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OXIDATION–REDUCTION POTENTIAL DEPENDENCE OF PYROPHOSPHATE-INDUCED CYTOCHROME AND BACTERIOCHLOROPHYLL REACTIONS IN *RHODOSPIRILLUM RUBRUM*

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SUMMARY

The oxidation–reduction potential dependence of pyrophosphate-induced reactions in chromatophores from the blue-green mutant of *Rhodospirillum rubrum* has been studied.

Two types of energy-requiring reactions are apparent: (1) Absorption changes resulting from bacteriochlorophyll interaction with a high energy state within the membrane. This reaction is independent of the oxidation–reduction potential poise between −475 mV and +430 mV; the reaction is attenuated from +430 mV to 530 mV as the bacteriochlorophyll is chemically bleached. (2) Oxidation–reduction reactions of cytochrome *b* (λ_{max} 562–563 nm; $E_{m7.0}$ −105 mV) and a cytochrome *c* (λ_{max} 552–553 nm; $E_{m7.0}$ +295 mV).

Antimycin A abolishes the pyrophosphate-induced cytochrome oxidation–reductions but not the bacteriochlorophyll reactions, whereas uncoupler abolishes both cytochrome and bacteriochlorophyll reactions.

INTRODUCTION

Sodium pyrophosphate or ATP, when added to coupled chromatophores of *Rhodospirillum rubrum*, induces oxidation–reduction reactions which can be ascribed to energy-requiring reverse electron flow of the type first demonstrated by Chance and Hollunger[1] in mitochondria. In this way, the reduction of cytochrome *b* and concurrent oxidation of cytochrome *c* has been demonstrated in *R. rubrum* by Baltscheffsky[2]. Further, the activation by pyrophosphate of the energy-requiring pyridine nucleotide transhydrogenase in *R. rubrum* shown by Keister and Yike[3] is analogous with that previously described in mitochondria by Danielson and Ernster[4].

Coupled hydrolysis of pyrophosphate has also been shown by Baltscheffsky[5] to effect red shifts in the absorbance properties of the carotenoids. The mechanism underlying these red shifts has been the subject of much discussion[5–8].

This paper is concerned with the description of the cytochromes involved in pyrophosphate-induced oxidation–reduction reactions in terms of an imposed poten-

tial. It also describes energy-linked bacteriochlorophyll absorbance changes in the visible region which appear similar to those associated with carotenoids.

MATERIALS AND METHODS

Cultures of *R. rubrum* (blue-green mutant, strain G9) were grown anaerobically in the light and harvested in late log phase after 1–2 days. The medium (see ref. 9) was as follows: (g/l) malate 6, $(NH_4)_2SO_4$ 1.25; (mg/l) KH_2PO_4 900, K_2HPO_4 600, $MgSO_4 \cdot 7H_2O$ 200, $CaCl_2 \cdot 2H_2O$ 75, ferrous sulfate $\cdot 7H_2O$ 11.8, ethylenediaminetetraacetic acid 20, biotin 0.015, nicotinic acid 1, and thiamine hydrochloride 1. Mn, Zn, Cu, Mo, Co and B were added as trace elements. The pH was adjusted to 6.8. Chromatophores were prepared by the alumina grinding method described by Baltscheffsky[10]. Bacteriochlorophyll concentration was measured at 880 nm using the *in vivo* extinction coefficient of Clayton[11]. Experiments were performed in an anaerobic cuvette system (described in ref. 12) which permits simultaneous readout of absorbance change (dual wavelength spectrophotometer) and oxidation–reduction potential (platinum electrode in conjunction with a standard calomel electrode). The chromatophore suspensions were stirred continuously under an atmosphere of Ar (Ultrapure, Matheson Co.) containing less than 1 ppm O_2. Oxidation–reduction mediating dyes were used to promote equilibrium between the electrode and the membrane bound carriers. They were: potassium ferricyanide, E'_0 approx. +430 mV (Baker Chemical Co.); diaminodurol E'_0 approx. +240 mV, a generous gift from Dr I. Bremmer; phenazine methosulphate, E'_0 +80 mV (Sigma Chemical Co.); phenazine ethosulphate E'_0 +55 mV (K and K Laboratories, Plainview, New York); duroquinone, E'_0 approx. + 5 mV (Eastman Organic Chemicals, Rochester, New York); pyocyanine, E'_0 —34 mV (K and K Laboratories, Plainview, N.Y.); 2-hydroxy-1,4-naphthaquinone, E'_0 —145 mV (Eastman Organic Chemicals, Rochester, N.Y.); anthraquinone-2,6-sulphonate, E'_0 —184 mV (Aldrich Chemical Co., Milwaukee, Wis.); anthraquinone-2-sulphonate, E'_0 —225 mV (Fisher Scientific Co., Fair Lawn, N.J.) and methyl viologen E'_0 —430 mV (Mann Research Labs N.Y.). Ferricyanide was used to adjust the oxidation–reduction potential to more positive values, and a freshly prepared, dilute solution of sodium dithionite was used to adjust the potential to more negative values.

RESULTS

Pyrophosphate-induced spectrophotometric changes of bacteriochlorophyll

Contributions from carotenoids in these studies can be ruled out since chromatophores from the carotenoid deficient, blue-green mutant of *R. rubrum* were used. Fig. 1 shows that, on addition of an anaerobic solution of sodium pyrophosphate, spectrophotometric changes occur at potentials chosen (—475 mV or +430 mV) such that all the electron transport components are either fully oxidized (at +430 mV the exception is bacteriochlorophyll) or fully reduced. Under the influence of these extreme potentials no "reverse electron flow" would be expected, unless at +430 mV bacteriochlorophyll oxidizes under this influence. (The changes shown are corrected for a dilution artifact (———) caused by the addition of the pyrophosphate solution which becomes significant in regions of high absolute absorbance. This artifact was controlled for each pyrophosphate addition by adding the same amount of buffer;

all other spectra reported in this paper are corrected for in this manner.) The spectrum generated is essentially the same at both potentials: there is an absorbance increase maximum at approximately 433 nm, and in the α-band region there is a shift to longer wavelengths producing a trough at 575 nm and a peak at 595 nm. Little or no absorbance changes are detectable when the chromatophores are poised above +520 mV; at these potentials the bacteriochlorophyll is essentially oxidatively bleached which suggests that the pyrophosphate-induced absorbance changes are due to bacteriochlorophyll. The changes appear unaffected by the presence of the electron transport inhibitor, antimycin A (see Figs. 2 and 3), but are abolished by uncoupler.

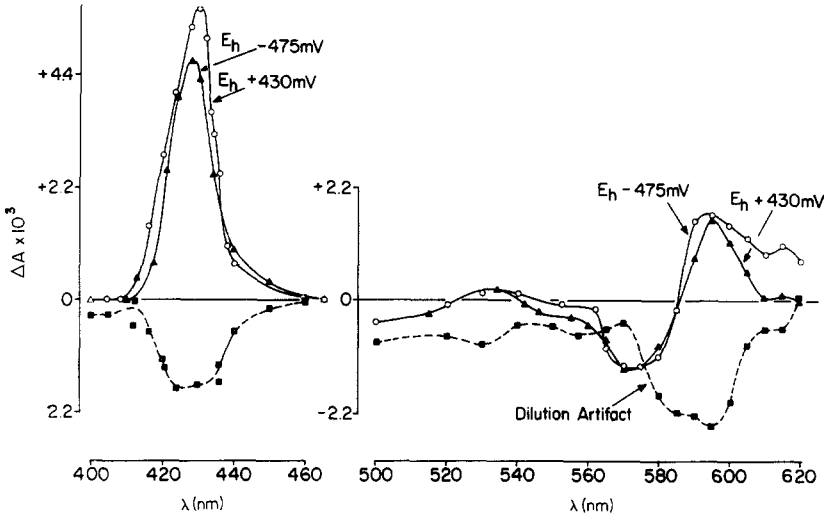

Fig. 1. Pyrophosphate-induced spectra at high and low oxidation–reduction potentials in *R. rubrum* chromatophores. The chromatophores (bacteriochlorophyll 130 μM) were suspended in 0.2 M glycylglycine buffer pH 7.2 (final volume 4.5 ml) containing 5 mM $MgCl_2$. The material was poised at −475 mV in the presence of 10 μM methyl viologen, and at +430 mV using potassium ferricyanide (approx. 200 μM). The added sodium pyrophosphate (7 μl of an anaerobic 100 mM solution to give 130 μM final concentration) was sufficient to saturate the reaction; 15 such additions could be made without very much deterioration of the reaction. Since there was a dilution artifact, 7 μl of buffer was added after each pyrophosphate addition; this is shown by — — —. The difference spectra shown are corrected for this dilution artifact. The reference wavelength was 465 nm. The chromatophores were continuously stirred under an atmosphere of Ar.

These facts suggest that bacteriochlorophyll is responding in some manner to a "high energy" state within the chromatophore. The inability to detect a significant absorbance decrease at about 600 nm (a change characteristic of bacteriochlorophyll oxidation) is consistent with the conclusion that the spectrum is not the result of oxidation–reduction events. However, a minor contribution to the spectrum from bacteriochlorophyll oxidation induced by pyrophosphate at +430 mV cannot entirely be ruled out: it is apparent when comparing this spectrum with that constructed at −475 mV (where all relevant components are presumed reduced so no reverse electron flow can occur) there is a slightly increased absorbance at 433 nm and a decreased absorbance at 605 nm which are changes characteristic of oxidized bacteriochlorophyll.

Pyrophosphate-induced oxidation of cytochrome c and of cytochrome b

After allowing a chromatophore suspension to become anaerobic (no added substrate or redox dyes) such that the *c*-type cytochromes are just reduced, addition of pyrophosphate produced the spectrum(○—○) shown in Fig. 2. Apart from the bacteriochlorophyll shift there is the oxidation of a *c*-type cytochrome; the absorbance minimum is at 552 to 554 nm. This is similar to that already described[2]. Addition of 4 μM antimycin A abolished the reaction. The presence of redox mediating dyes used to poise the system at known oxidation–reduction potentials greatly diminished the reaction; addition of 20 μM phenazine methosulphate (Fig. 2) can be

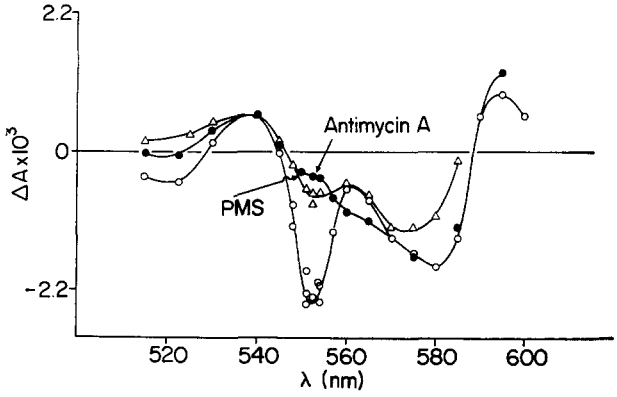

Fig. 2. Pyrophosphate-induced cytochrome *c* oxidation in *R. rubrum* chromatophores. The procedures were as described in Fig. 1 except that for (○) no redox mediators were added and cytochrome *c* was adjusted with dithionite to an almost fully reduced state (E_h approx. +150 mV) before pyrophosphate addition; (●) were obtained as described for (○) but in the presence of 4 μM antimycin A. (△) represents points obtained when the chromatophores were poised at E_h +100 mV, in the presence of 20 μM phenazine methosulphate (PMS).

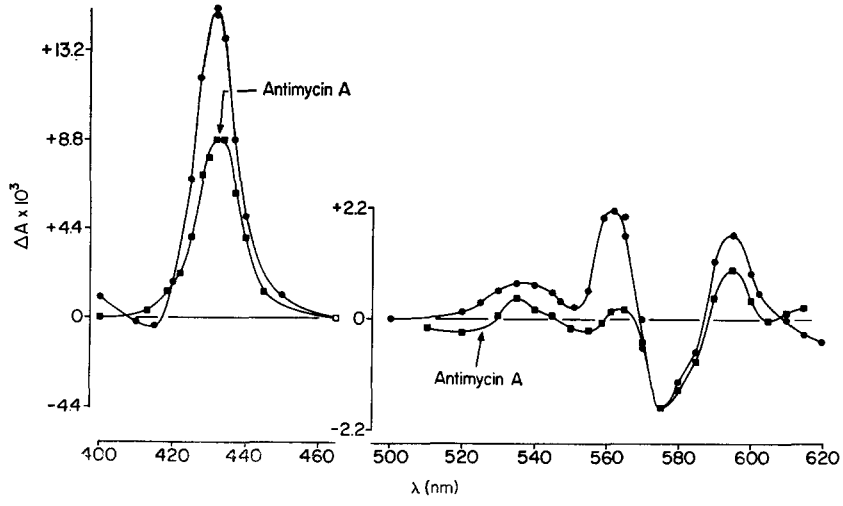

Fig. 3. Pyrophosphate-induced cytochrome *b* reduction in *R. rubrum*. The procedures were as described in Fig. 1 except that the chromatophores were poised using 20 μM duroquinone and 10 μM pyocyanine at E_h −55 ± 5 mV in the absence (●) and presence (■) of 4 μM antimycin A.

regarded as a potential clamp, which being in equilibrium with cytochrome *c* holds the state of cytochrome at a value dictated by the oxidation–reduction potential poise.

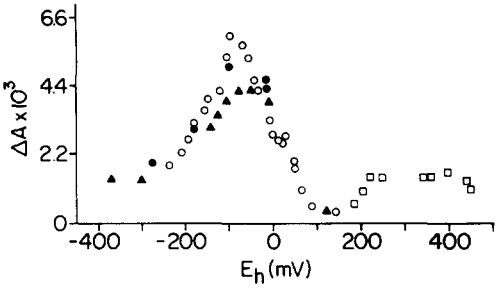

Fig. 4. Oxidation–reduction potential dependence of pyrophosphate-induced cytochrome *b* reduction. The procedures were as described in Fig. 1 except that the chromatophores were poised at the various potentials from +150 mV to −400 mV in the presence of the following mediators: 60 μM diaminodurol, 30 μM phenazine methosulphate, 30 μM phenazine ethosulphate, 6 μM pyocyanine, 20 μM duroquinone, 50 μM 2-hydroxy-1,4-naphthaquinone, 20 μM anthraquinone-2-sulphonate and 20 μM anthraquinone-2,6-sulphonate. ●, points taken during a reductive sequence; ○, points taken during an oxidative sequence; and △, points taken during a second reductive sequence on the same sample. From 450 mV to +200 mV (□) 60 μM diaminodurol and potassium ferricyanide were used. Pyrophosphate additions were usually made every 2–4 min. The measuring wavelengths were 562 *minus* 575 nm.

The same was not true for cytochrome *b*. Addition of high concentrations of several mediating dyes did not diminish the extent of pyrophosphate-induced cytochrome *b* reduction observed under anaerobic conditions in the presence of succinate suggesting the dyes cannot clamp the oxidation–reduction state of the *b*-cytochrome in question. Fig. 3 describes the spectrum generated when the chromatophores were poised at −55 mV; in addition to the bacteriochlorophyll contributions, absorbance maxima in the Soret region at approximately 430 nm and in the α-band region 562 nm describe the reduced cytochrome *b*. As was observed with cytochrome *c*, antimycin A inhibits the reaction. Fig. 4 describes the dependence of the reaction on the oxidation–reduction potential poised before addition of pyrophosphate. The extent of cytochrome *b* reduction is maximal at approximately −80 mV, and is relatively insignificant below −300 mV or above +100 mV. The background change observed at potentials more negative than −400 mV and more positive than +400 mV is due to the bacteriochlorophyll change, since in this experiment 575 nm was used a reference wavelength.

DISCUSSION

The *in situ* oxidation–reduction potentials of cytochromes in *R. rubrum* recently measured by Dutton and Jackson[13] has revealed the *c*-type cytochrome(s) to have a midpoint potential of +295 mV at pH 7.0 in close agreement with previously determined values[14]. No other *c*-cytochromes with more electronegative potentials were detected in measurable quantities [*cf.* for example *Chromatium* D (refs 12, 15)]. This would suggest that the *c*-cytochrome which undergoes pyrophosphate-induced oxidation has a midpoint of +295 mV and that this cytochrome acts as the electron acceptor at an energy conservation site in *R. rubrum* in an analogous way to cyto-

chrome c_1 at Site II in mitochondria. Dutton and Jackson also reported three other cytochromes which absorb at 560 nm, have approximate midpoint potentials (pH 7.2) of +170 mV, —5 mV and —105 mV. The latter cytochrome has an absorbance maximum at approximately 562 nm (Ushman and Dutton, unpublished results; see also ref. 14) and hence has the characteristics of a *b*-type cytochrome. It is similar to the one observed in this paper to undergo energy-linked reduction; furthermore, the fact that this reaction is maximal at —80 mV (when the —5 mV and +170 mV cytochromes are already chemically reduced) supports the view that the *b*-cytochrome involved has a midpoint potential at —105 mV. The behavior exhibited by this cytochrome is that expected by a component which occupies a position at the low-potential end of an energy-conserving site in photophosphorylation.

In studies on energy-conserving Site II in the respiratory chain of pigeon heart mitochondria and submitochondrial particles[16–18] Wilson and Dutton have shown that a *b*-cytochrome (designated cytochrome b_T) undergoes an apparent energy-dependent change in measured midpoint potential (changing from —30 mV to approximately +240 mV) on addition of ATP. The midpoint potentials of cytochromes $c + c_1$ appear unaltered. The familiar ATP-induced cytochrome *c* oxidation in mitochondria preparations is essentially abolished in the presence of redox dyes (see also ref. 19); this contrasts with that of ATP-induced cytochrome b_T reduction which appears independent of the concentration of the several redox dyes added. This apparent "energy dependence" of the midpoint potential of cytochrome b_T led to the consideration[16] that this cytochrome acts as the transducer of electrochemical potential energy into that of chemical potential energy (the currency required for phosphorylation of ADP) at Site II of the respiratory chain.

In qualitative terms, the analogous nature of the results obtained with *R. rubrum* chromatophores is clear. It is conceivable that the pyrophosphate-induced cytochrome *b* reduction may therefore be a manifestation of an energy-linked formation of a more electropositive form of this cytochrome. However, since the oxidation–reduction potential (approx. —80 mV) for maximal cytochrome *b* reduction (Fig. 3) is close to the midpoint potential of this cytochrome, the extent of the apparent change is probably less than 30 mV depending on how much of the cytochrome *b* is "active" (see ref. 17). We have not attempted to make any quantitative correlations of the extent of the postulated apparent midpoint potential change and the estimated free energy available from pyrophosphate hydrolysis under the experimental conditions. This cannot be done until further experimentation has established the apparent midpoint potential change is not the result of a slow rate of interaction between the *b*-cytochrome and mediating dyes, relative to the rate of reverse electron flow induced by the pyrophosphate.

The major point made here lies in the identification of what are probably the thermodynamic limits of the site(s) of energy conservation in the cyclic electron transfer system of *R. rubrum*. The midpoint potential values of the *b*-cytochrome (—105 mV) and cytochrome *c* (+295 mV) are thermodynamically appropriate in relation to the oxidation–reduction potential span of the light reaction: the midpoint of reaction center bacteriochlorophyll is approximately +440 mV (ref. 20), and that of the primary electron acceptor as determined at pH 8.0 by Cramer[21] is —145 mV (*cf.* ref. 20). We have confirmed this value; our own determination (midpoint potential at pH 7.4, —140 mV; $n = 1$) was done by assay at 10 °K of the light-induced

ESR signal at $g = 2$ in chromatophore samples poised before freezing at potential values ranging from $+150$ mV to -450 mV (J. S. Leigh and P. L. Dutton, unpublished results).

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