterocyst differentiation, and these cells rely on fixed carbon, imported from adjacent vegetative cells, as a source of reductant for N_2 fixation [2].

Heterocysts have high activities of enzymes in the oxidative pentose phosphate pathway [3,4], and the NADPH generated by these reactions is presumed to be a major source of electrons for nitrogenase [5]. NADPH-supported acetylene reduction in the dark has been demonstrated, but rates were slow [6,7]. The immediate electron donor to nitrogenase is ferredoxin [8], but there is lack of evidence supporting the ability of NADPH to reduce ferredoxin directly at appreciable rates in vivo.

Although early reports found that NADH-generating enzymes of the glycolytic pathway were

Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DSPD, disalicylidenepropanediamine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HOQNO, 2-(*n*-heptyl)-4-hydroxyquinoline *N*-oxide; P-700, reaction center of Photosystem I; sulfo-DSPD, disulfodisalicylidenepropanediamine.

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absent from heterocysts [3,4], Bothe et al. [8] recently reported comparable levels of activity in heterocysts and vegetative cell extracts for the NAD-linked glyceraldehyde-phosphate dehydrogenase. The ability of NADH to react with the electron-transfer chains of heterocysts deserves further study.

This paper examines the pathways by which NADH and NADPH can donate electrons and compares these donors to H_2, which is known to provide electrons to the respiratory and photosynthetic electron-transfer chains and to nitrogenase in heterocysts [7,9].

Methods

8-l cultures of *Anabaena* sp. strain 7120 (ATCC 27893, *Nostoc muscorum*) were grown at 28°C in quarter-strength Allen and Arnon medium [10] containing 2 mM K_2HPO_4. Cultures were sparged with 0.5% CO_2 in air, and the final pH of the medium was 7.2. Continuous illumination was provided by six 15 W cool white fluorescent tubes giving an average intensity of 12 W/m^2 at the surface of the vessel. Cells were harvested at a density of 2–2.4 μg Chl/ml.

Heterocysts were isolated from freshly harvested filaments by a procedure similar to that of Peterson and Wolk [11] with the following exceptions. Cell wall digestion was carried out at pH 7.5 in 40 mM Hepes, 10 mM Na_2EDTA, and 4 mg/ml lysozyme. After 30 min, the cell suspension was centrifuged and resuspended in 40 mM Hepes, 1 mM $MgCl_2$, pH 7.5, containing 0.2 mg/ml DNAase. After a 5 min incubation, the suspension was sonicated in a sonic cleaning bath for 3–6 min to disrupt vegetative cells. Heterocysts were freed of vegetative cell debris by three centrifugations and washings in Hepes-$MgCl_2$ buffer. All operations were conducted under H_2. Isolated heterocysts were resuspended in buffer and stored at 0°C under Ar.

For experiments in which the use of freeze-thawed heterocysts is indicated, isolated heterocysts were subjected to three cycles of freezing in a dry ice/acetone bath and thawing at room temperature. This procedure greatly increased permeability to small hydrophilic molecules but allowed the release of only a small amount of cellular protein. Supernatant and membrane fractions from heterocysts were prepared from frozen heterocysts, which had been stored in liquid N_2, by breaking the cells with two passes through a French pressure cell at 138 MPa. The broken cell suspension was centrifuged at $2000 \times g$ for 5 min to remove unbroken cells and wall fragments. The whitish-green pellet was discarded and the supernatant was centrifuged at $45000 \times g$ for 1.5 h. The pellet from this centrifugation was used as heterocyst membranes. The supernatant was further centrifuged at $150000 \times g$ for 1 h, and the resulting supernatant was used as the soluble heterocyst extract. Membranes used in Table II were isolated anaerobically under H_2; cell extracts used in Table III–V were isolated under air.

Vegetative cell membranes were prepared by washing freshly harvested filaments with Hepes-EDTA buffer and resuspending the washed filaments in Hepes-$MgCl_2$ buffer containing 2 mg/ml lysozyme and 0.2 mg/ml DNAase. After a 30 min incubation at 30°C vegetative cells were broken with one pass through a French pressure cell at 40 MPa. Heterocysts and large wall fragments were removed by a 5 min centrifugation at $2000 \times g$. The supernatant was centrifuged at $45000 \times g$ for 90 min and the pellet from this centrifugation was resuspended in Hepes-$MgCl_2$ buffer and used as vegetative cell membranes.

Oxygen uptake was measured in an anaerobic chamber fitted with a combination O_2-H_2 electrode [12]. All reactions were conducted in 2 ml of Hepes-$MgCl_2$ buffer at 30°C. Where indicated reactions were illuminated with 900 W/m^2 of orange actinic light. The initial O_2 concentration was 30 μM for all assays. Stoichiometries of electron transfer were determined for the cyanide-sensitive dark reaction and for the cyanide-insensitive, light-dependent O_2-uptake reaction when H_2 was the electron donor: the dark, cyanide-sensitive reaction required four electrons/O_2 molecule consumed, and the light-dependent reaction required two electrons/O_2 molecule.

Horse heart cytochrome *c* can substitute for endogenous cytochrome *c*-553 in both respiratory and photosynthetic electron flow [13]. Its reduction was followed in a dual-wavelength spectrophotometer by measuring the absorbance change at 550 nm minus that at 540 nm, at 20°C. Samples

contained 25 μM cytochrome *c* in 1-ml Hepes-$MgCl_2$ buffer. Spinach ferredoxin, employed in the cytochrome *c* reduction assays, effectively substitutes for *Anabaena* ferredoxin in the assay of ferredoxin:NADP reductase from *Anabaena* [14].

For the measurement of nitrogenase activity (acetylene reduction), 1 ml heterocyst suspensions in Hepes-$MgCl_2$ buffer were incubated in 9-ml serum bottles at 30°C under 15% C_2H_2 (v/v) in Ar or H_2 as indicated. Illumination, when provided, was 500 W/m^2 of white (tungsten) light. A 0.5 ml gas sample was taken after 20 min and C_2H_4 content was quantitated by gas chromatography [15].

Chlorophyll was determined by extracting with 80% acetone and applying the extinction coefficient at 665 nm reported by Vernon [16]. Protein was assayed according to the method of Bradford [17]. A Chl/protein ratio of 0.014 was measured for isolated heterocysts. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and staining was conducted using the method of Chua and Bennoun [18].

Freeze-thawed heterocysts were provided with glucose 6-phosphate plus NADP, from which endogenous glucose 6-phosphate dehydrogenase generated NADPH. In all cases, reaction rates in freeze-thawed heterocysts with glucose 6-phosphate plus NADP were equal to or greater than those when NADPH was provided directly. NADPH was provided directly to all membrane and soluble fractions.

Cytochrome *c*-553 from *Phormidium luridum* was donated by Dr. H.W. Siegelman and was purified according to Ref. 14. Immunoglobulin (IgG) was prepared from antiserum raised in rabbit against spinach ferredoxin:NADP reductase, and was the generous gift of Dr. Nam-Hai Chua. Horse heart cytochrome *c* (type III) was purchased from Sigma. DBMIB and sulfo-DSPD were kindly given by Dr. A. Trebst. All other reagents were obtained from commercial sources.

Results

Freeze-thawed heterocysts

The isolated heterocysts used in this report are highly impermeable to small, hydrophilic molecules thus preventing the measurement of reactions requiring such exogenous additions. However, when these cells are subjected to three freeze-thaw cycles, permeability to small molecules greatly increases. Fig. 1 compares polypeptides from the soluble protein fraction of heterocysts with those from proteins released into the external medium before and after the freeze-thaw treatment. During the 3 h incubation after freezing, approx. 20% of the soluble heterocyst protein was

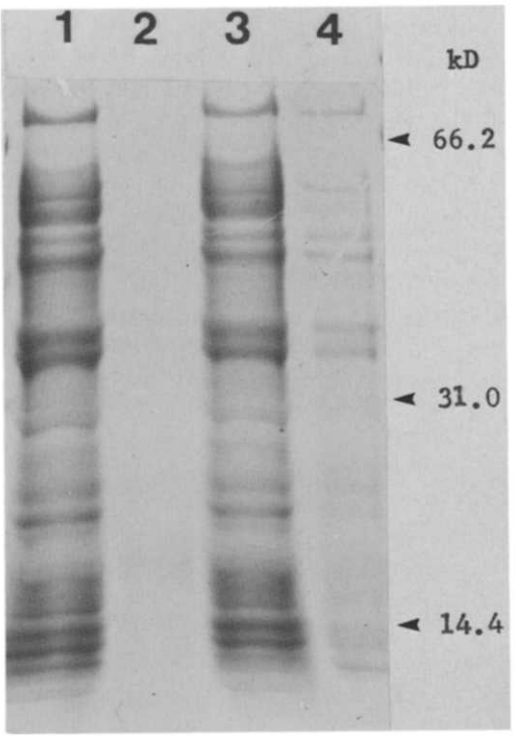

Fig. 1. Polypeptide composition resolved on sodium dodecyl sulfate polyacrylamide gel, of soluble extracts from heterocysts and of extracellular proteins before and after the freeze-thaw treatment. One half of a preparation of isolated heterocysts (250 μg Chl/ml) was subjected to three freeze-thaw cycles, the other was used as a control. After a 3 h incubation at 0°C, heterocysts were removed from both samples by centrifugation; these supernatants are designated as extracellular medium (lanes 2 and 4). The pelleted heterocysts were resuspended in a volume of buffer equal to that of the extracellular medium, broken with the French press and centrifuged at 45000 × *g* for 75 min. The 45000 × *g* supernatants were applied to the gel (lanes 1 and 3). Equal volumes of extract were applied to all lanes. Lane 1, soluble extract from untreated cells (48 μg protein); lane 2, extracellular medium from untreated cells (2 μg protein); lane 3, soluble extract from freeze-thawed cells (40 μg protein); lane 4, extracellular medium from freeze-thawed cells (14 μg protein). kD, kilodalton.

released into the medium. Protein release did not occur in the untreated sample. Fig. 1 reveals that the polypeptide composition of the released protein is similar to that of the protein retained within the cells, and that no preferential release of small protein molecules occurs.

Two possible sites for the reduction of O_2, using electrons from pyridine nucleotide, are: (1) at the cyanide-sensitive cytochrome a-a_3 respiratory complex [19] and (2) at the reducing side of Photosystem I, in a cyanide-insensitive light-driven reaction; therefore, O_2 uptake was measured in both dark and light, with and without cyanide.

Table I shows these and comparable data in which H_2, rather than pyridine nucleotide, was the electron donor. Both H_2 and NADH support cyanide-sensitive O_2 uptake in the dark. The level of KCN used in these experiments is sufficient to block cytochrome oxidase completely [19a]. After inhibition of this respiratory O_2 uptake with cyanide, significant light-dependent O_2 uptake occurred owing to its reduction by Photosystem I, and this activity was further enhanced by the addition of the autooxidizable mediator, methyl viologen. When NADPH was provided as electron donor, cyanide was much less inhibitory and illumination had little effect on O_2 uptake. Considerably more enhancement of O_2 uptake by methyl viologen was observed in this case than with H_2 or NADH as reductant. These results suggest that like H_2, NADH donates electrons both to the respiratory and photosynthetic electron-transfer chains. NADPH, in contrast, is oxidized primarily via a light-independent pathway capable of reducing the low-potential mediator, methyl viologen. This cyanide-insensitive, NADPH-dependent O_2 uptake is probably catalyzed by ferredoxin:NADP reductase.

Heterocyst membranes

Studies of electron-transport pathways catalyzed by a preparation of heterocyst membranes were made in order to establish the role of soluble carriers. Ferredoxin:NADP reductase is largely released during preparation of membranes [20], as is the soluble cytochrome c-553. The latter mediates

TABLE I

O_2 UPTAKE IN FREEZE-THAWED HETEROCYSTS

Complete reactions contained buffer, O_2, 2.6–6.6 μg Chl and an electron donor. Other concentrations were: 20 μM H_2, 1 mM NADH, 25 μM KCN, 0.5 mM methyl viologen. NADPH was provided as 1 mM NADP plus 3 mM glucose 6-phosphate. Rates are corrected for O_2 uptake in the absence of added electron donors. Control dark rates were between 0.66 and 1.3 and light rates were between 5.8 and 16 μmol O_2 consumed/mg Chl per h.

Electron donor	Additions	Rate (μmol O_2 consumed/mg Chl per h)	
		Dark	Light
H_2	none	250	250
	KCN	46	96
	KCN + methyl viologen	40	220
NADH	none	72	94
	KCN	21	78
	KCN + methyl viologen	28	100
NADPH	none	190	170
	KCN	130	120
	KCN + methyl viologen	860	880

TABLE II

O_2 UPTAKE BY HETEROCYST MEMBRANES

Complete reactions contained buffer, O_2, 0.5 mM methyl viologen, an electron donor and 38 μg Chl as heterocyst membranes. When present, NADPH concentration was 1 mM and cytochrome c-553 from *Phormidium luridum* was 5 μM. Other concentrations are as in Table I. The rates have been corrected for O_2 uptake which occurred when no electron donor was provided.

Donor	Additions	Rate (μmol O_2 consumed/mg Chl per h)	
		Dark	Light
H_2	none	59	100
	cytochrome c-553	61	250
	cytochrome c-553 + KCN	6.6	220
NADH	none	10	30
	cytochrome c-553	11	55
	cytochrome c-553 + KCN	3.0	61
NADPH	none	26	27
	cytochrome c-553	26	25
	cytochrome c-553 + KCN	30	26

electron flow between cytochrome *f* and both oxidized P-700 and cytochrome oxidase [13].

Table II shows that in heterocyst membranes, H_2 and NADH again behaved similarly with respect to cyanide sensitivity and light enhancement of O_2 uptake, and with either of these donors the addition of cytochrome *c*-553 increased the rate of O_2 consumption in the light. *Phormidium luridum* cytochrome *c*-553 reacts rapidly with P-700 but slowly with cytochrome oxidase from *Anabaena* (unpublished data); this explains its failure to stimulate respiration. NADPH-dependent O_2 uptake was not inhibited by cyanide, and illumination or the addition of cytochrome *c*-553 did not enhance the rate. Approx. 97% of the NADPH-dependent O_2 uptake was eliminated in the membrane fraction, confirming that this reaction is catalyzed by a soluble enzyme. NADPH is incapable of donating electrons to the membrane-bound electron-transfer chains in this preparation.

The oxidation pathway for NADH in the membrane fraction was explored further to determine the comparative levels of activity in vegetative cells and heterocysts and the degree of specificity for NADH over NADPH. Electron transfer from NADH or NADPH to equine or cyanobacterial cytochrome *c*, catalyzed by heterocyst or vegetative cell membranes, provides a convenient assay for this activity, since NADH supplies electrons to both the respiratory and photosynthetic electron-transfer chains and cytochrome *c*-553 is an intermediate common to both [13]. Cyanide must be added, for in its absence no cytochrome *c* reduction occurs owing to reoxidation of the reduced cytochrome *c* by cytochrome oxidase in the membrane.

Table III shows that in heterocyst membranes, ferredoxin markedly improves the rate of equine cytochrome *c* reduction with NADPH as electron donor, but is without effect with NADH as donor. HOQNO inhibits electron flow in mitochondria at the level of cytochrome *b* [21] and appears to act similarly in heterocysts. NADH-dependent cytochrome *c* reduction is strongly inhibited by HOQNO. In contrast, NADPH-dependent cytochrome *c* reduction is not inhibited by HOQNO and thus does not involve cytochrome *b*. The enhancement of this reaction by added ferredoxin suggests mediation by ferredoxin:NADP reductase and a pathway external to the membrane. The rate of NADPH-dependent cytochrome *c* reduction could be decreased more than 80% by washing membranes with buffer containing 5 mM EDTA and 150 mM KCl, confirming that this activity is due to residual enzyme bound to the membranes. NADH-dependent cytochrome *c* reduction was not affected by this treatment (data not shown).

Vegetative cell membranes also have NADH: cytochrome *c* oxidoreductase activity although rates are less than in heterocyst membranes. Because vegetative cells have more chlorophyll molecules per electron-transfer chain than heterocysts [22], calculations of activity on a chlorophyll basis can be misleading. However, if specific activity is calculated based on the number of cytochrome *f* molecules present, heterocyst membranes are still about twice as active as vegetative cell membranes.

NADH-dependent cytochrome *c* reduction catalyzed by heterocyst membranes displayed Michaelis-Menten saturation kinetics with a K_m for NADH of 6.2 μM. Table IV shows the effect of a number of inhibitors on this reaction. Of the compounds tested, DCCD, HOQNO and DSPD were effective inhibitors, A DCCD-sensitive site near the plastoquinone pool has been identified in spinach thylakoids [23]. DSPD blocks electron flow in chloroplasts at or before plastocyanin [24]. Sulfo-DSPD, however, was not inhibitory at this

TABLE III

REDUCTION OF HORSE HEART CYTOCHROME *c* BY VEGETATIVE CELL AND HETEROCYST MEMBRANES

Reactions contained buffer, 25 μM cytochrome *c*, 25 μM KCN, 100 μM NADH or NADPH, 5–8 μg Chl, and where indicated 6 μM ferredoxin (from spinach) and 45 μM HOQNO.

Additions	Rate (μmol cytochrome *c* reduced/mg Chl per h)			
	Heterocysts		Vegetative cells	
	NADH	NADPH	NADH	NADPH
None	58	10	11	3.4
Ferredoxin	57	24	13	12
Ferredoxin +HOQNO	22	25	4.1	11

TABLE IV

INHIBITORS OF NADH-DEPENDENT CYTOCHROME *c* REDUCTION IN HETEROCYST MEMBRANES

Reaction mixtures were as in Table III. Inhibitors were added in small volumes of ethanol or (DSPD, sulfo-DSPD, amytal) dimethyl sulfoxide. Equivalent solvent volumes were added to control reactions. The control rate was 30 μmol cytochrome *c* reduced/mg Chl per h.

Inhibitor	None	HOQNO		DCCD		DSPD		DBMIB		Amytal	Rotenone	Sulfo-DSPD
Concentration (μM)	–	0.5	10	25	100	60	600	2	50	5000	200	600
Relative rate	100	64	34	70	10	60	13	117	431	97	74	103

site, as also shown in spinach thylakoids [24]. Rotenone and amytal, which block NADH oxidation in mitochondria [25] and membranes of *Anabaena variabilis* [26], were only weak inhibitors of cytochrome *c* reduction in heterocysts. DBMIB was not inhibitory below 2 μM and it increased reaction rates several-fold at higher concentrations.

Heterocyst soluble fraction

NADPH-dependent O_2 uptake in freeze-thawed heterocysts appeared to be catalyzed by the soluble protein ferredoxin:NADP reductase, therefore O_2 uptake and cytochrome *c* reduction were examined in a heterocyst soluble fraction. The activity is compared with that of purified spinach ferredoxin-NADP reductase in Table V. Both preparations showed enhancement of cytochrome *c* reduction by ferredoxin. Although DSPD and sulfo-DSPD are both inhibitors of spinach ferredoxin:NADP reductase [24], no inhibition was observed with the heterocyst extract. Antibody against the spinach enzyme also failed to inhibit activity in the heterocyst extract. NADPH-dependent O_2 uptake by spinach ferredoxin:NADP reductase and the heterocysts supernatant, like that

TABLE V

REDUCTION OF HORSE HEART CYTOCHROME *c* AND O_2 UPTAKE BY A SOLUBLE HETEROCYST EXTRACT AND BY SPINACH FERREDOXIN:NADP REDUCTASE

Complete reactions contained buffer, 40 μm NADPH and either 25 μM cytochrome *c* or 30 μM O_2. DSPD and sulfo-DSPD were present at 600 μM. Antibody to spinach ferredoxin:NADP reductase was provided as 0.48 mg (for spinach enzyme) or 2.4 mg (for heterocyst supernatant) immunoglobulin. Other concentrations were as in Tables II and III.

	Rate (μmol substrate reduced/mg protein per h)	
	Spinach ferredoxin:NADP reductase	Heterocyst soluble extract
Cytochrome *c* reduction		
no additions	112	9.8
+ ferredoxin	1890	38.2
+ ferredoxin + HOQNO	1990	37.4
+ ferredoxin + sulfo-DSPD	555	38.7
+ ferredoxin + DSPD	1350	38.2
+ ferredoxin + antibody	820	38.5
O_2 uptake		
no additions	28	1.3
+ methyl viologen	284	56.3
+ methyl viologen + ferredoxin	625	108

in frozen heterocyst (Table I), is strongly dependent on added methyl viologen. When NADH rather than NADPH was provided as electron donor to the soluble heterocyst extract, rates of O_2 uptake and cytochrome *c* reduction were less than 10% of the comparable rates with NADPH.

Acetylene reduction in heterocysts

The ability of whole and freeze-thawed heterocysts to reduce acetylene in the presence of different electron donors is displayed in Table VI. Isolated heterocysts are starved of reductant and reduce acetylene at slow rates unless provided with a reductant such as H_2. In the presence of H_2, whole heterocysts reduce acetylene in a reaction that depends entirely on endogenous phosphorylation and cofactors. Little activity is observed in the dark upon addition of dithionite plus an ATP-generating system, owing to the inability of these reagents to penetrate the cells. However, when this preparation is subjected to three freeze-thaw cycles, small hydrophilic molecules can freely penetrate allowing utilization of exogenous ATP and reductant.

Freeze-thawed heterocysts have lost the ability to reduce acetylene when provided with H_2 and light (Table VI). Addition of ADP and P_i under these conditions, however, restores approx. 20% of the acetylene reduction seen in intact heterocysts, showing that loss of phosphorylation substrates is partially responsible for inactivity. Some uncoupling of the photosynthetic membranes has apparently occurred, since the provision of an ATP-generating system rather than ADP and P_i yields a rate close to that given by intact heterocysts.

When acetylene reduction in whole or freeze-thawed heterocysts depends on phosphorylation of endogenous or added ADP, it is sensitive to uncoupling by valinomycin plus nigericin (Table VI). Inclusion of these ionophores together with an exogenous supply of ATP provides a means of recognizing effects of light which are now solely due to promotion of electron flow to nitrogenase. Thus, in the presence of uncoupler and ATP, H_2-dependent acetylene reduction is strictly light dependent; hydrogenase cannot reduce ferredoxin directly but requires the participation of Photosys-

TABLE VI

ACETYLENE REDUCTION BY WHOLE AND FROZEN HETEROCYSTS

Assays contained 50 mM KCl, 11 μg Chl and when indicated 10 mM $Na_2S_2O_4$, 1 mM NADH or an NADPH-generating system (3 mM glucose 6-phosphate plus 1 mM NADP). The values are the means of duplicate assays.

Electron donor	Other additions	Rate (μmol C_2H_4 produced/mg Chl per h)	
		Dark	Light
Whole heterocysts			
none	none	0.0 –	2.8 –
$Na_2S_2O_4$	ATP [a]	3.8 –	20 –
H_2	none	0.6 –	92 (0.8) [b]
Freeze-thawed heterocysts			
H_2	none	– –	0.8 –
H_2	ADP [c]	0.8 (0.9)	17 (1.1)
H_2	ATP	9.2 (0.5)	82 (70)
NADH	ATP	6.3 (2.8)	41 (42)
NADPH	ATP	77 (67)	74 (67)
$Na_2S_2O_4$	ATP	118 (102)	128 (106)

[a] Indicates an ATP-generating system containing 5 mM ATP, 7 mM $MgCl_2$, 20 mM creatine phosphate and 0.05 mg creatine phosphokinase.

[b] Values in parentheses were obtained with 10 μM valinomycin plus 5 μM nigericin present.

[c] Included 5 mM ADP, 5 mM P_i and 7 mM $MgCl_2$.

tem I [9,27]. In the absence of uncoupler, ATP drives some reverse electron flow and supports slow acetylene reduction in the dark. Electron donation from NADH to nitrogenase is similarly light dependent. In contrast, either glucose 6-phosphate/NADP or dithionite can support rapid acetylene reduction that is independent of light and of coupled membranes.

Discussion

The ultimate source of reductant for N_2 fixation in heterocysts is fixed carbon imported from vegetative cells [2]. The pathways of electron transfer and the metabolic intermediates involved are less clear. NADPH-generating systems have been shown to support dark acetylene reduction in isolated heterocysts at about half the rate with dithionite [6,7]; however, the activities of the isolated heterocyst preparations used in those studies were low. Dark NADPH-dependent acetylene reduction requires electron transfer through ferredoxin:NADP reductase and ferredoxin to nitrogenase; this pathway and its regulation have been described in detail [5]. The thermodynamically unfavorable electron transfer from NADPH to ferredoxin would demand a high NADPH/NADP ratio which might not be maintained in vivo [28]. This has prompted some workers to invoke participation of energized membranes and reverse electron flow to account for the transfer of electrons from NADPH to nitrogenase [28,29]. It is shown here that when heterocysts are made permeable by freezing, glucose 6-phosphate/NADP supports rapid acetylene reduction in the dark even in the presence of nigericin plus valinomycin and K^+ which abolish both the proton gradient and membrane potential [30]. This demonstrates that generation of NADPH by the oxidative pentose phosphate pathway and electron flow to nitrogenase via ferredoxin:NADP reductase can proceed simultaneously, at a physiologically meaningful rate, without the participation of energized membranes.

Frozen heterocysts and soluble heterocyst extracts catalyze NADPH-dependent reduction of cytochrome *c* or of O_2 and these reactions are enhanced by ferredoxin or methyl viologen, respectively. In the presence of methyl viologen the rate of electron transfer from NADPH to O_2 by this route is several times the maximum rate of acetylene reduction in isolated heterocysts. This pathway is sufficiently active in the absence of viologen to be partially or entirely responsible for the cyanide-insensitive respiration that occurs in cyanobacteria [31,32].

Ferredoxin:NADP reductase from heterocyst extracts is unlike that of the spinach enzyme in several ways: its ferredoxin-dependent cytochrome *c* reduction activity is insensitive to antibody raised against the spinach enzyme and is not inhibited by either DSPD or sulfo-DSPD. Inhibition by DSPD, which blocks the spinach enzyme [24], has been used as evidence for ferredoxin involvement in cyanobacterial reactions [32,33]; but since DSPD and sulfo-DSPD failed to inhibit ferredoxin:NADP reductase from heterocysts, using a ferredoxin-dependent assay, such conclusions are unwarranted. DSPD has also been shown to inhibit photosynthetic electron transport before or at plastocyanin in spinach chloroplasts [24], the oxyhydrogen reaction in heterocyst membranes [9] and (in work presented here) electron flow from NADH to cytochrome *c* in heterocyst membranes.

Respiratory oxidation of pyridine nucleotide has been reported in a number of cyanobacteria and has been correlated with ATP synthesis [26,32,34]; NADPH usually supported about twice as rapid an O_2 uptake as did NADH. In contrast to those results, we find no evidence for an NADPH-specific respiratory enzyme in *Anabaena* 7120. The apparent presence of an NADH-specific dehydrogenase in heterocyst membranes is nevertheless noteworthy, since the role of NADH in heterocyst metabolism is uncertain [8]. The possibility that NADH dehydrogenase may be a functionless enzyme in heterocysts, which is degraded only slowly following differentiation, seems unlikely since heterocysts have twice the activity per molecule of cytochrome *f* compared to vegetative cells.

Electron flow from NADH to cytochrome *c* is inhibited by HOQNO, which blocks the reoxidation of cytochrome *b* [21], and by DCCD, which inhibits both the reduction and reoxidation of plastoquinone [23]. Cytochrome *b*-563 and plastoquinone are proposed to be part of a common segment in the respiratory and photosyn-

thetic electron-transfer chains in cyanobacteria [7]. NADPH-specific cytochrome *c* reduction does not involve the membrane-bound cytochrome *b*-563, since no HOQNO inhibition of this reaction was observed; ferredoxin enhancement, however, supports involvement of residual membrane-bound ferredoxin:NADP reductase, as suggested above.

Although light enhances acetylene reduction by heterocysts, the role of light in the provision of reductant to nitrogenase has been difficult to assess because the enzyme requires a source of ATP in addition to a source of reductant for activity. Since freeze-thaw treatment renders heterocysts permeable to ATP (probably by disruption at the polar region of the cell envelope [35]), this can be supplied exogenously so that effects of light on electron flow can be independently observed. H_2-supported acetylene reduction was previously believed to be strictly light dependent, requiring electron flow through Photosystem I [9,27]. Table VI shows that in uncoupled heterocysts, acetylene reduction supported by H_2 or NADH is indeed almost entirely light dependent, and maximal rates are only observed during light-driven electron transfer. However, H_2 and NADH can support slower rates of acetylene reduction in the dark through ATP-driven, uncoupler-sensitive reverse electron flow. Electron donation from NADPH to nitrogenase in the dark is clearly unaffected by uncoupler, in contrast to the results with H_2 and NADH.

The present study demonstrates that NADH, like H_2, can donate electrons either to the respiratory electron-transfer chain or to the photosynthetic chain on the oxidizing side of P-700. Although the two electron-transfer chains contain some common electron carriers, it is not clear

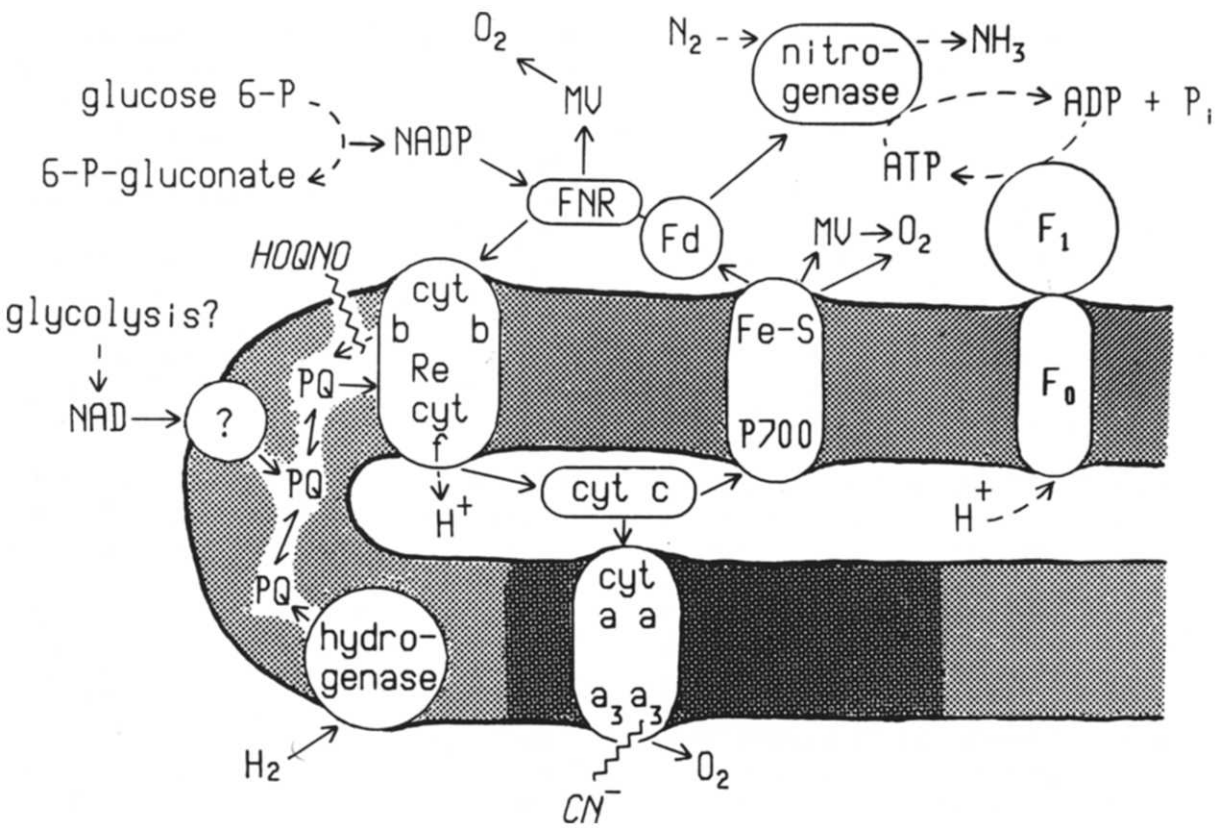

Fig. 2. Hypothetical scheme for the interaction of pyridine nucleotides and H_2 with the respiratory and photosynthetic electron-transfer chains in *Anabaena* heterocysts. Depicted is one edge of a flattened membrane vesicle, seen in cross-section. Two distinct lipid domains are indicated (see text). Electron-transfer reactions are shown by solid arrows; dashed arrows indicate metabolic conversions or ion fluxes. Fd, ferredoxin; P_i, phosphate; cyt, cytochrome; PQ, plastoquinone; MV, methyl viologen.

whether segments of the chain coincide or whether the two chains exist on different membranes; the results presented in this study are consistent with either proposal. Electron micrographs reveal that heterocysts contain two very distinct types of membranes: concentric lamellar membranes occurring at the center of the cell and contorted tubular membranes found at the cell periphery [36]. Arrhenius plots of photosynthetic and respiratory activities in *A. variabilis* [37] and *Anacystis nidulans* [38] suggest that the lipid environment of cytochrome oxidase differs from that of Photosystem I; however, differential fractionation of these membrane components and activities has not been reported.

Fig. 2 shows a possible arrangement of the electron-transport pathways in a heterocyst membrane vesicle. Proteins and reactions are shown with a membrane sidedness which is based on findings for similar components in chloroplasts, mitochondria and photosynthetic bacteria. Trans-membrane electron transfer from NADH to added cytochrome *c* could not occur in sealed vesicles, since only one reaction site would be accessible to exogenously added reactants. The ability of both NADH and externally added cytochrome *c* to react simultaneously with heterocyst membranes suggest that both sides of the membrane are exposed in at least part of the membrane fraction and that the isolated membranes may exist as a mixture of sheets and closed vesicles. The measured electron flow rates may thus be presumed minimum values, possibly limited by membrane topology.

The location of ferredoxin:NADP reductase and endogenous cytochrome *c*-553 on opposite sides of the membrane also explains why NADPH cannot donate electrons to P-700 or cytochrome oxidase at appreciable rates in heterocysts, even though in soluble extracts, NADPH can reduce added cytochrome *c* via ferredoxin:NADP reductase. The slight inhibition by cyanide of NADPH-dependent O_2 uptake (Table I) may suggest slow donation by NADPH to the membrane-bound electron-transfer chain via ferredoxin:NADP reductase and cytochrome *c*: a reaction perhaps made possible by membrane damage during the freeze-thaw treatment.

Fig. 2 suggests that photosynthetic and respiratory pathways share a common cytochrome *b-f* complex: this is the simplest assumption, for which proof is being sought by kinetic studies. Reduced cytochrome *c*-553 would interact with P-700$^+$ or cytochrome oxidase by diffusion in the lumen. Its relative affinity for these oxidants and their relative abundance in the membrane would determine the balance between respiratory and photosynthetic activities at the prevailing light intensity and O_2 tension. Lateral communication between membrane particles by diffusion of plastoquinone in the hydrophobic phase is also indicated in Fig. 2. Here again, the simplest assumption is that all reductases supply a common acceptor pool. If, however, cytochrome oxidase resided on a separate membrane, it would be associated with a distinct pool of the cytochrome *b-f* complex, plastoquinone, and some or all of the reductases.

The present study demonstrates that NADH and NADPH donate electrons to nitrogenase via different pathways. Although NADH supported substantial rates of acetylene reduction under the conditions employed in this study, it is uncertain whether this donor provides electrons for N_2 reduction in vivo. Further study is required of the metabolic pathways leading to generation of NADH in heterocysts.

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References

1 Stewart, W.D.P. (1973) Annu. Rev. Microbiol. 27, 283–316
2 Wolk, C.P. (1968) J. Bacteriol. 96, 2138–2143
3 Winkenbach, F. and Wolk, C.P. (1973) Plant Physiol. 52, 480–483
4 Lex, M. and Carr, N.G. (1974) Arch. Microbiol. 101, 161–167
5 Apte, S.K., Rowell, P. and Stewart, W.D.P. (1978) Proc. R. Soc. B 200, 1–25
6 Lockau, W., Peterson, R.B., Wolk, C.P. and Burris, R.H. (1978) Biochim. Biophys. Acta 502, 298–308

7 Eisbrenner, G. and Bothe, H. (1979) Arch. Microbiol. 123, 37–45

8 Bothe, H., Neuer, G., Kalbe, I. and Eisbrenner, G. (1980) in Nitrogen Fixation (Stewart, W.D.P. and Gallon, J.R., eds.), pp. 83–112, Academic Press, London

9 Houchins, J.P. and Burris, R.H. (1981) Plant Physiol. 68, 712–716

10 Allen, M.B. and Arnon, D.I. (1955) Plant Physiol. 30, 366–372

11 Peterson, R.B. and Wolk, C.P. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 6271–6275

12 Jones, L.W. and Bishop, N.I. (1976) Plant Physiol. 57, 659–665

13 Lockau, W. (1981) Arch. Microbiol. 128, 336–340

14 Susor, W.A. and Krogmann, D.W. (1966) Biochim. Biophys. Acta 120, 65–72

15 Peterson, R.B. and Burris, R.H. (1976) Arch. Microbiol. 108, 35–40

16 Vernon, L.P. (1960) Anal. Chem. 32, 1144–1150

17 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254

18 Chua, N. and Bennoun, P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2175–2179

19 Peschek, G.A. (1981) Biochim. Biophys. Acta 635, 470–475

19a Peschek, G.A. (1981) Biochem. Biophys. Res. Commun. 98, 72–79

20 Rowell, P., Diez, J., Apte, S.K. and Stewart, W.D.P. (1981) Biochim. Biophys. Acta 657, 507–516

21 Convent, B. and Briquet, M. (1978) Eur. J. Biochem. 82, 473–481

22 Almon, H. and Böhme, H. (1980) Biochim. Biophys. Acta 592, 113–120

23 Sane, P.V., Johanningmeier, U. and Trebst, A. (1979) FEBS Lett. 108, 136–140

24 Laasch, N., Kaiser, W. and Urbach, W. (1979) Plant Physiol. 63, 605–608

25 Ikuma, H. and Bonner, W.D. (1967) Plant Physiol. 42, 1535–1544

26 Leach, C.K. and Carr, N.G. (1970) J. Gen. Microbiol. 64, 55–70

27 Eisbrenner, G., Distler, E., Floener, L. and Bothe, H. (1978) Arch. Microbiol. 118, 177–184

28 Haaker, H., Laane, C. and Veeger, C. (1980) in Nitrogen Fixation (Stewart, W.D.P. and Gallon, J.R., eds.), pp. 113–138, Academic Press, London

29 Hawkesford, M.J., Reed, R.H., Rowell, P. and Stewart, W.D.P. (1981) Eur. J. Biochem. 115, 519–523

30 Padan, E. and Schuldiner, S. (1978) J. Biol. Chem. 253, 3281–3286

31 Wolk, C.P. (1973) Bacteriol. Rev. 37, 32–101

32 Peschek, G.A. (1980) Arch. Microbiol. 125, 123–131

33 Tel-Or, E. and Stewart, W.D.P. (1977) Proc. R. Soc. B 198, 61–86

34 Biggins, J. (1969) J. Bacteriol. 99, 570–575

35 Fay, P. and Lang, N.J. (1971) Proc. R. Soc. B 178, 185–192

36 Lang, N.J. and Fay, P. (1971) Proc. R. Soc. B 178, 193–203

37 Scherer, S., Stürzl, E. and Böger, P. (1981) Z. Naturforsch. 36c, 1036–1040

38 Peschek, G.A., Muchl, R., Kienzl, P.F. and Schmetterer, G. (1982) Biochim. Biophys. Acta 679, 35–43