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Two NADH:ubiquinone oxidoreductases of *Azotobacter vinelandii* and their role in the respiratory protection

Yulia V. Bertsova, Alexander V. Bogachev, Vladimir P. Skulachev *

Department of Bioenergetics, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia

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Abstract

Initial steps of the *Azotobacter vinelandii* respiratory chain have been studied on the inside-out subcellular vesicles. Two NADH:ubiquinone oxidoreductases were revealed: (i) proton-motive, capsaicin-sensitive and oxidizing dNADH as well as NADH enzyme and (ii) enzyme non-coupled to the energy conservation, capsaicin-resistant and oxidizing only NADH. The level of the oxidoreductases strongly depends upon $[O_2]$ and $[NH_3]$ in the growth medium. Increase in $[O_2]$ results in lowering of the coupled-enzyme level and in rise of the non-coupled one. Exclusion of NH_3 from the growth medium increases the level of the non-coupled enzyme whereas that of the coupled enzyme remains constant. The O_2-linked control of NADH:ubiquinone oxidoreductases requires CydR, a Fnr-like regulatory protein. Summarizing the above observations with those made in this group on the terminal steps of the *A. vinelandii* respiratory chains, one can assume that the respiratory protection of nitrogenase could be carried out by co-operation of the non-coupled NADH:ubiquinone oxidoreductase and the ''partially coupled'' quinoloxidase of the *bd*-type. Efficiency of this chain seems to be five-fold lower than that of the usual proton-motive chain (the coupled NADH:ubiquinone oxidoreductase, the Q-cycle and cytochrome oxidase of the *o*-type) which is also present in *A. vinelandii* and operates at low $[O_2]$.

Keywords: Respiratory protection; NADH:quinone oxidoreductase; NADH dehydrogenase I; NADH dehydrogenase II; Protonic potential; Energy coupling; CydR; (*Azotobacter vinelandii*)

1. Introduction

Respiration, as a molecular oxygen consuming process was postulated to be employed by aerobic cells to maintain safely low levels of intracellular $[O_2]$. It was hypothesized that such a function is carried out by non-coupled or uncoupled respiratory chains to avoid such a restriction as ADP availability, inherent to the energy-coupled respiration [1–3].

As a precedent of such kind of system, the *Azotobacter* respiratory protection mechanism has been considered [2]. In late sixties, Dalton and Postgate postulated [4,5] that this bacterium consumes vast amounts of oxygen to protect the nitrogenase complex, an enzyme extremely sensitive to O_2. The presence of such a respiratory protection mechanism presumably explains why *Azotobacter*, in contrast to other nitrogenase-containing bacteria, can reduce N_2

Abbreviations: $\Delta\bar{\mu}H^+$, transmembrane H^+ electrochemical potential difference; ΔpH, transmembrane pH difference; CCCP, *m*-chlorocarbonylcyanide phenylhydrazone; dNADH, reduced nicotinamide hypoxanthine dinucleotide; NDH, NADH dehydrogenase

* Corresponding author. Fax: +(7) 095 939 03 38; E-mail: skulach@head.genebee.msu.su

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even at high ambient oxygen concentrations [6,7]. Later it was found that deletions in genes encoding the *bd*-type oxidase impairs the N_2 reduction by *A. vinelandii* at high $[O_2]$ [8,9], although the mutant cells still contain another (*o*-type) oxidase [10]. It was concluded that it is the *bd* oxidase that is involved in the nitrogenase respiratory protection mechanism [8,9]. In line with this, it was shown that contribution of the *bd* oxidase to the oxygen consumption is strongly induced in NH_3-depleted media [11] or with the increase in $[O_2]$ [12]. In a study on the *A. vinelandii* subcellular vesicles, it was suggested that the *bd* oxidase is not coupled to energy conservation [13]. Recently, we have reinvestigated this option on both intact *A. vinelandii* cells and their inside-out vesicles. It was found that the *A. vinelandii bd* oxidase, like the *Escherishia coli bd* oxidase [14], is, in fact, coupled to the protonic potential generation but its efficiency (H^+/e^- ratio) is 1 i.e. two-fold lower than that of the oxidases belonging to the haem-copper family [15].

Another aspect to be revealed was the type of mechanism by means of which ubiquinol is produced to be later oxidized by the *bd*-type oxidase operating at high rate. If the respiratory protection hypothesis is correct, a fast non-coupled enzyme must operate in the initial part of the *A. vinelandii* respiratory chain. As it has been demonstrated, the *A. vinelandii* respiratory chain possesses several dehydrogenases, i.e. NADH dehydrogenase, NADPH dehydrogenase, malatedehydrogenase, succinatedehydrogenase and hydrogenase [16]. Nevertheless, it is known that the main respiratory substrate in bacteria is NADH, so it would be tempting to investigate the possibility of existence in *A. vinelandii* electron-transport chain of a non-coupled NADH:ubiquinone oxidoreductase. In *E. coli*, NADH:quinone oxidoreductase reaction can be catalyzed by two enzymes differing in their mechanisms and function i.e. the energy coupled NADH dehydrogenase (NDH) I and the non-coupled NDH II (for review, see [17]). On the other hand, there are some examples of bacteria possessing only one of this activities. For instance, *Paracoccus denitrificans* has NDH I, not NDH II, whereas *Bacillus subtilis* possesses NDH II as the only NADH-oxidizing respiratory chain enzyme [18].

In *E. coli*, the proton-motive NADH:quinone oxidoreductase consists of 14 subunits. It contains FMN and 5–7 FeS clusters as cofactors [19,20]. The operation of this enzyme is coupled to translocation of more than one H^+ per e^- across the membrane [21]. The *E. coli* non-coupled NADH:quinone oxidoreductase is a single subunit enzyme possessing FAD as the only redox group [22].

Ackrell and Jones suggested [13] that NADH:ubiquinone oxidoreduction in *A. vinelandii* membrane vesicles is coupled to the $\Delta\bar{\mu}H^+$ generation. However, co-existence of a non-coupled enzyme in this bacterium was not questioned and remained obscure up to date.

In this paper, we present the results showing that *A. vinelandii* employs two NADH:ubiquinone oxidoreductases, namely the coupled NDH I-type and the non-coupled NDH II-type enzymes, the latter being involved in the respiratory protection.

2. Materials and methods

The strains used in this study are listed in Table 1. *A. vinelandii* UW136 and MK8 strains were a gift from Professor R.K. Poole, the *A. vinelandii* 55

Table 1
Bacterial strains used in this study

Strain	Phenotype	Genotype	Reference or source
A. vinelandii UW136	Wild type	Rif^R	[8]
A. vinelandii MK8	$CydR^-$	UW136 *cydR*::Tn5 Rif^R Km^R	[8]
A. vinelandii 55	Wild type	Wild type	a
E. coli GR70N	Wild type	F^- *thi rpsL gal*(?) Str^R	[23]
E. coli MWC215	NDH II^-	GR70N *ndh*::Cm Str^R Cm^R	[23]
E.coli ANNO91	NDH I^-	F^- *thi* Δ*nuoI* Str^R Km^R	H. Weiss

[a] Department of Microbiology, Moscow State University.

strain was obtained from the Department of Microbiology, Moscow State University, the *E. coli* GR70N and MWC215 were kindly provided by Professor R.B. Gennis and *E. coli* ANNO91 was generously supplied by Professor H. Weiss.

The *A. vinelandii* cells were grown in the modified Burke's medium BS and BSN [24]. The *E. coli* cells were grown in the modified M9 medium [25]. In all cases, a thermostated shaker (200 rpm, 37°C) was used.

The effects of $[O_2]$ variation on growing *A. vinelandii* cells was studied in a Techne BR-6 fermenter (the medium volume, 800 ml; temperature, 37°C) with controlled pH and $[O_2]$ conditions. Oxygen concentration was calculated assuming $[O_2]$ in air-saturated water solution at 37°C to equal 185.5 μM [26]. Cells were grown in BS or BSN media without nutrients limitation. In all the cases, the cell concentration was kept constant at 1×10^8 cells/ml with respective continuous dilution with fresh growth medium (turbidistat culture). Dilution rates were around 0.25/h for *A. vinelandii* UW136 cells growing in BSN medium, 0.15/h in the case of BS medium and 0.125/h for *A. vinelandii* MK8 cells in BSN medium.

To obtain cell lysate and subcellular vesicles, the cells were harvested by centrifugation ($10\,000 \times g$, 10 min) and washed twice with medium 1 (50 mM NaCl, 10 mM KH_2PO_4 and 1 mM $MgSO_4$, pH 7.5). The sediment was suspended in medium 2 (20 mM HEPES, 5 mM $MgSO_4$, 25 mM K_2SO_4, 0.5 mM dithiothreitol and 0.5 mM EDTA, pH 7.5) and passed through a French press (16 000 psi). Non-damaged cells and cells debris were removed at $22\,500 \times g$ (10 min). Supernatant was used as cell lysate or further centrifuged at $50\,000 \times g$ (90 min) to obtain subcellular vesicles. The final sediment in this case was suspended in medium 2 (35–40 mg protein/ml) and stored in liquid nitrogen.

A ΔpH formation by the vesicles was monitored using acridine orange fluorescence quenching (a MPF-4 fluorimeter; excitation, 492 nm; emission, 530 nm). Incubation mixture contained 20 mM HEPES (pH 7.5), 60 mM KCl, 2 mM $MgSO_4$, 4 μM acridine orange and vesicles (40–60 μg protein/ml). A 2 ml sample was incubated for 5 min. Respiration was initiated by adding NADH or dNADH.

NADH and dNADH oxidation by vesicles was measured with a Hitachi 557 spectrophotometer (340–400 nm absorbance difference). Incubation mixture contained 20 mM HEPES (pH 7.5), 60 mM KCl, 2 mM $MgSO_4$ and vesicles (1–5 μg protein/ml). The reaction was initiated by adding of 0.12 mM NADH or dNADH. ε_{340} taken for NADH and dNADH was 6.22×10^3/M/cm. In some experiments, the cell lysate was used instead of vesicles. In this case, the mixture was supplemented with alamethicin (17 μg/ml) with protein concentration increased to 20 μg/ml.

NADH:Q_1 and dNADH:Q_1 oxidoreductions by vesicles was measured as described for NADH and dNADH oxidases but in the presence of 1 mM NaCN and 20 μM Q_1.

Protein concentration was measured with microbiuret method using bovine serum albumin (Serva, type V) as a standard.

Q_1 was obtained from Product Manager Eisai, Japan.

Table 2
The NADH- and dNADH-oxidase activities of the *A. vinelandii* [a] and *E. coli* subcellular vesicles

Strains	Oxidase activities, μmol NADH or dNADH/min/mg protein		dNADH Oxidase/NADH oxidase
	dNADH	NADH	
E. coli MWC215 (NDH II$^-$)	0.19	0.15	1.27
E. coli ANNO91 (NDH I$^-$)	0.00	0.34	0
E. coli GR70N (wild type)	0.34	0.65	0.53
A. vinelandii 55 (wild type)	0.97	1.82	0.51

[a] The *A. vinelandii* 55 cells were grown in BSN medium at atmospheric O_2 pressure.

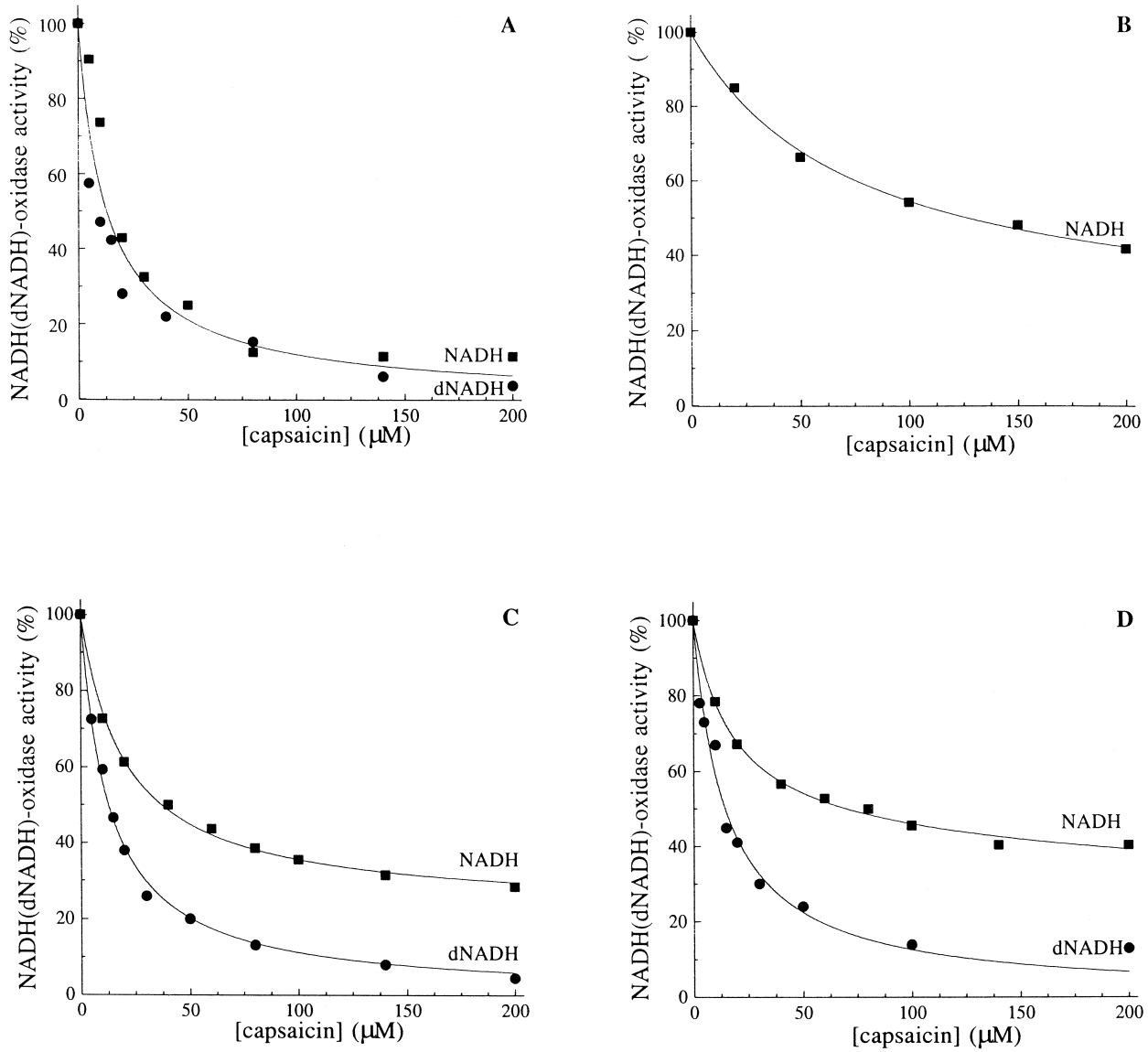


Fig. 1. The capsaicin inhibition of NADH (squares) and dNADH (circles) oxidation by *E. coli* (A–C) and *A. vinelandii* 55 (D) subcellular vesicles. (A), *E. coli* MWC215 (NDH II$^-$); (B), *E. coli* ANNO91 (NDH I$^-$); (C), *E. coli* GR70N (wild type). The NADH- and dNADH-oxidase activities in the absence of capsaicin (100% level) refer to the activities presented in Table 2. The *A. vinelandii* cells were grown in BSN medium at atmospheric O_2 pressure.

3. Results and discussion

In the majority of bacterial respiratory chains, NADH can be oxidized by enzymes of the NDH I- or the NDH II-type. NDH I oxidizes NADH as well as dNADH and its activity is inhibited by low capsaicin concentrations. On the other hand, NDH II consumes only NADH, but not dNADH and is resistant to low concentrations of capsaicin [22]. In line with this data, subcellular vesicles from the *E. coli* MWC215 strain (possessing only NDH I) oxidized either NADH or dNADH with similar rates (Table 2) and was sensitive to low capsaicin concentrations ($I_{0.5} = 18.6 \pm 2.3\,\mu M$, Fig. 1(A)). In the case of the *E. coli* ANNO91 strain (containing only NDH II), the subcellular vesicles oxidized only NADH (Table 2) and this NADH-oxidase activity was resistant to low capsaicin concentrations (Fig. 1(B)). At the same time,

subcellular vesicles from the wild type *E. coli* strain GR70N (possessing both NDH I and NDH II) consume NADH more rapidly than dNADH (Table 2). The dNADH oxidase activity of these vesicles is completely inhibited by low capsaicin concentrations ($I_{0.5} = 12.7 \pm 0.4\,\mu M$) whereas the NADH oxidase activity was only partially sensitive to this inhibitor (Fig. 1(C)). Thus, analysing the inhibition of NADH and dNADH oxidase activities by capsaicin with respect to these three described model systems we can determine the enzymatic composition of NADH:quinone oxidoreductase respiratory chain segments in other bacteria, possessing NDH I and/or NDH II.

From Fig. 1(D) and Table 2 one can see that subcellular vesicles from *A. vinelandii* 55 oxidized NADH more rapidly than dNADH, the NADH oxidase activity was only partially sensitive to capsaicin whereas the dNADH oxidase activity was completely inhibited by low concentrations ($I_{0.5} = 14.5 \pm 1.0\,\mu M$) of this inhibitor. These data resemble the results obtained on wild type *E. coli* strain harbouring both NDH I and NDH II. Thus, our results suggest that two different NADH:quinone oxidoreductases (NDH I- and NDH II-type) operate in the respiratory chain of *A. vinelandii*.

We would like to point out that under conditions used, the rate of dNADH oxidation was 51% of the NADH oxidation rate (Table 2). On the other hand, using the capsaicin inhibition analysis (non-linear regression calculation using GIM programme, Fig. 1(D)) the contribution of NDH I in the total NADH oxidase activity was estimated to be $\approx 54\%$. One can conclude from these data that NDH I from *A. vinelandii* oxidizes dNADH and NADH at similar rates (see also the data on the *E. coli* NDH I, Table 2). Hence, from here on we shall regard the level of the dNADH oxidase activity to be equal to NDH I activity and the difference between the NADH- and dNADH-oxidase levels as NDH II activity.

In Fig. 2 generation of ΔpH by the *A. vinelandii* vesicles has been studied. As seen, the addition of dNADH to *A. vinelandii* subcellular vesicles resulted in a ΔpH formation reported by the quenching of acridine orange fluorescence. This ΔpH was completely abolished by high concentrations of KCN, inhibiting the *A. vinelandii* oxidases. Subsequent addition of Q_1 partially restored ΔpH (Fig. 2(A)).

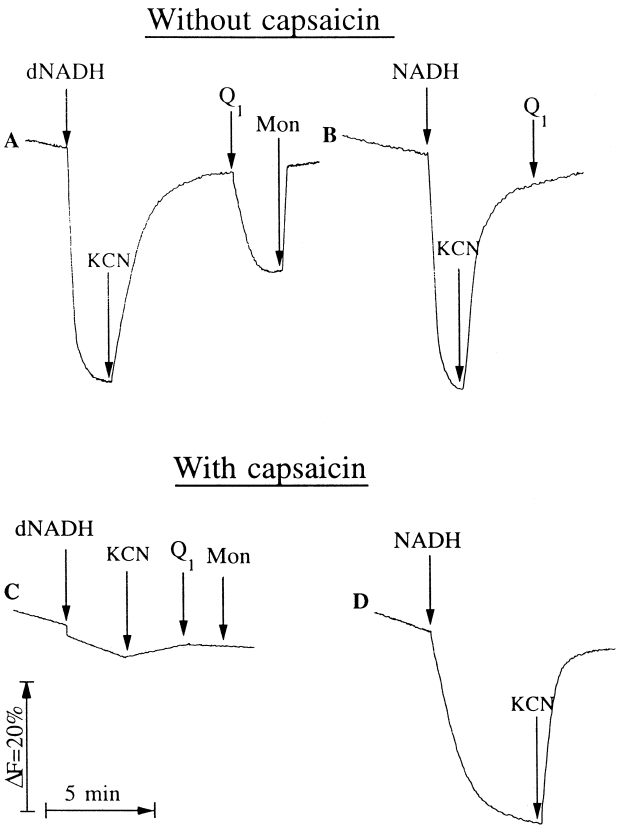


Fig. 2. The ΔpH formation by *A. vinelandii* UW136 (wild type) subcellular vesicles. Additions: 0.1 mM dNADH, 0.6 mM NADH, 0.3 mM KCN, 80 μM Q_1, 2.5 μM monensin (Mon) and 0.1 mM capsaicin. The NADH- and dNADH-oxidase and Q_1 reductase activities of these vesicles are listed in Table 3.

Since the dNADH:Q_1 oxidoreductase activity is catalyzed by NDH I only, this observation indicates that *A. vinelandii* NADH dehydrogenase-I is proton-motive. As Fig. 2(B) shows, NADH oxidation by Q_1 fails to produce measurable ΔpH. We would like to point out that in subcellular vesicles used in this experiment the contribution of NDH I to total NADH:Q_1 oxidoreduct$_{ase}$ activity was only 9% (Table 3), since Q_1 proved to be a much better oxidant for NDH II than for NDH I. It means that the

Table 3
The NADH and dNADH oxidase and Q_1 reductase activities of *A. vinelandii* UW136 subcellular vesicles

	Oxidase activity μmol/min/mg protein	Q_1 reductase activity μmol/min/mg protein
NADH	9.3	6.0
dNADH	2.1	0.54

$NADH:Q_1$ oxidoreductase activity was catalyzed almost solely by NDH II and thus, the activity of *A. vinelandii* NDH II is not coupled to $\Delta\bar{\mu}H^+$ generation. Moreover, in the presence of capsaicin, dNADH failed to energize the particles (Fig. 2(C)), whereas the oxidation of NADH resulted in ΔpH generation in a cyanide-sensitive fashion (Fig. 2(D)). In the latter case, ubiquinol, produced by the non-coupled NDH II, is oxidized via the energy-coupled terminal oxidases [14].

The above mentioned non-coupled NDH II seems to be a good candidate for the initial step of the respiratory protection chain terminated by the *bd* oxidase. If such is the case, then the NDH II activity

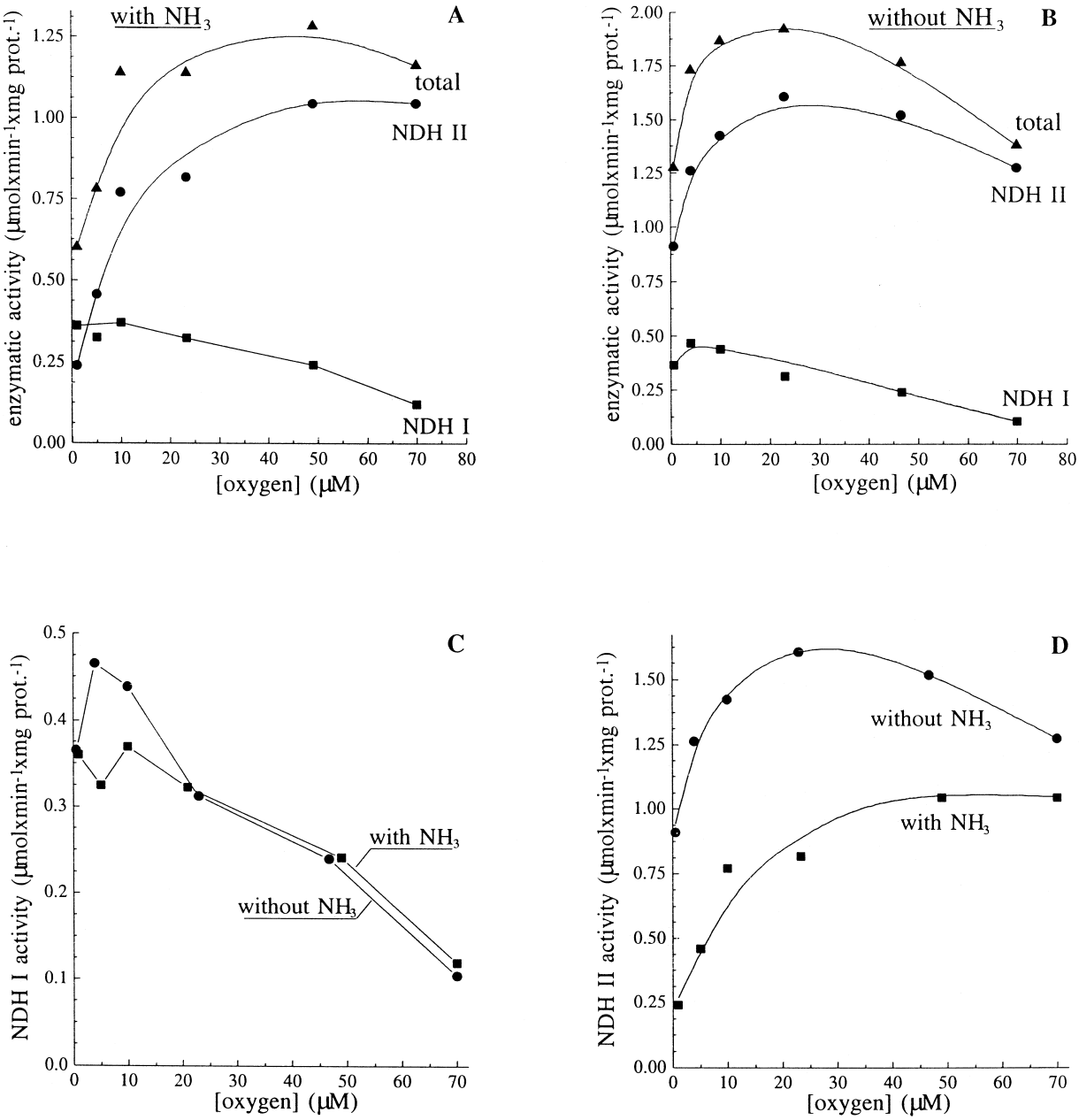


Fig. 3. NDH activities of lysates of *A. vinelandii* UW136 cells grown with NH_3 (the BSN medium) or without NH_3 (the BS medium) at different $[O_2]$.

must be induced by NH_3 deficiency as well as by increased oxygen concentration i.e. by conditions where respiratory protection is necessary to survive. As our experiments showed, exclusion of NH_3 from the growth medium caused an increase in the NDH II activity of the *A. vinelandii* 55 cell lysate from 0.3 to 0.55 μmol NADH/min/mg protein. As for NDH I, it remained constant (about 0.2 μmol NADH/min/mg protein). Similar relationships were revealed for another *A. vinelandii* wild-type strain, UW136.

In Fig. 3 the effect of $[O_2]$ variation on the chemostat-grown culture of *A. vinelandii* UW136 is shown. It can be seen that in the NH_3-containing medium, the rise in $[O_2]$ resulted in a three-fold decrease of the NDH I level, a four-fold increase in NDH II, and a more than two-fold increase in the total NADH-oxidase activity (Fig. 3(A)). In the NH_3-depleted medium, the $[O_2]$ effect on NDH I was similar to that in the NH_3-supplemented medium (Fig. 3(B), (C)). As for the NDH II level, it was initially almost four-fold higher than in the NH_3-containing medium, and the $[O_2]$ rise caused its further increase (Fig. 3(B), (D)).

Thus, NDH I activity is increased in cells grown under O_2-depleted conditions, the effect being independent of the NH_3 availability. The NDH II activity responds by increase to both O_2 rise and NH_3 deficiency as would be expected of an enzyme involved in respiratory protection.

Induction of the NDH II activity is observed under the same conditions where the induction of the *bd*-type oxidase (an enzyme clearly involved in respiratory protection [16]) is shown to take place [11,12]. It is known that the latter effect is mediated by a regulatory *A. vinelandii* protein, CydR (synonymous to FNR in *E. coli*) [27]. Thus, it was shown that the *A. vinelandii* strains lacking this protein overproduce the *bd*-type oxidase even during growth in low oxygen concentration conditions [8,27]. As seen from Fig. 4, regulatory protein CydR is also required for the O_2-linked regulation of the NDH I/NDH II ratio. It was found that this ratio is constant and very low in a $CydR^-$ mutant strain, being independent of the O_2 level. In fact, at low $[O_2]$, this ratio is as low as at high $[O_2]$.

The very fact that the *A. vinelandii bd*-type oxidase and NDH II are controlled by one and the same

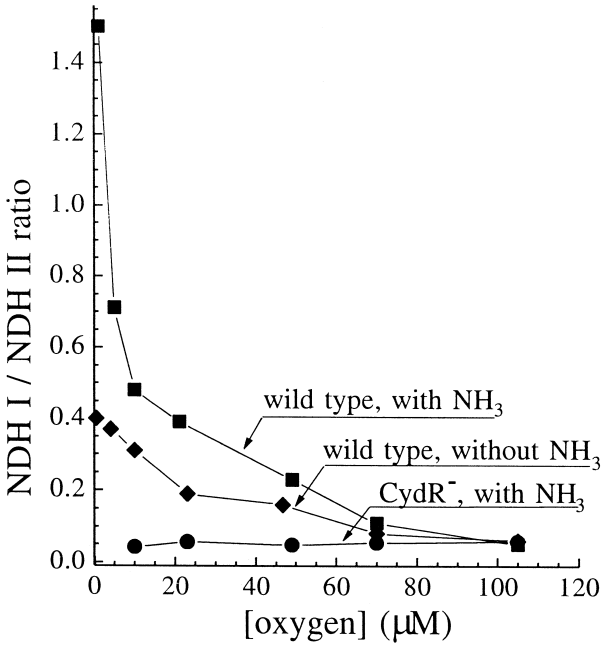
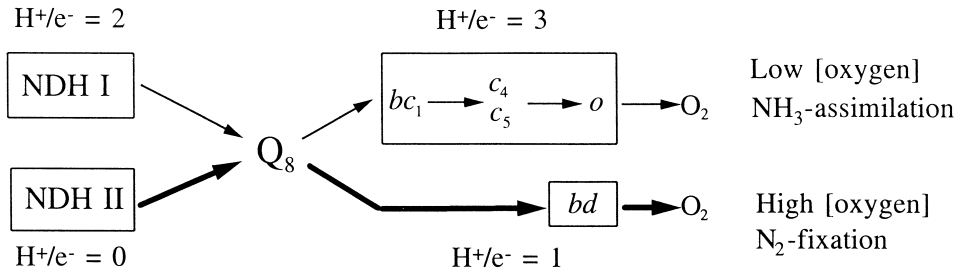


Fig. 4. The NDH I/NDH II ratio as a function of the ambient oxygen concentration in two *A. vinelandii* strains, i.e. UW136 (wild type) and MK8 ($CydR^-$).

regulatory system suggests the co-operative induction mechanism of both enzymes is presumably involved in the respiratory protection.

The results of current and accompanying paper [14] are summarised in Scheme 1.

It is assumed that *A. vinelandii* cells possess at least two respiratory chains differing in enzyme composition and physiological function. One of them completely coupled (light arrows). It includes NDH I (described in this paper), the Q-cycle [14] and the *o*-type oxidase [28]. Assuming the H^+/e^- ratios to be equal to 2 for NDH I [29], and 3 for bc_1-complex (Q-cycle) and the *o*-type oxidase [30], we can estimate the efficiency of this chain at 5 H^+/e^- passing from NADH to O_2 [31]. The other chain (bold arrows) is much simpler and includes the non-coupled NDH II (this paper) and the ''partially coupled'' ($H^+/e^- = 1$ instead of 2) *bd*-oxidase [14]. In total, this chain translocates only one H^+ per e^-. It is the latter chain that is activated at high $[O_2]$ and low $[NH_3]$ i.e. under conditions when respiratory protection of nitrogenase becomes vital. Its maximal activity is much higher than that of the completely coupled chain. When $[O_2]$ lowers, an increase in level of



Scheme 1.

the coupled-respiratory chain and decrease in that of the ''partially coupled'' one is controlled by the same regulatory system (CydR).

It is remarkable that the respiratory protection chain does not include either the first or the second energy coupling sites whereas these are *the* enzymes that provide respiratory control and also inevitably produce bulk cellular $O_2^{-\cdot}$ [2]. The third coupling site cannot form $O_2^{-\cdot}$ since the redox potential of the one-electron O_2 reduction (about -0.15 V) is much more negative than that of the terminal oxidase intermediates. Apparently, should the respiratory protection mechanism be provided by a completely coupled chain, the rates of oxygen consumption would be much lower and the rates of the $O_2^{-\cdot}$ formation would be much higher.

The very fact that some energy is still conserved by the respiratory protection chain can be accounted for by the fact the that *Azotobacter* is a typical respirer obtaining all the necessary energy from oxidation of organic substrates by O_2. In this respect, it differs from, e.g. plant cells possessing in addition photosynthetic and glycolytic mechanisms of energy production. According to the data recently obtained in our and Wagner's groups, the respiratory protection function in plant mitochondria is carried out by the non-coupled alternative quinoloxidase [32,33] which apparently co-operates with the non-coupled NAD(P)H:quinone oxidoreductases [2].

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