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GENERATION OF REDUCING POWER IN CHEMOSYNTHESIS

V. THE MECHANISM OF PYRIDINE NUCLEOTIDE REDUCTION BY NITRITE IN THE CHEMOAUTOTROPH *NITROBACTER AGILIS*

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SUMMARY

1. Cell-free extracts from Nitrobacter catalyzed an energy-dependent reduction of NAD^+ by NO_2^-; the process could be driven either at the expense of added ATP or by the energy generated from NO_2^- oxidation.

2. The reduction of cytochrome *c* by NO_2^- was observed to be an energy-dependent reaction which involved reversal of electron transfer from cytochrome a_1. The subsequent energy-linked reduction of the flavoproteins and pyridine nucleotides occurred concomitantly with the oxidation of cytochrome *c*.

3. The reduction of each mole of NAD^+ by NO_2^- required the utilization of approx. 5 moles of ATP. These observations are in harmony with the calculated energetics of the overall reverse electron flow process which involves a free energy gap of some 35 kcal.

4. Under conditions when ATP was limiting, the energy-linked reduction of 1 molecule of NAD^+ by nitrite required the concomitant oxidation of 2 molecules of cytochrome *c*. However, when the process was driven by an optimal ATP concentration (0.7 mM), the rate of NAD^+ reduction was about 1.5-fold compared to the rate of cytochrome *c* oxidation.

5. The process of energy-linked reversal of electron transfer in Nitrobacter was markedly sensitive to the inhibitors of the flavoprotein systems as well as to antimycin A or 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. In addition, CN^- was observed to be a potent inhibitor. The uncouplers of oxidative phosphorylation caused a strong inhibition of the ATP-linked reverse electron transfer, and the energy transfer from ATP to drive reverse electron flow was also inhibited by oligomycin.

INTRODUCTION

In chemosynthetic bacteria the oxidation of an inorganic substrate provides energy as well as reduced pyridine nucleotides for the cellular biosynthetic reactions involving CO_2 reduction[1,2]. The chemoautotroph Nitrobacter offers an unique case

Abbreviations: HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; CCCP, *m*-chlorocarbonyl-cyanide phenylhydrazone.

whereby the electrons from NO_2^- (E'_0 NO_3^-/NO_2^- = + 0.43 V) must reach the level of pyridine nucleotides (E'_0 $NAD^+/NADH$ = −0.32 V) in order to generate the obligately required reducing power. The experimental evidence for NAD^+ reduction coupled to NO_2^- oxidation was first provided by KIESOW[3]. Simultaneously ALEEM *et al.*[4] demonstrated for the first time that the reduction of NAD^+ in Nitrobacter was an energy-linked process which involved an ATP-dependent reversal of electron transfer from ferrocytochrome *c* to flavoprotein(s) and the pyridine nucleotides. KIESOW[5] reported a year later that the intracellular reduction of pyridine nucleotides in Nitrobacter was inhibited by 2,4-dibromophenol, an observation which also reflected the energy-linked nature of the pyridine-nucleotide reduction by NO_2^-.

The pathway of electron transport from NO_2^- to O_2 mediated by cytochrome *c* and cytochrome a_1, originally reported by ALEEM AND NASON[6] was later modified by ALEEM[7] in that NO_2^- oxidation involved the participation of cytochrome a_1 and a_3-like components, and that the reduction of cytochrome *c* by NO_2^- was energy-dependent. Similar results were also reported independently by KIESOW[8], and in addition, he made an important observation that the reduction of cytochrome *c* by cytochrome a_1 was ATP-dependent and that this reaction was also inhibited by 2, 4-dibromophenol. Further work concerning the mechanism of NO_2^- oxidation by Nitrobacter has been reported by ALEEM[9] who has been able to show that: (1) NO_2^- oxidation catalyzed by the Nitrobacter electron-transport particle was mediated by cytochrome a_1 and cytochrome oxidase components and that the process was coupled to ATP generation in only the terminal segment of the electron-transport chain. (2) With NO_2^- as the electron donor, the coupled ATP synthesis was independent of the participation of the pyridine nucleotides or flavins. (3) The possibility for the existence of two energy-coupling sites in the cytochrome *c*: O_2 oxido–reductase region was ruled out because with either NO_2^- or ascorbate as electron donors, the Nitrobacter system could yield, at best, P/O ratios of 1.0. Although cytochrome *c* may participate in the reaction, the net energy yield in this case would be zero because the reduction of cytochrome *c* by NO_2^- would be driven by ATP, although the reoxidation of cytochrome *c* by cytochrome oxidase may be coupled to ATP synthesis.

Thus the above experimental findings of ALEEM are not in harmony with the postulations of KIESOW that the electron transport from NO_2^- to O_2 mediated by cytochrome a_1 is not coupled to ATP synthesis and that NO_2^- oxidation consumes energy which is provided by the oxidation of NADH[4,6].

In spite of the preliminary reports by ALEEM *et al.* as well as KIESOW, the observed energy-linked reactions in the chemoautotroph Nitrobacter remain to be characterized. This report, therefore, deals with the energetics, stoichiometry of energy consumption, and pathways of energy-dependent reversal of electron transfer from NO_2^- to NAD^+.

MATERIALS AND METHODS

The methods for growth and preparation of cell-free extracts from *Nitrobacter agilis* have been described in a previous report[9]. The cell-free fraction 10 000 × *g* supernatent was used as the enzyme source in all the experiments reported in this article.

The energy-dependent reduction of pyridine nucleotide by NO_2^- was measured

in a dual split-beam spectrophotometer capable of recording difference absorption changes at two desired wavelengths in the same reaction mixture at the same time. Thus the oxido–reduction changes in cytochrome a_1 and c, cytochrome c and flavoprotein, and cytochrome c and NAD^+ could be followed by monitoring the respective wavelength couples, *e.g.*, at 438 and 550 nm, 550 and 450 nm, and 550 and 340 nm.

The reaction was performed in thunberg-type cuvettes of 1-cm light path. The reaction mixture, unless otherwise specified, contained in a total volume of 3.27 ml, cell-free extract containing 7.5 mg protein, 50 nmoles of cytochrome c (horse heart, Sigma), 5 μmoles of $MgCl_2$, 250 μmoles of Tris–HCl (pH 8.0), 10 μmoles of KNO_2 and 2 μmoles of NAD^+. The treatment cuvette in addition was supplied with 2 μmoles of ATP. The side arm of the cuvettes contained enzyme, Mg^{2+} and cytochrome c. After evacuating and gassing the cuvettes with O_2-free N_2 (repeated 3 times), the contents of the side arm were tipped in, and absorbance changes were recorded at the two desired wavelengths.

RESULTS AND INTERPRETATION

ATP-dependent reduction of NAD^+ by NO_2^- in Nitrobacter cell-free extracts

The data of Fig. 1 show that the treatment of the Nitrobacter cell-free extracts with 3.3 mM NO_2^- and 800 μM NAD^+ followed by the addition of 700 μM ATP resulted in the initial reduction of cytochrome c but no reduction of NAD^+. The latter

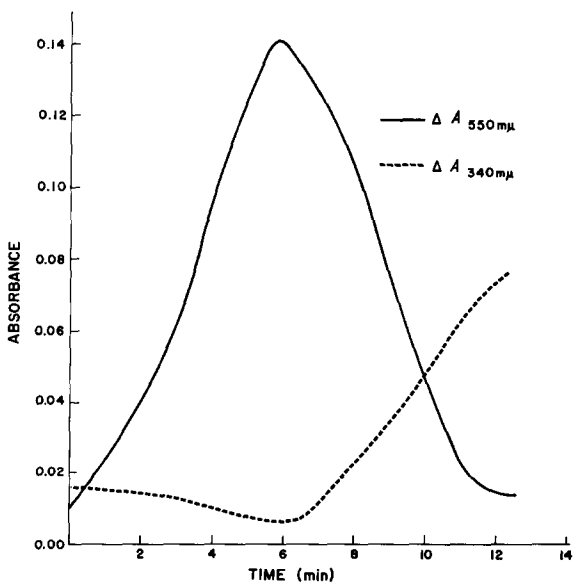

Fig. 1. Energy-dependent oxidation of cytochrome c and coupled reduction of NAD^+ by NO_2^- catalysed by Nitrobacter cell-free preparations. The reaction mixture in a total volume of 3.1 ml contained Nitrobacter cell-free extract containing 7.5 mg of enzyme protein, 250 μmoles of Tris–HCl (pH 8.0), 2 μmoles of NAD^+, 10 μmoles of NO_2^-, 5 μmoles of Mg^{2+}, 2 μmoles of ATP, and 2.0 mg of cytochrome c (Sigma, horse heart, Type II). The reaction was carried out in thunberg-type cuvettes of 1 cm light path. The side arm of the cuvette contained enzyme, ATP, Mg^{2+}, and cytochrome c. The control cuvette contained all the components except ATP. The cuvettes were evacuated and the contents of the side arm were tipped in to start the reaction. The absorbance was recorded at 550 and 340 mμ in a dual wavelength double-beam spectrophotometer attached to a dual-pen recorder.

was reduced concomitantly with the oxidation of cytochrome *c* under anaerobic conditions. Thus the reduction of cytochrome *c* by NO_2^- and of NAD^+ by reduced cytochrome *c* may be seen as ATP-dependent phenomena. Under the experimental conditions the oxidation of only 21 nmoles of cytochrome *c* could be observed coupled to the reduction of 32 nmoles of NAD^+. Thus apparent lack in the stoichiometry is due to the fact that cytochrome *c* is an intermediary electron carrier between NO_2^- and NAD^+.

Carriers involved in the energy-linked transfer of electrons from NO_2^- to NAD^+

It was reported earlier that in the Nitrobacter electron-transport chain, NO_2^- enters at the level of cytochrome a_1 (refs. 5, 6). Therefore, the observed ATP-dependent reduction of cytochrome *c* by NO_2^- (Fig. 1) must involve energy-linked reverse electron flow from cytochrone a_1 to cytochrome *c*. The data in Fig. 2 illustrate that concomitant to the ATP-dependent reduction of cytochrome *c* by NO_2^- there was in fact oxidation of cytochrome a_1 (as indicated by the decrease in absorption at 438 nm). It may also be seen that the energy-linked oxidation of ferrocytochrome *c* resulted in the initial reduction of the flavoprotein system (decrease in absorption at 450 nm), and its subsequent oxidation caused the coupled reduction of the exogenously added NAD^+ (increase in absorption at 340 nm). It was also observed that cytochrome a_1 underwent cyclic oxidation–reduction sequence depending upon the redox state of cytochrome *c*; thus, a_1 was oxidized coupled to the reduction of *c*,

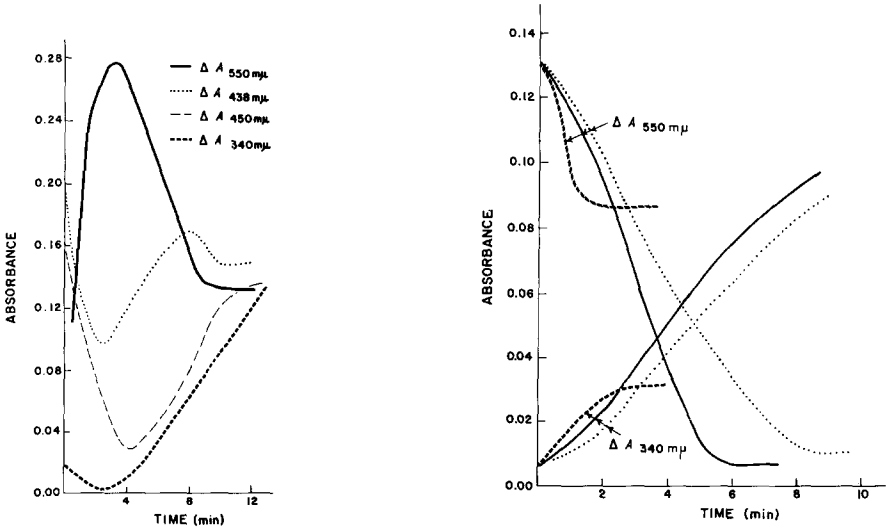

Fig. 2. Pathways of ATP-dependent reversal of electron transfer from NO_2^- to NAD^+. Experimental conditions were similar to those described in Fig. 1 except the enzyme contained 7.0 mg of protein. Absorbance changes corresponding to the redox states of cytochrome a_1, cytochrome *c*, FMN and NAD^+ were measured at 438, 550, 450 and 340 mμ respectively in the presence of added 50 nmoles of FMN.

Fig. 3. Effect of NO_2^- concentration on the ATP-dependent reduction of NAD^+. The experimental conditions were the same as described in Fig. 1 except that the reaction was carried out in the absence of NO_2^- as indicated by (------), in presence of 5 μmoles of NO_2^- (·····) and 10 μmoles of NO_2^- (———). The absorption was followed at 550 and 340 mμ in the same reaction mixture at the same time.

and when the transfer of electrons from *c* to the flavoprotein and pyridine nucleotide system caused the oxidation of *c*, the transfer of electrons from nitrite again caused the reduction of a_1, which was oxidized once again under the pressure of ATP. Thus the ATP hydrolysis may be visualized as the driving force for the reverse electron flow involving reversal of the sequence of the reactions of oxidative phosphorylation.

Substrate and ATP optima for the energy-linked reduction of NAD$^+$

The optimal nitrite concentration for the ATP-driven oxidation of cytochrome *c* and coupled reduction of NAD$^+$ was found to be 3.4 mM and at pH 8.0 (Fig. 3). A 10 mM NO_2^- concentration was somewhat inhibitory. However, at 3.4 mM NO_2^- 41 nmoles of NAD$^+$ were reduced although only the oxidation of 20 nmoles of cytochrome *c* could be observed. The reaction was somewhat slower in the presence of 1.7 mM NO_2^-. In case when the electrons were donated by the endogenous substrate (in the absence of added NO_2^-), the ATP-dependent NAD$^+$ reduction did occur concomitant to the oxidation of cytochrome *c*, but the reaction leveled off after the reduction of 12 nmoles of NAD$^+$.

No reduction of NAD$^+$ by NO_2^- was observed in the presence of 0.1 mM ATP; higher concentrations of ATP resulted in the increased reduction of NAD$^+$ in the presence of up to 1 mM ATP (Fig. 4). Concentrations more than 1 mM ATP were

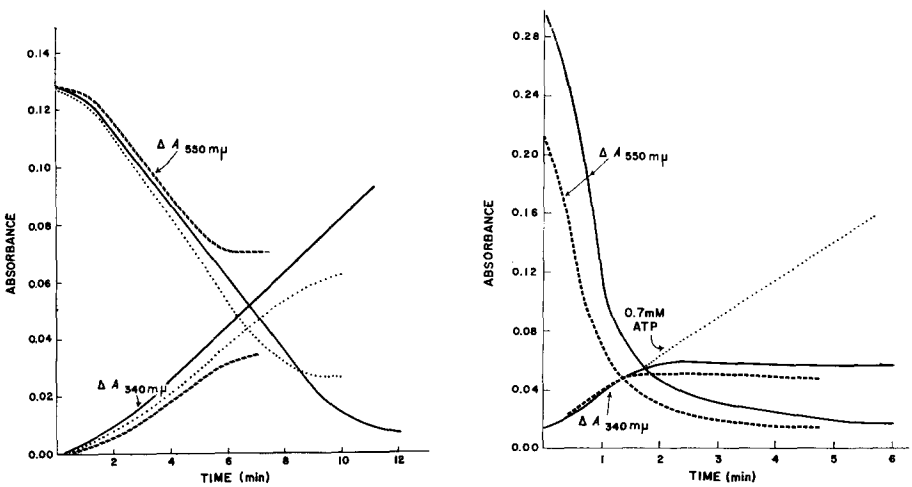

Fig. 4. Effect of ATP concentration on the energy-dependent reduction of NAD$^+$ by NO_2^-. The experimental conditions were the same as described in Fig. 1 except that the ATP concentration was varied: 1 μmole (------), 2 μmoles (.....), and 3 μmoles (———). NAD$^+$ reduction and cytochrome *c* oxidation is indicated by the increase in absorbance at 340 mμ and the decrease in absorbance at 550 mμ respectively.

Fig. 5. Reduction of NAD$^+$ by NO_2^- driven by the energy of NO_2^- oxidation. The enzyme preparations were preincubated aerobically for 10 min at 26° with 10 μmoles of NO_2^-, 3 μmoles of phosphate and 2 μmoles of ADP prior to evacuation. The reference cuvette contained a complete reaction mixture but was evacuated immediately without aerobic preincubation. The experimental conditions were similar to those described in Fig. 1 except that no ATP was added to the reaction mixture. The energy-linked oxidation of cytochrome *c* and coupled reduction of NAD$^+$ was followed at 550 and 340 mμ (———). Upon the addition of a 0.7 mM concentration of ATP, the reduction of NAD$^+$ proceeded at a linear rate (.....). The energy-linked reversal of electron transfer from cytochrome *c* to NAD$^+$ when the enzyme preparation was preincubated aerobically with 10 μmoles of NO_2^- alone followed by anaerobiosis (------).

somewhat inhibitory. The data also indicated that in the presence of 0.33 mM ATP, the reaction leveled off after the reduction of 15 nmoles of NAD^+. However, NAD^+ reduction proceeded at linear rate when 1 mM ATP was present, in which case 47 nmoles of NAD^+ were reduced concomitant to an observed oxidation of 20 nmoles of cytochrome *c*.

Reduction of NAD^+ driven by the energy of NO_2^- oxidation

Aerobic preincubation at 26° of the Nitrobacter cell-free extracts with 3.3 mM NO_2^- for 10 min followed by anaerobiosis, resulted in the rapid reduction of added NAD^+ concomitant to the oxidation of cytochrome *c* (Fig. 5). The energy-linked oxido–reduction reaction yielded a complete stoichiometry in that the oxidation of 30 nmoles of cytochrome *c* was coupled to the reduction of 15 nmoles of NAD^+. In the case when the cell-free extracts were preincubated with NO_2^- in the presence of ADP and phosphate, the energy-linked reversal of electron transfer from cytochrome *c* to NAD^+ yielded similar results, *e.g.*, 44 nmoles of cytochrome *c* were oxidized concomitant to the reduction of 21 nmoles of NAD^+. Since no ATP was added initially to the reaction mixture, the energy of NO_2^- oxidation may be visualized as the driving force for the reverse electron flow observed in this system. As a limited amount of energy was generated by NO_2^- oxidation, the reaction leveled off after about 2 min; however, upon a further addition of 0.7 mM ATP, the reduction of NAD^+ resumed at a linear rate (Fig. 5).

Determination of ATP/NADH quotient

The data concerning the stoichiometry of ATP-driven NAD^+ reduction by NO_2^- is presented in Table I. It may be seen that between 4–5 ATP equivalents are utilized per equivalent of NAD^+ reduced. These observations fit in well with the calculated energetics which correspond to a thermodynamic gap of some 35 kcal/mole for the reduction of NAD^+ by NO_2^- (assuming $\Delta F'$ for ATP hydrolysis = —7 kcal).

Unfortunately, the stoichiometry of NO_2^- disappearance coupled to NAD^+ reduction could not be established due to the complexity of the 'reversible' nitrate reductase in Nitrobacter. Attempts to gain this stoichiometry in the presence of pyruvate–lactate dehydrogenase trap were unsuccessful because the NO_3^-, produced upon removal of electron from NO_2^-, could be reduced again by the reduced cytochrome a_1 and *c* components.

TABLE I

STOICHIOMETRY OF ATP-DEPENDENT REDUCTION OF NAD^+

Experimental conditions were the same as in Fig. 1 except that various ATP levels were employed as indicated in the table. 'Net ATP used' corresponds to the P_i released with respect to the presence and absence of added NAD^+.

Total ATP employed (*μmoles*)	*Net ATP used* (*μmoles*)	*Net NAD^+ reduced* (*μmoles*)	*Ratio ATP/NADH*
1	0.670	0.169	4.0
2	1.515	0.283	5.3
3	2.370	0.414	5.7

Sensitivity of the ATP-dependent reverse electron flow to various inhibitors

Since the electrons donated by NO_2^- enter the electron-transport chain of Nitrobacter at the cytochrome a_1 level[5,6] (Fig. 2), the process of energy-linked electron reversal should be sensitive to the inhibitors which specifically block electrons at various segments of the respiratory chain. The data presented in Table II did in fact indicate that the energy-linked oxidation of cytochrome *c* as well as the coupled reduction of pyridine nucleotide were inhibited by CN^-, antimycin A or 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), and inhibitors of the flavoprotein systems such as atabrine, thenoyltrifluoroacetone, rotenone and amytal. Moreover, the ATP-dependent reverse electron flow was also markedly sensitive to uncouplers of the energy-transfer reactions such as *m*-chlorocarbonyl-cyanide phenylhydrazone (CCCP), pentachlorophenol, 2,4-dibromophenol, and 2.4-dinitrophenol. In addition, oligomycin was found to be a potent inhibitor of the ATP-linked cytochrome *c* oxidation and coupled NAD^+ reduction.

TABLE II

EFFECT OF INHIBITORS ON THE ATP-DEPENDENT REVERSE ELECTRON FLOW FROM NO_2^- TO NAD^+

The enzyme preparation was preincubated for 3 min with various inhibitors. The experimental conditions were similar to those described in Fig. 1. In the absence of added inhibitor, 46 nmoles of cytochrome *c* were oxidized with the concomitant reduction of 43 nmoles of NAD^+.

Inhibitor	*Concn.*	*Inhibition* (%)	
		Cytochrome c oxidized	*NAD^+ reduced*
Atabrine	100 μM	77	77
Rotenone	10 μM	45	34
Thenoyltrifluoroacetone	1.7 mM	64	71
Amytal	2 mM	40	41
Antimycin A	1.5 μg/mg protein	64	63
HQNO	5.3 μg/mg protein	67	68
CN^-	0.3 mM	77	76
	0.7 mM	90	97
CCCP	17 μM	55	82
Pentachlorophenol	20 μM	100	100
2,4-Dibromophenol	10 μM	100	97
2,4-Dinitrophenol	33 μM	100	95
Oligomycin	0.8 μg/mg protein	43	73
	2 μg/mg protein	61	81

DISCUSSION

The process of energy-linked reduction of NAD^+ by succinate in mammalian mitochondria involving reversal of electron transfer was initially demonstrated by CHANCE AND HOLLUNGER[10,11] and independently reported by KLINGENBERG and co-workers[12,13]. Since then the partial reactions of the reversal of the sequence of oxidative phosphorylation have been investigated in detail in several laboratories[14–24].

The physiological importance of energy-linked electron reversal in chemosynthetic bacteria lies in the fact that with the exception of H_2-oxidizing bacteria, the redox potentials of the inorganic energy-yielding substrates utilized by the chemo-

autotrophs are several-fold greater than the redox potentials of the pyridine nucleotides[25] which are essential in the reduction of CO_2[1,2,26]. Therefore all the autotrophic forms of life must generate reduced pyridine nucleotides to achieve the cellular biosynthetic reactions. We have reported earlier that in the chemoautotrophs, Nitrosomonas, Ferrobacillus and Thiobacilli the entry of the inorganic substrates is effected at the cytochrome *c* level and the conservation of energy takes place only in the terminal site or the cytochrome: O_2 oxido-reductase segment of the electron-transport chain[2,27–29]. The energy thus generated can then be utilized to effect the reversal of electron transfer from cytochrome *c* to the pyridine nucleotide[2,30], and the pathway of reverse electron transfer is mediated by the flavoprotein systems in these organisms.

The Nitrobacter system appears to be unique in that the entry of NO_2^- in its electron-transport chain is effected at the cytochrome a_1 level and that the energy conservation takes place between cytochrome a_1 and O_2 with the possible mediation of iron and/or copper[9]. If NO_2^- were to couple with cytochrome *c*, then an energy gap of some 8 kcal must be bridged in view of the E'_0 of NO_2^-/NO_3^- system being + 0.43 V compared to the E'_0 of the cytochrome *c* system (+ 0.25 V)[31]:

$$NO_2^- + H_2O + 2 \text{ ferricytochrome } c \longrightarrow NO_3^- + 2 \text{ ferrocytochrome } c + 2\ H^+ \qquad (1)$$
$$\Delta F' = + 8.3 \text{ kcal}$$

A further investment of about 26 kcal of energy would be required for the reduction of pyridine nucleotides by ferrocytochrome *c*:

$$2 \text{ Ferrocytochrome } c + 2\ H^+ + NAD^+ \longrightarrow 2 \text{ ferricytochrome } c + NADH + H^+ \qquad (2)$$
$$\Delta F' = + 26.3 \text{ kcal}$$

While Reaction 1 appears to take place in one step (involving energy-linked reversal of electron transfer from cytochrome a_1 to cytochrome *c* (Fig. 2), Reaction 2 would probably involve at least two steps, since flavoproteins mediate the reduction of NAD^+ by ferrocytochrome *c*. We have also observed the presence of Q-10 in the Nitrobacter electron-transport particles and possibility cannot be excluded that Q-10 is also involved as an electron carrier in this process. Thus the pathway of electron transport from NO_2^- to NAD^+ may therefore be presented as follows:

$$NO_2^- \longrightarrow \text{cytochrome } a_1 \xrightarrow{ATP} \text{cytochrome } c \xrightarrow{ATP} \text{cytochrome } b\ (Q_{10}) \longrightarrow \text{flavoproteins} \xrightarrow{ATP} NAD^+$$

The proposed pathway of energy-linked reverse electron flow from NO_2^- to NAD^+ is in harmony with the observed inhibitory effects of various inhibitors acting specifically at various segments of the electron-transport chain (Table II). It may be of interest to mention that while in the chemoautotrophs Nitrosomonas and Thiobacillus, the ATP-dependent reduction of pyridine nucleotides is insensitive to oligomycin[2,27], the latter exerts a marked inhibition upon the energy-transfer reactions in Nitrobacter, an observation which is analogous to the one observed in mammalian systems. Moreover, the electron transport and coupled phosphorylation with NADH as the electron donor is inhibited by rotenone or amytal, antimycin A or HQNO, and CN^- (ref. 9); these compounds are also potent inhibitors of the energy-linked reverse electron flow from NO_2^- to NAD^+. In addition the ATP-dependent reversal of electron transfer in Nitrobacter is also very sensitive to the uncouplers of the energy-transfer reactions. In view of these observations it is reasonable to conclude, therefore, that the process

of energy-linked electron transport from NO_2^- to NAD^+ involves a reversal of the complete sequence of the reactions of oxidative phosphorylation in the chemoautotroph *Nitrobacter agilis*.

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