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ENERGY-LINKED ELECTRON TRANSFER REACTIONS IN *RHODOPSEUDOMONAS VIRIDIS*

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SUMMARY

The particulate fraction of *Rhodopseudomonas viridis* when supplied with succinate catalyses the reduction of NAD^+ by light; this reaction is inhibited by uncouplers of oxidative phosphorylation but not by oligomycin. Formation of NADH takes place in the dark when ATP or PP_i is supplied. Both light and dark reactions are inhibited by valinomycin and nigericin, when added together, but not by either separately. NADH formation in *R. viridis* appears to take place by an energy-dependent reversal of electron flow and energy may be conserved in the form of a membrane potential. The addition of ATP caused the oxidation of both C553 and C558 in chromatophores; carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and oligomycin abolished this oxidation.

The NAD^+ and NADH concentrations at equilibrium in the light-dependent reaction were determined and the oxidation–reduction potential of this couple calculated. From this value it was calculated that under these experimental conditions the energy requirement to form NADH from the succinate/fumarate couple at E_h = 0 V was 9.4 kcal.

Particles of *R. viridis* contained an active transhydrogenase, driven by either light or ATP, that was sensitive to uncouplers of oxidative phosphorylation; the light-driven reaction was insensitive to oligomycin and was inhibited by antimycin A and 2-heptyl-4-hydroxyquinone-*N*-oxide.

R. viridis did not grow aerobically but particles contained NADH oxidase activity that was cyanide sensitive. There was no spectroscopic evidence for cytochromes of the *b*-type in reduced-*minus*-oxidised spectra of particles or in pyridine haemochrome spectra of whole cells.

INTRODUCTION

Rhodopseudomonas viridis differs from other purple non-sulphur bacteria in that the absorption bands of its bacteriochlorophyll are at longer wavelengths than the corresponding bands of the usual bacteriochlorophyll[1] and it is unusual in having no capacity for dark aerobic growth. Olson and Nadler[2] examined light-induced ab-

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinone-*N*-oxide.

sorbance changes in anaerobic whole cells of this organism and detected the oxidation of three *c*-type cytochromes (C550.5, C553 and C558) and possibly of a *b*-type or a cytochrome *cc'*. A reaction centre preparation contains a photochemically active bacteriochlorophyll called P960, and two *c*-type cytochromes, C558 and C553 of relatively high and low potential, respectively, either of which may donate electrons to oxidised P960[3,4]. We have examined electron transport reactions in chromatophore preparations of this organism and found that they carry out photophosphorylation and the light-induced reduction of NAD^+. NADH formation appears to proceed only by an energy-linked reversed electron flow process and we have attempted to determine the relationship between energy coupling and reduced pyridine nucleotide metabolism in this organism. Other photosynthetic bacteria in which this mechanism for NADH formation has been found are capable of aerobic growth and so might logically be expected to have a coupling site between NADH and the electron transport pathway[5], as is found in mitochondrial respiration, but such a coupling site would not be expected in the obligate anaerobe *R. viridis*.

MATERIALS AND METHODS

Growth of organisms

The strain of *R. viridis* used in this work (Pfennig's original strain) was a kind gift from Dr C. S. Dow, Department of Microbiology, University of Edinburgh. It was grown anaerobically in the light in the medium of Sistrom[6] at 30 °C.

Preparation of particles from Rhodopseudomonas viridis

Cells were harvested, washed in 100 mM Tris–HCl (pH 7.5) suspended in 10 mM Tris–HCl (pH 7.5) containing deoxyribonuclease (approx. 10 μg/ml) and disrupted by two passages through a French pressure cell at 10 tons/inch^2. Unbroken cells and large debris were removed by centrifugation at 30000 $\times$ *g* for 14 min and the particles collected and washed by centrifugation at 100000 $\times$ *g* for 90 min.

Measurement of NADH formation

The method used is essentially that given by Keister and Yike[5]. Chromatophores (approx. 0.06 mg bacteriochlorophyll) were suspended in a cuvette, in 3 ml of 50 mM Tris–HCl (pH 7.5) containing 1.3 mM $MgCl_2$, 3.3 mM succinate and 6.6 mM NAD^+. When ATP was added, a concentration of 0.33 mM was used. Incubations were carried out at 30 °C either in normal fluorimeter cuvettes with a 5-min preincubation to reduce dissolved oxygen before addition of NAD^+, or in anaerobic Thunberg cuvettes in an atmosphere of oxygen-free nitrogen. NADH formation was measured fluorimetrically. Actinic illumination was provided by 100 W quartz tungsten–iodine lamp screened by a Kodak-Wratten 88A Filter and the photomultiplier was protected by a Corning 9782 blue glass filter. Fluorescence was collected from the front face of the cuvette, and the production of NADH confirmed by adding lactate dehydrogenase and pyruvate at the end of the incubation.

Transhydrogenase activity

The transhydrogenase activity was measured according to the method reported by Keister and Yike[7] with $NADP^+$ as the hydrogen acceptor. Chromatophores

(approx. 0.06 mg bacteriochlorophyll) were suspended in a cuvette in 3 ml 50 mM Tris–HCl (pH 7.5) containing 1.3 mM $MgCl_2$, 0.06 mM NADH, 0.26 mM $NADP^+$, 0.2 M ethanol and an excess of alcohol dehydrogenase. ATP, when added, was 0.33 mM. Incubations were carried out either in Thunberg cuvettes under N_2 or in normal fluorimeter cuvettes at 30 °C with a 5-min preincubation before addition of $NADP^+$. NADPH formation was measured fluorimetrically, using the filter combination described for NADH formation. NADPH formation was confirmed by the addition of oxidised glutathione and glutathione reductase at the end of the incubation.

Bacteriochlorophyll

Bacteriochlorophyll was determined spectrophotometrically at 790 nm after extraction into acetone–methanol, assuming the extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$ given by Clayton[8] for bacteriochlorophyll *a*.

Spectrophotometry

Split-beam spectra were recorded using a machine constructed according to the principles outlined by Yang and Legallais[9]. The monochromator used was a Hilger D.330 and slit widths were 0.5 mm. For light-*minus*-dark spectra the filter combination was that described for use in the fluorimeter. The dual-wavelength spectrophotometer[10] used in this work was constructed using two Hilger D.330 monochromators.

Photophosphorylation

The pH change following the formation of ATP was measured using a sensitive pH meter by the method of Nishimura *et al.*[11]. The reaction mixture contained in 5 ml: 100 mM KCl, 10 mM $MgCl_2$, 0.4 mM succinate, 5 mM P_i, 0.4 mM ADP and particles (approx. 0.12 mg bacteriochlorophyll). The pH of the final solution was adjusted to pH 7.4 with tetramethylammonium hydroxide. A quartz tungsten–iodine lamp screened with a Kodak Wratten 88A Filter was the source of illumination and the reaction was measured at 30 °C.

RESULTS

Illumination of particles of *R. viridis* resulted in the reduction of NAD^+, if succinate was supplied as electron donor. NADH was formed under both aerobic and anaerobic conditions at approximately equal rates. Rates were low if ascorbate or mercaptoethanol were electron donors. NADH formation took place in the dark if energy was supplied as ATP or pyrophosphate, and some stimulation of the rate was found in the presence of bovine serum albumin. This reaction had the properties of reversed electron flow previously described for *Rhodospirillum rubrum*. When ATP-treated incubation mixtures were illuminated, NADH formation was stimulated but not to a rate equal to the sum of the two separate reactions (Table I). The succinate-linked reaction was inhibited by the uncoupler carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) when either light or ATP was the energy source; antimycin A or 2-heptyl-4-hydroxyquinone-*N*-oxide (HQNO) inhibited the light reaction strongly (Table II) but had little effect on the ATP-driven reaction. Oligomycin af-

TABLE I

RATES OF SUCCINATE-LINKED NAD^+ REDUCTION BY PARTICULATE FRACTION OF *R. viridis*

NADH formation was measured fluorimetrically as described under Materials and Methods. The reaction mixture contained in 3 ml: 50 mM Tris–HCl (pH 7.5), 1.3 mM $MgCl_2$, 3.3 mM succinate, 6.6 mM NAD^+ and the particles (approx. 0.06 mg bacteriochlorophyll). ATP when added was 0.33 mM.

Reaction conditions	*Rate of NADH formation (μmole/min per mg bacteriochlorophyll)*
Anaerobic + light	0.15
Anaerobic + ATP	0.16
Anaerobic + ATP + light	0.185
Aerobic + light	0.13
Aerobic + ATP	0.15
Aerobic + PP_i (1 mM)	0.05
Aerobic + ATP + light	0.17
Aerobic + light omit succinate	0
Aerobic + ATP omit succinate	0
Aerobic + light omit NAD^+	0
Aerobic + light + albumin (1 mg)	0.17
Aerobic + ATP + albumin (1 mg)	0.2

TABLE II

INHIBITION STUDIES ON SUCCINATE-LINKED NAD^+ REDUCTION BY PARTICULATE FRACTION OF *R. viridis*

Experimental conditions were as described in Table I. Except that additions to the incubation mixture were made as indicated, and incubations were carried out anaerobically, under nitrogen.

Addition	*Rate of NAD^+ reduction (% of control)*	
	Light	*ATP*
None	100	100
FCCP (1.6 μM)	5	5
Antimycin A (1.6 μM)	0	90
HQNO (33 μM)	40	90
Rotenone (10 μM)	20	30
Oligomycin (4 μg/ml)	112	5
ADP (0.3 mM) + P_i (5 mM)	50	—

fected only the ATP-driven reaction but rotenone affected both the light and ATP reactions. Addition of ADP and P_i caused an inhibition of the light-dependent reduction of NAD^+. These are characteristics of a reaction driven by the high energy state. Oligomycin prevents the formation of this state from ATP but not from photosynthetic electron flow. Antimycin A and HQNO presumably act by inhibiting cyclic photophosphorylation (see later).

Some idea of the nature of the high energy state in *R. viridis* is given by the use of the ionophore antibiotics valinomycin and nigericin. Neither antibiotic alone inhibited NADH formation but the two in combination completely abolished the reduction of NAD^+ (Table III). We would interpret this result by suggesting that

TABLE III

EFFECT OF NIGERICIN AND VALINOMYCIN ON LIGHT-DEPENDENT SUCCINATE-LINKED NAD^+ REDUCTION BY PARTICULATE FRACTION OF *R. viridis*

NADH formation was measured fluorimetrically as described in Table I. Additions were made as indicated.; 20 mM KCl was present throughout.

Addition	*Rate of light-dependent NAD^+ reduction (μmole/min per mg bacteriochlorophyll)*
None	0.13
Valinomycin (4.5 μM)	0.13
Nigericin (1 μM)	0.13
Valinomycin (4.5 μM) + nigericin (1 μM)	0

a primary event in energy conservation in particles of *R. viridis* is the establishment of a proton gradient and consequent membrane potential, positive on the inside due to the inward movement of a proton. In the presence of valinomycin the small amount of endogenous K^+ moves out of the particles, but is insufficient to overcome the potential due to the proton movement. In the presence of nigericin the H^+ is exchanged[12] for external K^+ and so the energy of the membrane becomes purely a membrane potential due to a K^+ gradient but still sufficient to energise NAD^+ reduction. However, when both nigericin and valinomycin are present, then the K^+ gradient is dissipated and no energy is available for NADH formation[13].

Another energy-linked reaction that has been found in photosynthetic bacteria is the formation of NADPH by a transhydrogenase[7]. The transhydrogenase was driven either by light or ATP although the two rates were not additive (Table IV) and was affected by inhibitors in a way very similar to the energy-linked reduction of NAD^+ (Table V), except that rotenone was almost without effect. Oligomycin stimulated the light-dependent reaction but inhibited the ATP-driven reaction. No marked

TABLE IV

TRANSHYDROGENASE ACTIVITY OF PARTICULATE FRACTION OF *R. viridis*

NADPH formation was measured fluorimetrically as described under Materials and Methods. The reaction mixture contained in 3 ml: 50 mM Tris–HCl (pH 7.5), 1.3 mM $MgCl_2$, 0.26 mM $NADP^+$, 0.06 mM NADH, 0.2 M ethanol, an excess of alcohol dehydrogenase and the particles (approx. 0.06 mg bacteriochlorophyll). ATP when added was 0.33 mM, and incubation was anaerobic.

Reaction conditions	*Rate of NADPH formation (μmole/min per mg bacteriochlorophyll)*
Anaerobic + light	0.55
Anaerobic + ATP	0.5
Aerobic + light	0.45
Aerobic + ATP	0.4
Aerobic + light omit NADH	0
Aerobic + light + ATP	0.55

TABLE V

INHIBITION STUDIES ON TRANSHYDROGENASE ACTIVITY BY PARTICULATE FRACTION OF *R. viridis*

Experimental conditions were as described in Table IV, except that additions to the incubation mixture were made as indicated.

Addition	*Rate of $NADP^+$ reduction (% of control)*	
	Light	*ATP*
None	100	100
FCCP (1.6 μM)	0	0
Antimycin A (1.6 μM)	0	100
HQNO (33 μM)	50	90
Rotenone (10 μM)	100	100
Oligomycin (4 μg/ml)	150	0

TABLE VI

EFFECT OF INHIBITORS ON NADH OXIDATION BY PARTICULATE FRACTION OF *R. viridis*

NADH oxidation was measured at 30 °C in a dual-wavelength spectrophotometer (340 nm *minus* 390 nm). The incubation mixture contained in 3 ml: 50 mM Tris–HCl (pH 7.5) and chromatophores (approx. 0.02 mg bacteriochlorophyll). 0.03 mM NADH was added to start the reaction. Additions were made to the incubation mixture as indicated. The control rate of NADH oxidation was 0.3 μmole/min per mg bacteriochlorophyll.

Addition	*Rate of NADH oxidation (% of control)*
None	100
Antimycin A (5 μM)	60
HQNO (16 μM)	60
Rotenone (10 μM)	30
KCN (8 mM)	24
Azide (8 mM)	90

change in properties of the transhydrogenase was found when incubations were carried out anaerobically, under N_2.

Although *R. viridis* was incapable of aerobic growth, particles catalysed the oxidation of NADH. This reaction was partly inhibited by cyanide, antimycin A and HQNO (Table VI) although the oxidation of succinate, measured polarographically was relatively insensitive to antimycin A inhibition (25 % inhibition at 10 μM).

Photophosphorylation was catalysed by *R. viridis* particles at rates rather faster than photochemical NADH formation and was inhibited by the same compounds (Table VII).

Addition of either succinate or NADH to *R. viridis* particles caused a reduction of the two cytochromes, C553 and C558 (Fig. 1), without any spectroscopic evidence for the reduction of *b*-type cytochromes, nor was protohaem detected in spectra of the pyridine haemochromes of *R. viridis* (Fig. 2). Such a spectrum had a symmetrical α-band with a maximum at 551 nm characteristic of haem *c* and it appears that if cytochromes of the *b*-type are present, they are at concentrations very much below those of the photo-oxidised cytochrome *c*, in agreement with Thornber and Olson[14].

TABLE VII

EFFECT OF INHIBITORS ON PHOTOPHOSPHORYLATION BY PARTICULATE FRACTION OF *R. viridis*

Photophosphorylation was measured as described under Materials and Methods. The reaction mixture contained in 5 ml: 100 mM KCl, 10 mM $MgCl_2$, 0.4 mM succinate, 5 mM P_i, 0.4 mM ADP and chromatophores (approx. 0.12 mg bacteriochlorophyll). Additions were made as indicated.

Addition	*Rate of ATP formation (μmole/min per mg bacteriochlorophyll)*
None	1.0
FCCP (1.6 μM)	0
Antimycin A (2 μM)	0
HQNO (20 μM)	0.3
Rotenone (10 μM)	0.83
Oligomycin (4 μg/ml)	0.25

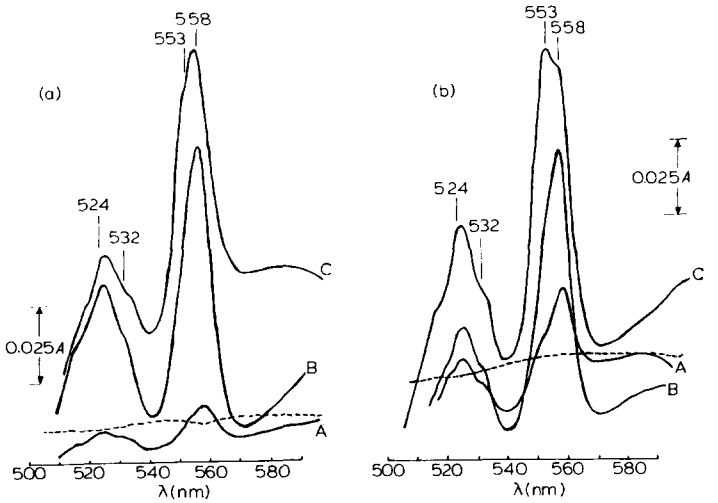

Fig. 1. Effect of addition of substrates to particulate fraction of *R. viridis*. Particles (approx. 0.24 mg bacteriochlorophyll) were suspended in 5 ml 10 mM Tris–HCl (pH 7.5) and divided between two cuvettes. 0.6 mM potassium ferricyanide was added to both test and reference cuvettes. Substrates were added either to normal cuvette or anaerobic Thunberg cuvette and spectra recorded at room temperature. (a) 3.3 mM succinate to test cuvette. Trace A recorded immediately, Trace B recorded after 5 min, Trace C recorded under anaerobic conditions using Thunberg cuvettes in an atmosphere of O_2-free nitrogen. (b) 0.3 mM NADH to test cuvette, Traces A, B and C recorded as for (a). ———, baseline.

The effect of the addition of ATP to particles of *R. viridis* is shown in Fig. 3. There was an oxidation of both C553 and C558 that was inhibited by FCCP or by oligomycin, suggesting that both cytochromes are located on the oxidising side of a coupling site.

The light-dependent reduction of NAD^+ by *R. viridis* proceeded to an equilibrium (Fig. 4) and this property was used to calculate the approximate energy requirements of NADH production. Particles were suspended in a medium where the potential was fixed by the succinate/fumarate couple and the ratio NAD^+/NADH

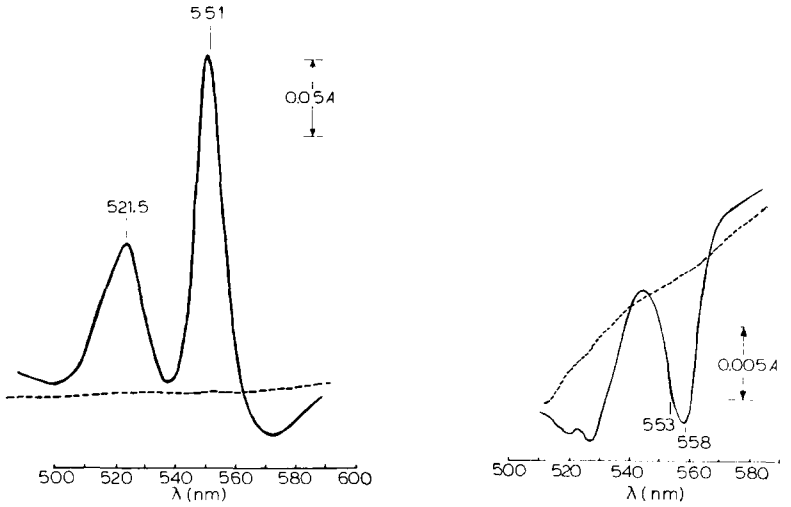

Fig. 2. Reduced-*minus*-oxidised pyridine haemochrome spectrum of whole cells of *R. viridis*. Bacteriochlorophyll and carotenoids of whole cells (approx. 0.02 mg bacteriochlorophyll) were removed by extraction with acetone–methanol, the residue was dissolved in 6 ml 20% (v/v) pyridine in 0.1 M NaOH and divided between two cuvettes. Dithionite was added to the test cuvette and the difference spectrum recorded at room temperature. – – – –, baseline.

Fig. 3. Energy-linked cytochrome oxidation in particulate fraction of *R. viridis*. Particles (approx. 0.12 mg bacteriochlorophyll) were suspended in 5 ml 10 mM Tris–HCl (pH 7.5) and divided between two cuvettes. 1.3 mM $MgCl_2$ was added to both test and reference cuvettes, which were evacuated and flushed with nitrogen; 0.33 mM ATP was added to test cuvette from the side-arm and the difference spectrum recorded at room temperature. – – – –, baseline.

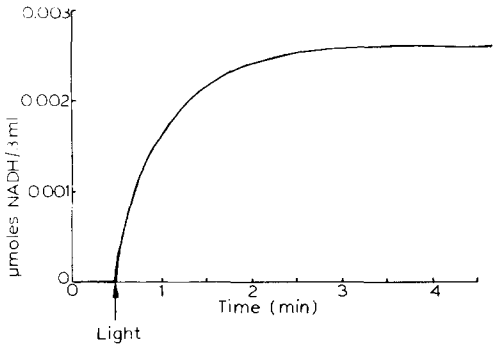

Fig. 4. Time course of light-driven NAD^+ reduction by particulate fraction of *R. viridis*. NADH formation was measured anaerobically described under Materials and Methods. The reaction mixture contained, in 3 ml: 50 mM Tris–HCl (pH 7.0), 1.3 mM $MgCl_2$, 3.3 mM succinate, 3.3 mM fumarate, 6.6 mM NAD^+ and chromatophores (approx. 0.06 mg bacteriochlorophyll). Light was switched on where indicated.

at the light-dependent equilibrium was determined. The potential of this NAD^+/NADH couple could be calculated using the standard Nernst equation.

$$E_h = E'_0 + \frac{RT}{nF} \ln \frac{[NAD^+]}{[NADH]}$$

The mid-point potential for the NAD^+/NADH couple was taken to be −0.32 V and for the succinate/fumarate couple, 0.0 V and the energy requirement, ΔG, to maintain this steady state reduction of NAD^+ is given by the equation

$$\Delta G = -nF\Delta E_h$$

n is the number of electrons lost or gained per molecule, F is the Faraday constant (23062 cal·V^{-1}·$equiv^{-1}$), ΔE_h is the difference in potential of the NAD^+/NADH couple and the succinate/fumarate couple. Using the experimental conditions described in Fig. 4 average values for ΔG of 9.4 kcal were determined.

Antimycin A has been shown to cause a cross-over point between cytochromes *b* and *c* in other purple non-sulphur bacteria[15,16] as in mitochondrial electron transport. The absence of detectable cytochrome *b* in reduced-*minus*-oxidised difference spectra caused us to examine more closely the site of action of antimycin A in *R. viridis*. The steady-state level of cytochrome *c* oxidation induced by steady illumination was not significantly affected by antimycin A, nor was the rate of dark reduction. However, in xenon-flash experiments, where there was a limited turnover of electron transport components, the rate of reduction of oxidised cytochrome *c* (measured at 555 nm) was much inhibited. The reaction of "bulk" cytochrome *c* appeared to mask this inhibitory effect upon reaction centre components in steady-state illumination experiments. No spectroscopic evidence was found for the accumulation of a component that was more reduced in the presence of antimycin A.

DISCUSSION

Because *R. viridis* is not capable of aerobic growth it might be expected to lack the energy-yielding pathway of electron flow from NADH to oxygen, or other high potential acceptors, that is found in mitochondria and in those photosynthetic bacteria capable of heterotrophic growth. In previously described systems NADH is coupled to the respiratory chain by cytochromes of the *b*-type[17,18], and this cytochrome appears to be lacking in *R. viridis*. Our results show clearly, however, that in *R. viridis* NADH formation by particles also proceeds *via* some high energy site of the electron transport pathway. It is driven either by light or by ATP and is abolished by treatment by uncouplers of oxidative phosphorylation. Photosynthetic formation of NADH by reversed electron flow is therefore not restricted to heterotrophic bacteria and could be important in strictly anaerobic bacteria such as *Chromatium*, where the mid-point potential of X, the primary electron acceptor is about −0.12 V, rather high for effective direct reduction of NADH[19]. Oligomycin inhibits the ATP-driven but not the light-driven formation of NADH and this indicates that a high energy precursor of ATP is utilised and not ATP itself. Since nigericin alone or valinomycin alone did not inhibit NADH formation whilst a mixture of the two was completely inhibitory, it appears that light energy may be transduced into the form of either a pH gradient or a membrane potential and then be utilised in energy conservation. Such a mechanism would be in accord with the chemiosmotic hypothesis of Mitchell[20] and with the observations of Jackson *et al.*[13] who showed that net energy-dependent ion fluxes in chromatophores of the photosynthetic bacterium *R. rubrum* were abolished by the combined addition of nigericin and valinomycin. Since the mid-point potential of C558 is near + 0.33 V (ref. 14), at least 2 molecules of ATP would be

needed to drive electrons from C558 to NADH. A more likely donor of electrons to NAD^+ must be C553 which has a mid-point potential near −0.02 V, or some other compound of about this potential; this would give a ΔG near that obtained in our experiments. It is interesting that when ATP is added to *R. viridis* particles an oxidation of both C553 and C558 appears to take place (Fig. 3) suggesting that each cytochrome is associated with the oxidising side of a coupling site, although energy-dependent changes in mid-point potential of these carriers could also explain these difference spectra[21].

Although *R. viridis* does not grow aerobically it does contain an NADH oxidase system that is sensitive to antimycin A, rotenone and cyanide. The oxygen electrode traces suggest that the terminal oxidase that is operating has a relatively high K_m for oxygen but it remains to be further characterised.

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