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Reduction of NAD^+ by the reversed respiratory electron flow in *Azotobacter vinelandii*

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Vesicles from *Azotobacter vinelandii* OP form NADH by reversed electron transport via site I of respiration. The reaction is strictly dependent on ATP as the energy source, on succinate or H_2 as the reductant and on two soluble protein fractions which also have ATPase activity. The ATP/ADP ratio in the assays is critical for maximal activity. Rates amount to 20–30 nmol NADH formed per min per mg protein. NADPH is also formed by the reversed respiratory electron flow with slightly higher activity. Succinate and NAD(P)H-dependent respiratory O_2-uptake by the vesicles are strongly affected by ATP and NAD(P)H-dependent O_2 uptake also by NAD^+. It is suggested that the reversed electron flow in *Azotobacter* acts as a regulatory valve and may provide reductant for N_2 fixation and nitrate reduction when the concentration of ATP is high and that of NAD(P)H is low in the cells.

Introduction

It has been known for some time that part of the respiratory electron flow is reversible. Chance and Hollunger [1,2] demonstrated a succinate-linked NAD^+ reduction which was strictly energy dependent in mitochondria from a variety of sources. This reversed respiratory electron flow has no obvious physiological role in mitochondria, whereas a function is likely in photosynthetic bacteria [3]. The experiments of Keister and Yike [4], using chromatophores from *Rhodospirillum rubrum*, indicated that this bacterium generates ATP either in the light by cyclic photophosphorylation or in the dark by respiration. The reduction of NAD^+ by succinate proceeds in the dark and uses site I of the respiratory electron-transport chain in the reversed direction. The reaction is strictly dependent on energy which can be provided by ATP. These results were extended to an energy-dependent NAD^+ reduction in chromatophores from *Rhodopseudomonas capsulata* using molecular H_2 as the reductant [5].

To our knowledge, the reversed respiratory electron flow has not yet been investigated in *Azotobacter*. Respiration of *Azotobacter* utilizes NADPH as well as NADH as electron donor. Electron transfer in the terminal region occurs via a branched cytochrome system. Remarkably, oxidative phosphorylation in *Azotobacter* is less sensitive to classical uncoupling than in mammalian mitochondria. Likewise, the respiratory control, defined as the stimulation of respiration by ADP and inorganic phosphate, is poorly expressed in particles and possibly also in intact cells of *Azotobacter* [6–8]. *Azotobacter* has been studied extensively with respect to its aerobic N_2

Abbreviations: P_i, inorganic phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PMS, phenazine methosulfate; DCIP, dichlorophenolindophenol; DBMIB, dibromothymoquinone.

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fixation. Many aspects of this latter reaction are, however, not understood currently. This is particularly true for the generation of the reductant for N_2 fixation by nitrogenase [9]. More recent experiments indicate a direct relationship between nitrogenase activity and the rate of the electron transfer through the respiratory chain [10]. The nature of this interaction between respiration and N_2 fixation remains uncertain. A possible role of the reversed respiratory electron flow in N_2 fixation has not yet been considered.

The present communication reports that NAD(P)H can be generated from succinate or H_2 by a reversed respiratory electron flow in a preparation from *Azotobacter*. The regulatory properties of the reversed electron flow indicate that the reaction may have impacts on N_2 fixation and assimilatory nitrate reduction in *Azotobacter*.

Materials and Methods

Lyophilized stock material of *Azotobacter vinelandii* OP was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, F.R.G. (DSM no. 366 = ATCC 13705). The organism was grown in a 10 l fermenter in batch cultures under N_2 fixing conditions and under vigorous aeration. The medium consisted of Burk salts [11] supplemented with 2% sucrose. After 24–36 h of growth, the cells were harvested by rapid centrifugation and stored at $-20\,^\circ$C prior to use.

The vesicles and the supernatant were prepared essentially in the same way as described [8]. 1–2 g cells (wet weight) were resuspended, twice washed in ice-cold water and centrifuged ($12\,000 \times g$, 10 min), and the cells were sonicated (2 min, power setting 7, microtip of an Ultrasonics sonicator) at 0–2 °C. The broken cells were centrifuged twice ($3000 \times g$, 10 min, then $16\,000 \times g$, 20 min). The supernatant was incubated for 20 min with 0.1 M KCl dissolved in 2 mM phosphate buffer (pH 7.0) in ice. When the effects of P_i were to be investigated, the phosphate buffer was substituted by 2 mM Hepes buffer (pH 7.0). The incubation was followed by a centrifugation ($140\,000 \times g$, 30 min), and the upper 2/3 of the supernatant was used as the supernatant fraction in the experiments. The lower 1/3 of the supernatant was discarded and the surface of the pellet was gently washed once with 1 vol. of distilled water. The red brownish sediment was homogeneously suspended in 1.5 ml of a 0.08 M ice-cold KCl solution and used as the vesicles (i.e. particulate fraction). The pH was monitored all over the procedures and ranged between 6.5 and 7.0.

The standard assay for the NAD(P)H formation by the reversed electron transport contained in a final vol. of 1 ml: vesicles, 0.15–0.25 mg protein, supernatant 0.5–1.0 mg and in μmol: Tris-HCl buffer (pH 7.5), 150; sodium succinate, 20; ATP, 2; $NAD(P)^+$, 1; $MgCl_2$, 10. The gas phase was either air or argon and the temperature 25 °C. NAD(P)H formation was measured spectrophotometrically at 340 nm or fluorometrically (in the initial experiments) by following the emission at 440 nm during the excitation at 340 nm. When succinate was replaced by H_2, the experiments were performed in sealed cuvettes under an atmosphere of H_2.

For determining the amounts of fumarate formed in the succinate-dependent reversed electron transport, a 0.2 ml aliquot of the assay mixture was supplemented with 0.8 ml ethanol and centrifuged (10 min, $10\,000 \times g$) to remove the protein. The supernatant was diluted with water to give a concentration of 20% ethanol. 25 μl were injected into a Waters model 510 high-pressure liquid chromatograph equipped with a C-18 Novapak column (10 cm × 0.7 cm, 5 μm mesh). Fumarate was eluted from the column with a mixture of methanol and 1% H_3PO_4 (1/20, v/v). The flow rate was 1 ml/min. The amount of fumarate was determined by the absorbance at 202 nm, and the retention time for fumarate was 170 s under the conditions employed.

For measuring the residual succinate concentration in this assay, 0.75 ml 1 M $HClO_4$ was added to a 0.25 ml aliquot of the standard assay mixture and the denatured protein was removed by centrifugation. The supernatant was neutralized with KOH. After standing for 20 min at 4 °C, the sample was centrifuged (10 min, $10\,000 \times g$) to remove the $KClO_4$ formed, and the amount of succinate in the supernatant was determined by the conversion to succinylcoenzyme A in the presence of ITP, coenzyme A and succinylcoenzyme A synthetase. The IDP formed reacted with phospho*enol*pyruvate to form ITP and pyruvate in the

presence of pyruvate kinase. Pyruvate was then converted to lactate with NADH and lactate dehydrogenase. The amount of NADH oxidized (determined by its absorbance at 340 nm) should be proportional to the concentration of succinate. More details of this assay are given in an information sheet from Boehringer [12]. However, under our assay conditions, the conversion of succinate to lactate took much longer than the 20 min indicated by Boehringer, and also did not reach completion. Therefore, the data were always corrected against an internal succinate standard.

The O_2 uptake by the vesicles was followed amperometrically with a conventional Clark-type electrode. The electrode chamber contained in a final vol. of 1.6 ml: vesicles with 0.003–0.06 mg protein and in μmol: Tris-HCl buffer (pH 7.5), 150; NAD(P)H, 2.0, or succinate, 10, and, when added, ATP, 2; $MgCl_2$, 10, or ADP. 4; K_2HPO_4/KH_2PO_4 buffer (pH 7.5), 20; $MgCl_2$, 15, or NAD^+ 2.5 (in this last case the amount of NADH in the chamber was 0.25 μmol). NAD(P)H-dependent reduction of $K_3Fe(CN)_6$ was measured spectrophotometrically at 420 nm in cuvettes containing in a final volume of 1 ml in μmol: potassium phosphate buffer (pH 7.5), 40; NAD(P)H, 0.705; $K_3Fe(CN)_6$, 0.5 and vesicles 0.01–0.05 mg protein. Succinate dependent reduction of phenazine methosulfate (PMS) coupled with 2.6-dichlorophenolindophenol (DCIP) was followed by the reduction of DCIP at 600 nm. The cuvettes contained in a final volume of 3 ml in μmol. Sodium succinate, 20; DCIP, 0.24; PMS, 3.3 and vesicles 0.01–0.05 mg protein. In separate assays, the NADH consumed was also determined photometrically at 340 nm and the ATP formed was measured luminometrically using the firefly method.

ATPase activity was determined in test tubes in a final vol. of 1 ml containing: protein, 0.01–1.5 mg; ATP, 1 μmol; $MgCl_2$, 10 μmol. After 30–60 min of incubation, a 0.3 ml aliquot was mixed with 0.5 ml 0.48 M $HClO_4$. After centrifuging and neutralizing, the amount of P_i was determined by the modified Fiske-Subbarow test [13]. The unspecific, nonenzymatic P_i release was always substracted from the data. Protein was determined by either the Biuret or the Bradford method. Chemicals were purchased from Boehringer or Sigma.

Results

The respiratory activities of the vesicles (particulate fraction) used in the present study were comparable to those of preparations from *Azotobacter* described by others [9,14]. The vesicles catalyzed an O_2 consumption dependent on NADH, NADPH or succinate (Table I). The O_2 uptake was accompanied by an ATP formation, but the ratio between ATP formation and O_2 uptake was smaller than 1 : 1 (see Table III) as described for similar preparations [8]. The O_2 consumption rates by the vesicles were only slightly enhanced by ADP and P_i (Table I) indicating that the coupling between electron transport and phosphorylation was not tight with such preparations.

TABLE I

REGULATION OF THE RESPIRATORY ELECTRON TRANSPORT IN VESICLES FROM *AZOTOBACTER* BY ATP, ADP + P_i AND NAD^+

The data refer to the respiratory forward reaction. Rates are given in nmol O_2 consumed, $K_3Fe(CN)_6$ or DCIP reduced/min per mg protein. O_2 uptake was followed amperometrically and $K_3Fe(CN)_6$ [37] or DCIP reduction [38] photometrically. The extinction coefficients used were for $K_3Fe(CN)_6$ 1 $mM^{-1} \cdot cm^{-1}$ at 420 nm [37] and for oxidized DCIP 21 $mM^{-1} \cdot cm^{-1}$ at 600 nm [37]. The reaction mixture was preincubated with the effectors (ATP, ADP + P_i or NAD^+) for 3 min in all cases before the reaction was started by adding the electron donors (NADH, NADPH or succinate). The preparation of the vesicles and the assay conditions are described under Materials and Methods.

Electron donor; effector	O_2 consumption	$K_3Fe(CN)_6$ reduction	DCIP reduction
1. NADH			
–	3760	11405	–
ADP + P_i	5400	14825	–
ATP	210	11421	–
NAD^+	1520	5201	–
2. NADPH			
–	978	2072	–
ADP + P_i	1141	2129	–
ATP	109	2112	–
NAD^+	94	326	–
3. Succinate			
–	185	–	536
ADP + P_i	212	–	516
ATP	34	–	494
NAD^+	161	–	527

The finding was striking that O_2 consumption with NADH, NADPH, and succinate was strongly affected by ATP (Table I). In all cases, the effects were more drastic when the reaction mixture was preincubated with ATP (routinely for 3 min) before adding the electron donors NAD(P)H or succinate (not documented). In contrast, NAD(P)H dependent $K_3Fe(CN)_6$ reduction and succinate-dependent reduction of dichlorophenolindophenol in the presence of phenazine methosulfate (PMS) was not affected by ATP (Table I). NAD^+ blocked O_2 uptake, $K_3Fe(CN)_6$ reduction (Table I) and NAD(P)H oxidation (not documented) with both NADH or NADPH as the electron donor. However, NAD^+ did not show effects on succinate-dependent O_2 uptake or DCPIP reduction.

The vesicles were prepared in the presence of 0.1 M KCl/2 mM phosphate buffer which was described to preserve most of the phosphorylating activity [8]. These particles could have lost succinate dehydrogenase which was reported to be either soluble [15] or particle bound [16] in *Azotobacter*. The vesicles used in the present study and not the supernatant converted succinate to fumarate with a specific activity of 180 nmol fumarate formed per min per mg protein under the standard conditions for the reversed electron transport (Fig. 1). The succinate-dependent respiratory O_2 uptake was severely blocked under these conditions due to the presence of ATP in the assay (Table I). The addition of supernatant to the vesicles slightly decreased the yield of the formation of fumarate from succinate (Fig. 1). Fumarate may have been partly metabolized further when the supernatant was present. The stoichiometry between succinate consumption and fumarate formation by the vesicles was 1 : 1.2 and 1 : 1.6 in two independent experiments in which the concentration of succinate was limiting (not documented). As described under Materials and Methods, the assay to determine succinate was critical and probably resulted in an underestimation of this compound. Therefore succinate dehydrogenase was also assayed in a different way. Succinate-dependent reduction of DCIP in the presence of PMS was detected only in the vesicles (Table I) and virtually not in the supernatant (not documented). All the data allow the conclusion that the vesicles convert succinate only to fumarate and

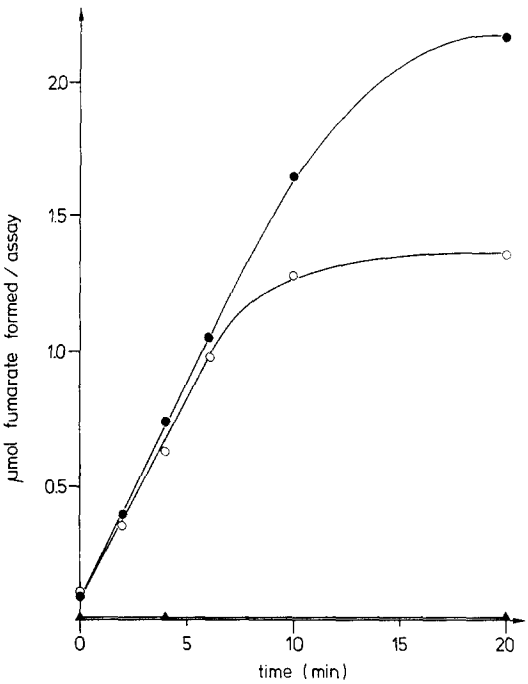


Fig. 1. The formation of fumarate from succinate by vesicles and soluble proteins from *Azotobacter vinelandii*. The experimental details are given in Materials and Methods. ●———●, activity of the vesicles (particulate fraction) (0.78 mg protein per assay); ▲———▲, of the supernatant from high speed centrifugation (1.3 mg protein per assay); ○———○, of the vesicles + supernatant.

that succinate dehydrogenase is bound to the vesicles in the preparation used.

The vesicles catalyzed very low rates of NADH formations by reversed electron flow. Significant activities were, however, observed when the assay contained both vesicles and a supernatant obtained at high speed centrifugation (Table II). NADH formation was strictly dependent on ATP, NAD^+ and succinate and therefore due to reversed respiratory electron flow. A boiled supernatant was ineffective. Rates were approx. 20–30 nmol NADH formed per min per mg protein and thus 5–10-times lower than the rate of O_2 consumption with succinate. The particles also performed a succinate-linked and ATP-dependent reversed electron flow with $NADP^+$ as the electron acceptor with slightly higher activity (Table II). The rates of the NAD(P)H formation by the reversed respiratory electron flow were not signifi-

TABLE II

ASSAY CONDITIONS OF THE $NAD(P)^+$ REDUCTION BY THE REVERSED ELECTRON FLOW OF *AZOTOBACTER VINELANDII*

The complete assay contained in a final volume of 1 ml: vesicles, 0.19 mg protein; supernatant, 0.4 mg protein and in μmol: Tris-HCl buffer (pH 7.3), 150; sodium succinate, 20; ATP, 2; $NAD(P)^+$ 1 and $MgCl_2$, 10. NADH formation was recorded photometrically. Rates are given in nmol $NAD(P)^+$ reduced/min per mg protein of the vesicles (with the exception of no. 6, where the protein concentration refers to the supernatant).

Assay condition	Rate (nmol $NAD(P)^+$ reduced per min per mg protein)
1. Complete	19.5
2. – Succinate	⩽ 0.8
3. – ATP	⩽ 0.9
4. – ATP, – succinate	⩽ 1.3
5. – Vesicles	⩽ 1.0
6. – Supernatant	⩽ 1.1
7. $NADP^+$ instead of NAD^+	26.0

TABLE III

DEPENDENCE OF THE REVERSED AND OF THE FORWARD RESPIRATORY ELECTRON FLOW ON THE REDUCTANT

Rates are given in nmol NADH formed/min per mg prpotein for the ATP-dependent reversed electron flow or in nmol O_2 consumed or ATP formed/min per mg protein for the respiratory forward reaction. NADH formation was measured in the presence of both vesicles and supernatant, and O_2 uptake and ATP formation with the vesicles alone. For other experimental conditions, see Materials and Methods. Ferredoxin (20 nmol/assay) and flavodoxin (20 nmol/assay) from *Azotobacter vinelandii* were isolated as described [31].

Electron donor	Reversed electron flow NADH formation	Forward electron flow	
		O_2 uptake	ATP formation
1. NADH	–	3663	1719
2. NADPH	–	978	838
3. Succinate	30.2	185	150
4. H_2	28.5	38	19
5. Succinate + H_2	30	190	150
6. NADH + flavodoxin	30.7	–	–
7. NADH + ferredoxin	27.3	–	–

cantly higher when the same assays were performed under argon and not in air (not documented).

It was suggested that the rate-limiting step in respiration of *Azotobacter* is at the level of the primary dehydrogenase(s) [17]. In the ATP-dependent NADH formation by the reversed respiratory electron flow, succinate could be substituted by H_2 with the same efficiency (Table III). NADH formation was not increased by adding both H_2 and succinate and was not stimulated by ferredoxin or flavodoxin from *Azotobacter* (Table III). Antibodies raised in rabbits against ferredoxin and flavodoxin did not show any effect on succinate and energy-linked NADH formation in *Azotobacter* (not documented). For comparison, the rates of the respiratory O_2 uptake and ATP formation by the vesicles using different electron donors are given in Table III. The respirtory activity was much lower with succinate or H_2 than with NADH or NADPH. The addition of H_2 did not enhance the rate of succinate-dependent O_2 uptake and ATP formation by the vesicles. Such an observation is not in accord with findings by others [17] but may be explained by differences in the preparations. H_2 uptake by the vesicles was 280 nmol/min per mg protein when PMS was the electron acceptor. Hydrogenase as well as succinate dehydrogenase apparently were not rate-limiting in the vesicles used in the present study.

Table IV gives the effects of several inhibitors on succinate-linked NADH-formation by the reversed electron flow. DBMIB (dibromothymoquinone) specifically blocks plastoquinone reactions in photosynthesis. Higher concentrations of this compound also affect the quinones of the respiratory chain from all other organisms investigated so far [18]. 50 μM DBMIB blocked the NADH formation approx. 50%, indicating that the transfer of electrons from succinate to NADH by the reversed electron flow involves the respiratory quinone (ubiquinone-8) of *Azotobacter* [19,20]. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone [11,21] and the ATP synthetase inhibitor oligomycin [21] were only slightly inhibitory (Table IV). It has already been noted by others [8,22] that these inhibitors are only poorly effective in energy-dependent reactions of *Azotobacter* for unknown reasons. The uncoupler FCCP

TABLE IV

EFFECTS OF INHIBITORS ON THE REVERSED ELECTRON FLOW IN *AZOTOBACTER*

For experimental conditions, see Materials and Methods and legend to Table II. DBMIB, 2,5-dibromothymoquinone; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

Inhibitor	Concentration	NADH formation (nmol/min per mg protein of the vesicles)
1. –		20
2. DBMIB		
	a. 10 μM	17
	b. 50 μM	8
	c. 100 μM	5
3. CCCP		
	a. 2.5 μM	17.7
	b. 5.0 μM	15.3
	c. 10.0 μM	14.3
4. Oligomycin		
	a. 1.9 μg per assay	17.9
	b. 7.5 μg per assay	16.2
	c. 15.0 μg per assay	15.8
	d. 30.0 μg per assay	15.4
5. FCCP		
	a. 0.1 μM	17.2
	b. 1.0 μM	15.1
	c. 5.0 μM	13.8
	d. 10.0 μM	11.9

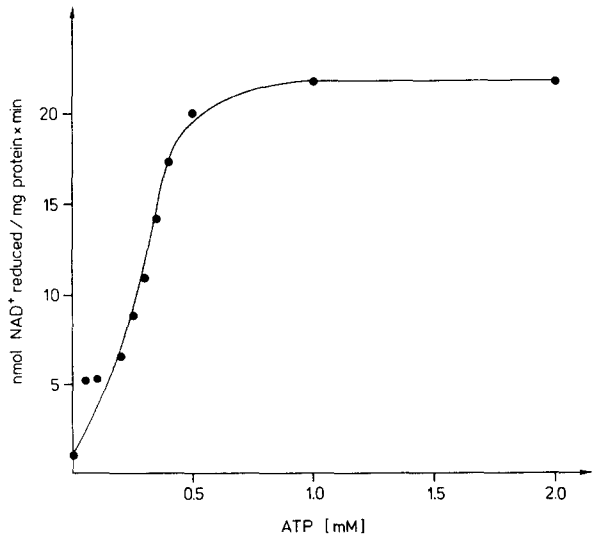


Fig. 2. The dependence of succinate linked NAD^+-reduction on ATP. The reaction mixture contained both vesicles and supernatant and the experiment was performed under standard assays conditions (see Materials and Methods).

[18] caused some inhibition, but did not totally block the reaction even at 0.01 mM. All the results with the inhibitors are in agreement with an energy-dependent reversed electron flow via site I of the respiratory chain.

The NADH formation activity by the succinate-linked reversed electron flow was strongly dependent on ATP in these assays (Fig. 2). ATP was, however, less stimulatory than a mixture of ATP and ADP (Fig. 3). The stimulation by ADP was more pronounced at lower concentrations of ATP in the vessels. Higher amounts of ADP were always inhibitory irrespectively of the concentration of ATP present. ADP alone was inactive. The

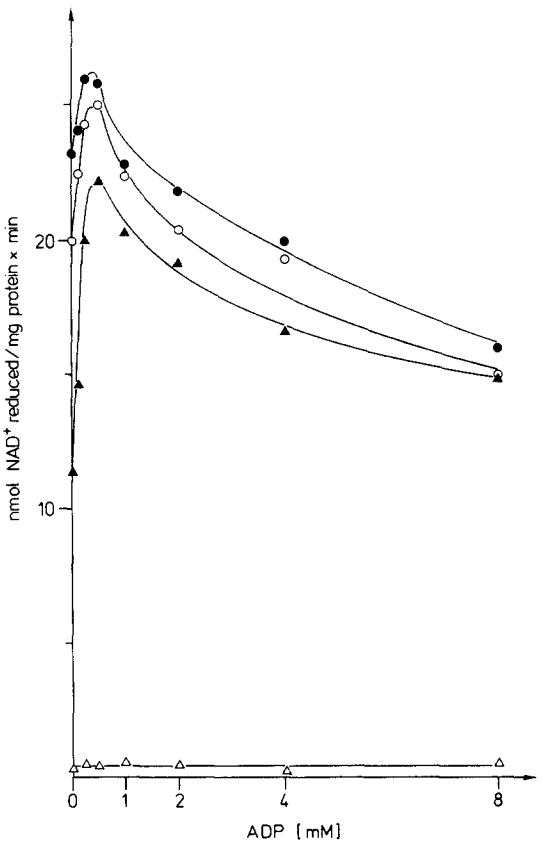


Fig. 3. The activity of the succinate-linked NAD^+ reduction in dependence on ADP. The experiment was performed under the same conditions as in Table II and Fig. 2. The values stand for the initial rates in the first five minutes. ▲———▲, assay performed with 0.25 mM ATP in the reaction mixture; ○———○, with 0.5 mM ATP; ●———●, with 1.0 mM ATP; △———△, control with ADP alone.

regulation of the succinate-linked reversed electron flow by ATP and ADP is thus complex. The adenosine nucleotides may have regulatory effects on parts of the respiratory chain or on the supply of energy for NAD(P)H-formation by the reversed electron flow. Myokinase may also be involved in the regulation. The specific activity of this enzyme was 19 nmol ATP formed per min per mg protein in the vesicles. AMP was virtually inactivate in the NADH formation assay, indicating that the ATP/ADP ratio rather than the energy charge was regulatory (not documented). ATP could be substituted by GTP to 40% of the maximal activity and by CTP to 20%, but not by pyrophosphate (not documented).

The dependence of the reversed electron transport on the addition of the supernatant obtained by high speed centrifugation was investigated further. Factors in the supernatant could be enriched by $(NH_4)_2SO_4$ precipitation. The fraction precipitating between 30 and 60% $(NH_4)_2SO_4$ was dialyzed and chromatographed on a DE-52 cellulose column using a linear NaCl concentration gradient (Fig. 4). ATPase showed two distinct activities in the elution pattern (Fig. 4) which were pooled in fraction 7 (eluting with about 0.18 M NaCl) and fraction 11 (eluting with about 0.28 M NaCl). These two fractions also stimulated the succinate- and ATP-dependent reversed electron flow by the vesicles from *Azotobacter* (Fig. 4, Table V). However, fraction 7 had lower ATPase activity but did better in stimulating NADH-formation by the reversed electron transport. The reverse was true for fraction 11. Both fractions stimulated the reversed electron transport synergistically (Table V). However, even when both were

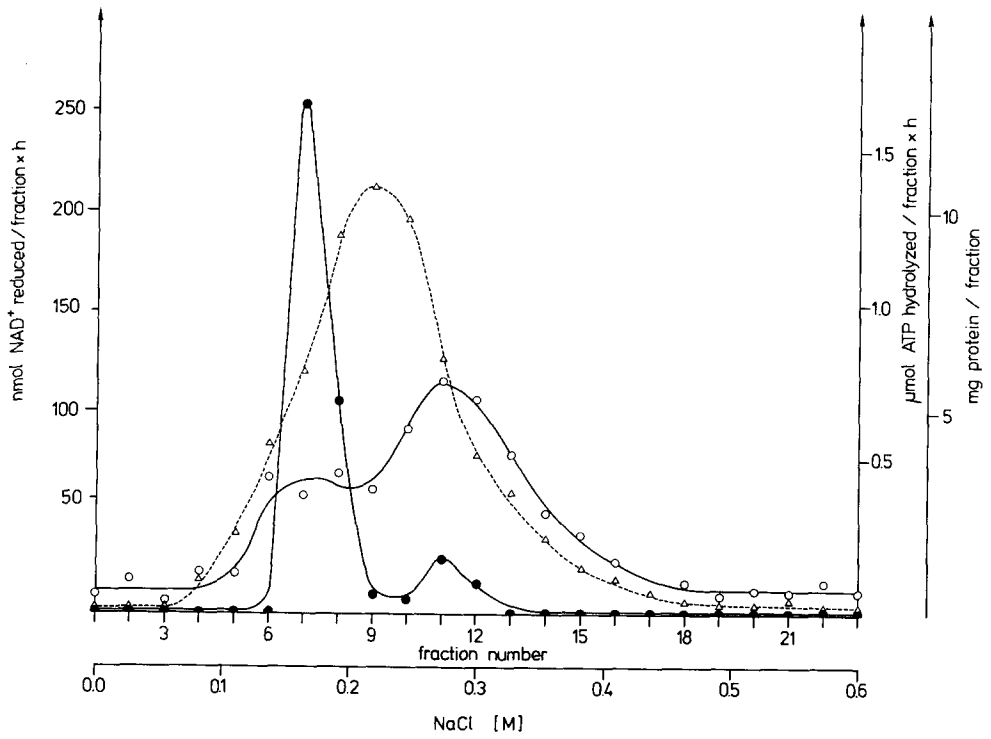


Fig. 4. The stimulation of the succinate-linked reversed electron flow by fractions isolated from the supernatant. The supernatant obtained after high speed centrifugation (140000 × *g*, 30 min, upper 2/3) was subjected to $(NH_4)_2SO_4$ precipitation. The fraction precipitating between 30 and 60% $(NH_4)_2SO_4$ was dialysed and chromatographed on a DE-52 cellulose column (0.8 × 3.5 cm) using a linear NaCl concentration gradient (0.0–0.6 M NaCl, 2 × 45 ml in Tris-HCl buffer, pH 7.5). The different eluting fractions (4 ml each) were assayed for activities. ●———●, succinate-dependent NAD^+ reduction by the reversed electron transport; ○———○, ATPase; △- - - - -△, protein.

TABLE V

STIMULATION OF THE REVERSED RESPIRATORY ELECTRON FLOW BY PROTEIN FRACTIONS ISOLATED FROM THE SUPERNATANT

The two fractions 7 and 11 were obtained by treating the supernatant with $(NH_4)_2SO_4$ and by chromatographing on DE-52 cellulose using a linear NaCl concentration gradient (see Fig. 4). The fraction numbers correspond to those in Fig. 4. The data refer to nmol NAD^+ reduced/min per mg protein of the vesicles. The test was performed under standard assay conditions as described under Materials and Methods.

Additions to vesicles	nmol NAD^+ reduced/min per mg protein
1. –	0.4
2. Supernatant (0.4 mg)	19.5
3. Fraction 7 (0.76 mg)	2.9
4. Fraction 11 (0.75 mg)	0.7
5. Fraction 7 (0.76 mg) + fraction 11 (0.188 mg)	3.0
6. Fraction 7 (0.76 mg) + fraction 11 (0.375 mg)	5.1
7. Fraction 7 (0.76 mg) + fraction 11 (0.750 mg)	7.7
8. Fraction 7 (0.19 mg) + fraction 11 (0.750 mg)	1.4
9. Fraction 7 (0.38 mg) + fraction 11 (0.75 mg)	5.4

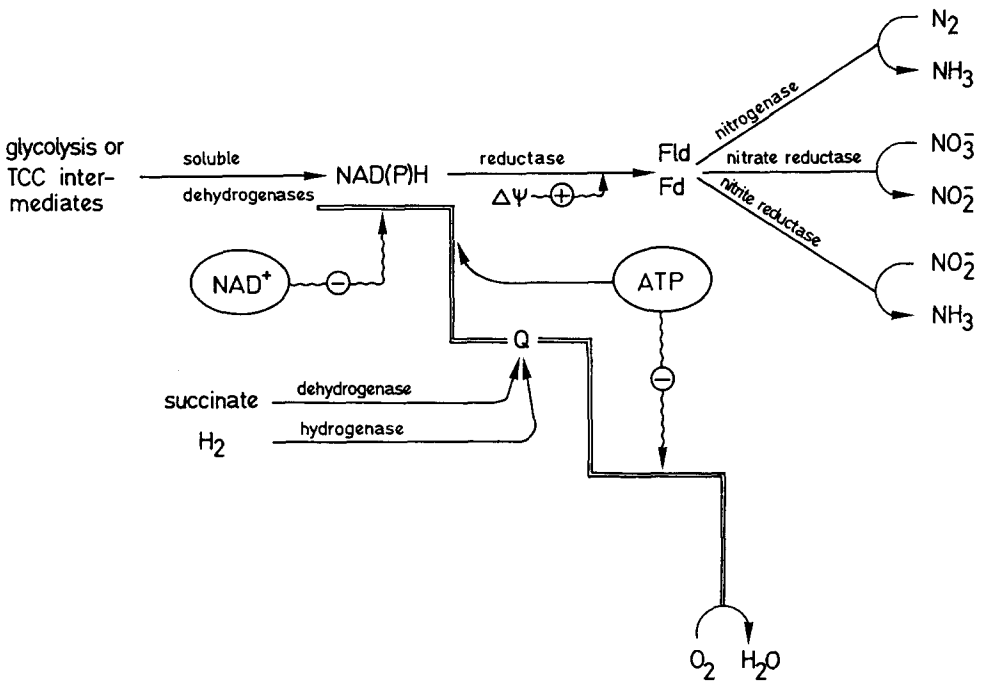
added in saturating concentrations, they could not fully restore the activity of the supernatant (Table V).

Discussion

The present communication defined the conditions for a respiratory site-I-dependent reversed electron transport in extracts from *Azotobacter*. NADH formation was strictly dependent on NAD^+, ATP and the electron donor succinate or H_2. The strict dependence of the NADH formation on the optimal ratio in the concentrations of ATP/ADP and on the addition of protein fractions having ATPase activity is worth mentioning. The vesicles performed also a NADPH formation by the reversed electron flow. In these experiments, $NADP^+$ may have either been electron acceptor alternative to NAD^+ or may have been reduced by transhydrogenation from internal NADH. A respiratory NADH dehydrogenase, a NADPH dehydrogenase and a transhydrogenase occur in *Azotobacter* [23,24]. Transhydrogenation was found to be energy dependent only when the transhydrogenase is membrane bound [25]. This was confirmed with the vesicles used here (unpublished results). Because small amounts of endogenous NAD^+ might have been present in these assays performed both with vesicles and supernatant, the requirements for the NADPH formation either directly or by transhydrogenation are met.

NADH formation from succinate by the reversed electron transport was only seen because the respiratory O_2 uptake was blocked by ATP and NAD^+. It had been reported some time ago that respiratory NADH dehydrogenase is subject to complex regulatory controls [26] and that the NADPH dehydrogenase is inhibited by ATP [14]. The data of this investigation indicated (i) that NAD(P)H dehydrogenation of the vesicles was affected by NAD^+, and (ii) that ATP blocked NADH, NADPH and succinate-dependent respiratory O_2 uptake. Therefore, NAD^+ acted at the NAD(P)H dehydrogenases, whereas regulation by ATP involved the whole respiratory electron flow of *Azotobacter* (see Scheme I). The inverse regulatory properties of the respiratory O_2 uptake with succinate or NAD(P)H as the electron donor and of the NADH formation by the reversed electron flow were so striking that physiological consequences seem to be implicit.

It could be argued that the rate of the NADH formation by the reversed electron transport is considerably lower than that of the respiratory NAD(P)H oxidation or O_2 uptake. NADH formation from succinate is thermodynamically unfavorable even though coupled with ATP hydrolysis. For comparison, the reversed respiratory electron flow in *Rhodospirillum rubrum* has undoubtedly a physiological function in the generation of reductant for photosynthetic CO_2 fixation. The rates of the ATP-driven NAD^+ reduction by chromatophore preparations from this bacterium are also not equivalent to those of the respiratory O_2 uptake or of the light-driven NADH formation [4]. Particles prepared from *Azotobacter* by others [8,14,28] and also used in the present study show a low P/O ratio. As mentioned above, the coupling between phosphorylation and electron flow may not be so tight in *Azotobacter* as in mitochondria. The NADH-formation activity by the reversed electron transport should be compared with the



Scheme I. Regulatory properties of the reversed respiratory electron transport in *Azotobacter vinelandii*.

yield in ATP in the forward reaction and not with rate of the O_2 uptake. In addition, the maximal activity of the reversed electron flow required the addition of two protein fractions isolated from the supernatant. Both had ATPase activity. There was, however, no direct correlation between their ATPase and their NADH formation activities and they were not pure. The stimulation of the reversed electron transport could be due to ATPase(s) or ATP synthetase subunits, but other factors cannot yet be ruled out. The components in the supernatant or in the fractions may only partly reassociate to the membranes when added back to the assays. This may result in a low coupling efficiency between ATP hydrolysis and reversed electron flow.

Thus the rate of NADH formation by the succinate- or H_2-linked respiratory electron transport could be much higher in vivo than in the present in vitro study. It is, however, probably smaller than the NAD(P)H formation activities catalyzed by soluble enzymes in *Azotobacter*. In crude extracts, NADH formation by glyceraldehyde-3-phosphate dehydrogenase amounted to 0.13 μmol and by pyruvate dehydrogenase to 0.227 μmol/min per mg protein. $NADP^+$-reduction rates were 0.98 μmol by isocitrate dehydrogenase and 0.014 μmol min per mg protein by glucose-6-phosphate dehydrogenase (Häger, K.-P., unpublished data). NADH formation by the reversed electron flow could, therefore, act as a regulatory valve in processes like N_2 fixation or assimilatory nitrate reduction in intact cells (Scheme I).

The regulation of the respiratory electron flow by ATP/ADP or NAD^+ indeed suggests such a function in the latter two reactions. N_2 fixation requires both ATP and NADPH [29] or, more likely, NADH [30] as the reductant in *Azotobacter*. The transfer of the electrons from NADH to nitrogenase involves the participation of a flavodoxin and a membrane-bound, unsufficiently characterized NADH:flavodoxin oxidoreductase [25,30]. The flow of electrons from NADH to flavodoxin was reported to be regulated by the membrane potential [25]. Nitrate reduction also requires NAD(P)H, reduced flavodoxin or ferredoxin [31] and ATP (ATP for the active uptake of nitrate) [22]. ATP for both N_2 fixation and

nitrate reduction is generated by respiration. Thus the regulation of respiration by ATP and NAD^+ controls the dispatch of the electrons from succinate, H_2 or other substrates. When ATP is limiting, electrons from succinate are transferred to the terminal oxidases. When the level of ATP is high and that of NADH is low, electrons from succinate are used to form NADH by the reversed respiratory electron transport to satisfy the requirements of N_2 fixation or nitrate reduction for reductant. Such a function of the reversed electron transport could explain the recently observed dependence of N_2 fixation on respiration in *Azotobacter* [10,32] and in the cyanobacterium *Gloeothece* [33].

Azotobacter does not grow autotrophically with H_2 as the sole source for energy and reductant [34] in contrast to *Rhizobium* [35]. The reversed respiratory electron flow must therefore have greater physiological importance in *Rhizobium*. In both bacteria, hydrogenase catalyzing the uptake of H_2 is membrane bound and couples to the quinone of the respiratory chain. Thus the reduction of NAD(P)H by H_2 must involve the reversed electron transport via site I of respiration in *Rhizobium* [36]. NAD(P)H is required in larger quantities as the reductant for CO_2-fixation by the reductive pentose phosphate cycle. A succinate-dependent and ATP-linked generation of NADH can, indeed, be shown in vesicle preparations of *Rhizobium japonicum* 122 DES [39].

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