Synthesis of pyrophosphate coupled to the reverse energy-linked transhydrogenase reaction in *Rhodospirillum rubrum* chromatophores

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Chromatophores from the photosynthetic bacterium *Rhodospirillum rubrum* contain a membrane-bound transhydrogenase catalyzing the transfer of a hydride ion between NADH and $NADP^+$. The reverse reaction, i.e. reduction of NAD^+ by NADPH, can furnish sufficient energy ($\Delta\tilde{\mu}H^+$) to drive the phosphorylation of inorganic orthophosphate (P_i) to pyrophosphate (PP_i). The rate of PP_i synthesis is 50 nmol PP_i formed/min per μmol Bchl which is 5% of the rate of light-induced PP_i synthesis. PP_i synthesis is inhibited by both the H^+-PPase inhibitor fluoride and the specific transhydrogenase inhibitor palmitoyl-CoA. The effects of both DCCD and uncouplers on the system provide additional evidence that the $\Delta\tilde{\mu}H^+$ generated by the reverse transhydrogenase reaction drives PP_i synthesis. The rate of PP_i synthesis can be partially inhibited by the addition of $NADP^+$, a substrate of the forward energy-consuming reaction. The $\Delta\tilde{\mu}H^+$ generated can also be used to drive ATP synthesis by the H^+-ATPase, but at a lower rate than the PP_i synthesis.

(Rhodospirillum rubrum) *Energy-linked transhydrogenase* *Pyrophosphate synthesis* *ATP synthesis* *Bioluminescence*

1. INTRODUCTION

The energy-linked nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) catalyses the reduction of $NADP^+$ by NADH. The enzyme functions as a proton pump and translocates protons across the membrane according to reaction 1 (reviews [1,2]).

$$nH^+_{out} + NADP^+ + NADH \rightleftharpoons nH^+_{in} + NADPH + NAD^+ \quad (1)$$

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Abbreviations: APS, adenosine 5′-phosphosulphate; Bchl, bacteriochlorophyll; BSA, bovine serum albumin; DAPP, diadenosine pentaphosphate (Ap_5A); DCCD, *N,N′*-dicyclohexylcarbodiimide; DTE, 1,4-dithioerythritol; FCCP, carbonyl cyanide *p*-trifluoromethoxyhydrazone; PCB^-, phenyl dicarbaundecarborane; ANS, 8-anilinonaphthalene-1-sulphonic acid; H^+-PPase, H^+-pyrophosphatase; H^+-ATPase, H^+-ATP Synthase

Here, the subscripts out and in refer to H^+ locations in mitochondria or bacteria; the opposite is the case for submitochondrial particles and chromatophores. The first direct evidence for the existence of this enzyme, in submitochondrial particles, was provided by Danielson and Ernster [3.] Later, it was also shown to exist in bacteria [4–7] including the photosynthetic bacterium *Rhodospirillum rubrum* [8,9]. The energy coupling of the enzyme has been demonstrated by an energy-dependent 5–10-fold increase in the rate [3] and a 500-fold increase in the extent [10,11] of the reduction of $NADP^+$ by NADH. In mitochondria the transhydrogenase reaction can be driven by electron transport generated energy through any of the coupling sites of the respiratory chain, or by ATP hydrolysis [3,12]. In *R. rubrum* chromatophores, Keister and Yike [8,9] have shown that the reaction can be driven by ATP or PP_i hydrolysis and by light.

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Reversibility of the energy-linked transhydrogenase shown in reaction 1 has been demonstrated using ATP production [13], uptake of the lipophilic anion PCB^- [14–16] and by enhancement of ANS fluorescence [17]. The reaction has also been shown to induce the energy-dependent red shift of cytochrome *b* in intact mitochondria [18,19].

In addition to the H^+-ATPase, *R. rubrum* chromatophores also contain a membrane-bound H^+-PPase which catalyses both PP_i synthesis [20] and PP_i hydrolysis [21].

In these chromatophores PP_i synthesis can be achieved in both continuous [20] and intermittent light [22]. In view of the fact that the reverse transhydrogenase reaction can drive phosphorylation of ADP to ATP in submitochondrial particles [13], it seemed possible that it could also be used to drive PP_i synthesis by the H^+-PPase in the chromatophore membrane. We show here that this is the case, by monitoring continuous PP_i synthesis by a sensitive luciferase technique [23].

2. MATERIALS AND METHODS

Trizma base, glycylglycine, DTE, FCCP, oligomycin, antimycin A, valinomycin, nigericin, magnesium acetate, BSA, APS, DAPP, palmitoyl-CoA, NADPH, NADH, $NADP^+$, NAD^+ and ATP-sulphurylase (ATP:sulphate adenylyl transferase; EC 2.7.7.4) were purchased from Sigma (St. Louis, USA). Purified luciferase (EC 1.13.12.7), D-luciferin and L-luciferin were obtained from LKB Wallac (Turku, Finland). DCCD was obtained from Fluka (Buchs, Switzerland). Other materials were reagent grade and obtained from commercial sources.

2.1. *Preparation of chromatophores*

R. rubrum (strain S1) was grown anaerobically in the medium described in [24]. After 40 h of growth, cells were harvested and washed, and chromatophores prepared as in [25] with the exception that the cells were washed once in 0.2 M glycylglycine buffer, pH 7.4. Bchl was determined using an in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ at 880 nm reported by Clayton [26].

2.2. *Continuous monitoring of PP_i synthesis*

Measurement of PP_i formation was performed as in [23]. All reactions were carried out at room temperature. The standard assay volume of 1 ml contained the following components: 0.1 M glycylglycine, pH 7.75 (NaOH), 2 mM EDTA, 10 mM $Mg(OAc)_2$, 0.1% BSA, 1 mM DTE, 0.1 mg D-luciferin, 8 μg L-luciferin, 5 μM APS, 0.3 units ATP-sulphurylase, 10 mM NaP_i, 1 μM DAPP, 10 μg oligomycin, 4 μM antimycin, 5 μM rotenone, purified luciferase (for amount see [23]), chromatophores corresponding to a Bchl concentration of 0.8 μM, 0.5 mM NAD^+ and 0.5 mM NADPH to start the reaction. Phosphate solutions were incubated with yeast PPase to remove endogenous PP_i (for details see [23]).

2.3. *Continuous monitoring of ATP synthesis*

The assay was performed as for PP_i determination with the exception that the standard assay contained 50 μM ADP. Oligomycin, APS and ATP-sulphurylase were omitted.

3. RESULTS AND DISCUSSION

3.1. *Measurement and characteristics of the transhydrogenase-driven PP_i synthesis reaction*

Table 1 lists the conditions needed to observe the

Table 1

Reverse transhydrogenase-driven synthesis of PP_i and its inhibition by various agents

Conditions	PP_i synthesis (nmol PP_i/min per μmol Bchl)
(1) No antimycin and rotenone	340
(2) With antimycin (4 μM) and rotenone (5 μM)	50
(a) − 10 mM NaPi	0
(b) + 10 mM NaF	0
(c) + 0.1 mM DCCD	0
(d) + 5 μM palmitoyl-CoA	5
(e) + 0.5 mM NADH	0

Continuous PP_i synthesis was measured as described in section 2. Variations were omission of antimycin and rotenone (1), omission of NaP_i (2a), addition of NaF 1–2 min after the start of the reaction (2b), preincubation with DCCD or palmitoyl-CoA for 5 min prior to addition of NADPH (2c,2d) and replacement of NADPH by NADH (2e). The data given have been corrected for PP_i synthesis in the dark (see text and fig.1)

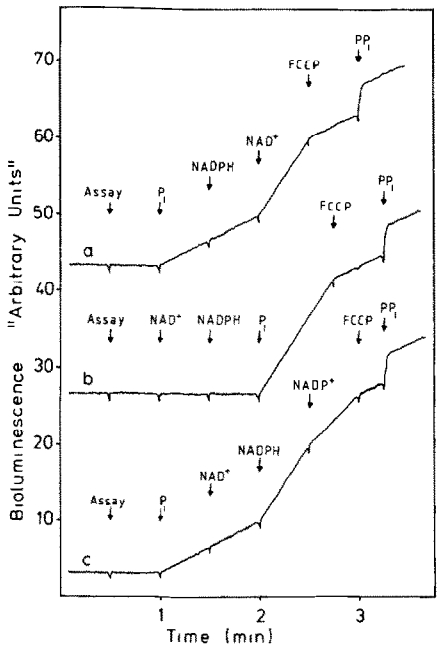

Fig.1. Profile of the reverse transhydrogenase-driven PP_i synthesis under different conditions. PP_i synthesis was measured under the conditions described in section 2 and table 1. The traces show the effect on the PP_i synthesis of (a) reversing the order of addition of NAD^+ and NADPH, (b) omitting P_i and (c) adding $NADP^+$ (0.4 mM). In all cases FCCP was added immediately before the PP_i standard. Note that the dark reaction PP_i synthesis is approx. 50% of the reverse transhydrogenase-driven reaction (a,c). The amounts of Bchl and PP_i standard added were 800 and 10 pmol, respectively.

reverse transhydrogenase-driven PP_i synthesis and the ways in which it can subsequently be inhibited. In fig.1 we show the time course of PP_i synthesis under various conditions. To eliminate ATP synthesis or hydrolysis by the H^+-ATPase, oligomycin was included in the assay system. Similarly, DAPP was included to avoid ATP synthesis by adenylate kinase. Any variations in luciferase and ATP-sulphurylase activity due to sample composition or reagent inactivation were compensated for by internal calibration with a PP_i standard in every assay.

To observe the true value of the reverse transhydrogenase-driven synthesis of PP_i, the electron transport inhibitors antimycin A and rotenone have to be included in the assay system (table 1). In the absence of both inhibitors, PP_i synthesis was 340 nmol PP_i/min per μmol Bchl. In the presence of antimycin this was inhibited 35% and in the presence of rotenone alone or of both inhibitors, by 85%. A possible source of energy for the PP_i produced might be the NADH generated in the transhydrogenase reaction, which subsequently is oxidized through the rotenone-sensitive NADH-ubiquinone reductase complex. However, addition of NADH alone in the presence of antimycin and rotenone produced no PP_i synthesis (table 1). Therefore, when these inhibitors are present to eliminate this oxidation, the true reverse transhydrogenase-driven PP_i synthesis is observed, i.e. 50 nmol PP_i/min per μmol Bchl. This value is approx. 5% of the light-driven synthesis under the same conditions (cf. [22]). The order of addition of NADPH or NAD^+ has no effect on the profile of the reverse transhydrogenase-driven PP_i synthesis (see fig.1). Confirmation that the PP_i synthesis is catalysed by the H^+-PPase is provided by absence of PP_i synthesis when the substrate P_i is omitted or when the enzyme is specifically inhibited using NaF (table 1). Similarly, blocking the proton channel of both enzymes using DCCD also leads to inhibition of reverse transhydrogenase-driven PP_i synthesis (table 1). In addition to blocking proton channels [27], DCCD has also been reported to inhibit the catalytic activity of the transhydrogenase [28].

The results in figs 1 and 2a also provide direct evidence that the driving force for this PP_i synthesis by the H^+-PPase is the $\Delta\tilde{\mu}H^+$ generated by the reverse transhydrogenase reaction. PP_i synthesis is inhibited by preincubation with the specific transhydrogenase inhibitor palmitoyl-COA (table 1). Rydström [29] first showed that palmitoyl-CoA inhibits both the forward and reverse transhydrogenase reactions in submitochondrial particles. Our observation that 5 μM palmitoyl-CoA causes 90% inhibition of PP_i synthesis is in good agreement with his results [30]. Also, the rate of PP_i synthesis is inhibited by $NADP^+$, one of the products of the reaction, with 50% inhibition at 0.4 mM $NADP^+$ (figs 1c,2a).

The PP_i synthesis observed prior to initiation of the reverse transhydrogenase-driven PP_i synthesis (typically 50%, see fig.1) is assumed to be a dark reaction which bypasses the requirement for $\Delta\tilde{\mu}H^+$ and is due to the high $\Delta G_0'$ of -11.4 kcal/mol for the ATP-sulphurylase-catalysed reaction [31],

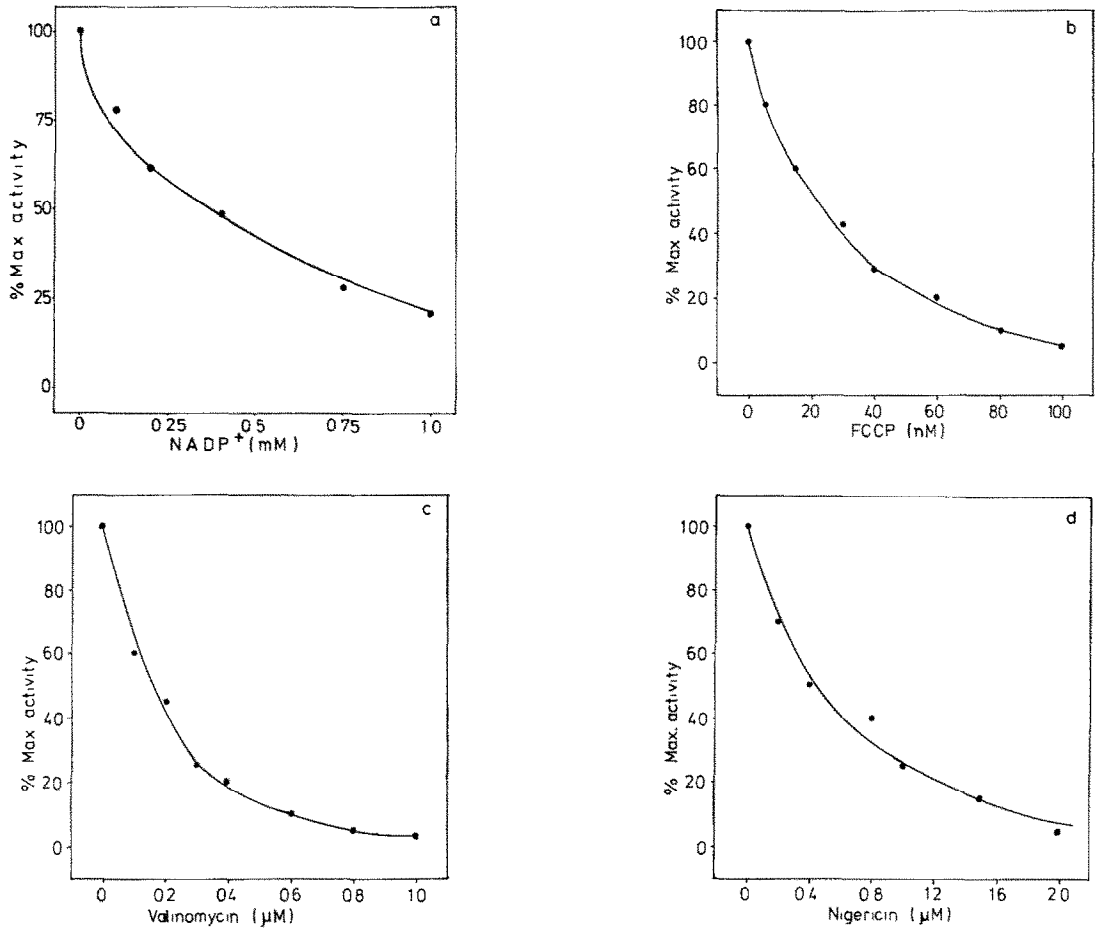

Fig.2. Titration of the reverse transhydrogenase-driven PP_i synthesis with product uncoupler and ionophores. PP_i synthesis was titrated with (a) $NADP^+$, (b) FCCP, (c) valinomycin and (d) nigericin. 100% activity values were measured as described in section 2 and have been corrected for dark reaction PP_i synthesis. Valinomycin and nigericin were incubated for 5 min prior to the addition of NADPH. $NADP^+$ and FCCP were added after the reverse transhydrogenase-driven PP_i synthesis had been established. The amounts of Bchl and PP_i added were as in fig.1.

which drives the H^+-PPase-catalysed reaction ($\Delta G_0' = -4$ kcal/mol) [32]. This is supported by the observation that inhibition of the H^+-PPase (by NaF) causes inhibition of this dark reaction.

3.2. *Dissipation of the $\Delta\tilde{\mu}H^+$ set up by the reverse transhydrogenase reaction*

Fig.2b–d shows the results of titrating the reverse transhydrogenase-driven PP_i synthesis in chromatophores with the uncoupler FCCP and with the ionophores valinomycin and nigericin, the last two in the presence of 20 mM KCl. 50% inhibition was obtained using 20 nM FCCP, 0.15 μM valinomycin and 0.4 μM nigericin. It should be noted that the capacity to form a $\Delta\tilde{\mu}H^+$ is low in this system under these conditions when compared to the conditions which lead to light-induced PP_i synthesis [22].

3.3. *ATP synthesis driven by the reverse transhydrogenase reaction*

In fig.3 is shown the time course of reverse transhydrogenase-driven ATP synthesis during which the rate reached 35 nmol/min per μmol Bchl. This is significantly lower than the rate of PP_i synthesis observed. In this experiment the $\Delta\tilde{\mu}H^+$ generated is being used both for PP_i synthesis by the H^+-PPase (although this PP_i is not

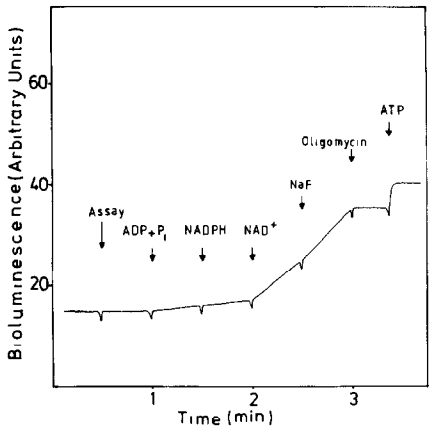

Fig.3. Profile of the reverse transhydrogenase-driven ATP synthesis. ATP synthesis was measured under conditions described in section 2. The trace shows the effect of NaF and oligomycin on ATP synthesis. The amounts of Bchl and ATP standard added were 800 and 10 pmol, respectively. Note that the response of the luciferase system is inhibited by 20% after addition of 10 mM NaF. This effect is not shown in the trace.

detected because there is no ATP-sulphurylase and APS present), and for ATP synthesis by the H^+-ATPase. Addition of NaF inhibits the H^+-PPase and so the $\Delta\tilde{\mu}H^+$ is used to a greater extent for ATP synthesis, hence the increase of 30% in synthesis of ATP. Consequently, addition of oligomycin leads to complete inhibition of ATP synthesis.

In conclusion, we have shown that it is possible to reverse the energy-linked transhydrogenase enzyme of *R. rubrum* chromatophores and use the $\Delta\tilde{\mu}H^+$ generated to drive PP_i synthesis by the H^+-PPase and ATP synthesis by the H^+-ATPase.

It is worthwhile to note that at the low energy levels obtainable by the reversal of the transhydrogenase reaction, the rate of ATP synthesis is only 70% of the rate of PP_i synthesis. This is in striking contrast to the situation when saturating light is the energy source. The rate of ATP synthesis is then 7–10-times greater than that of PP_i synthesis. On the other hand, at very low light intensity, or after single light flashes, where the energy levels also are low, the PP_i synthesis is significantly higher than the ATP synthesis [22,33].

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