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The membrane potential of intact *Rhodospirillum rubrum* cells in the absence of light-dependent and oxygen-linked electron transfer

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In the absence of oxygen-linked and light-dependent electron transfer, the steady-state membrane potential of intact *Rhodospirillum rubrum* cells was usually between 65 and 75% of that of dark aerated cells, as indicated by the relative extent of the bacteriochlorophyll electrochromic changes that were induced by oxygen and by uncouplers. That potential was not due to residual levels of oxygen or light, because its value was not significantly altered by the presence of oxygen-trapping systems or by exhaustive gassing with Ar, and because it was also exhibited by a reaction-center-less mutant. The dark anaerobic potential was unaffected by 0.11 M K^+; that seemed to exclude a diffusion potential generated by dissipation of a previously built K^+ gradient. In contrast, it was largely abolished by 0.5 mM *N,N'*-dicyclohexylcarbodiimide, suggesting its dependence on ATP hydrolysis by the proton-translocating ATPase of the bacterial membrane. That was not expected because *R. rubrum* did not grow fermentatively under the conditions used. Low concentrations of protonophores were more effective in dissipating the anaerobic than the aerobic membrane potential. That observation indicated a lower activity of the electrogenic system responsible for the anaerobic potential. In consequence, the addition of uncouplers at low levels resulted in a marked enhancement of the membrane potential decrease which followed the transition between the aerobic and the anaerobic steady states.

Introduction

According to the chemiosmotic hypothesis, a protonmotive force or protonic potential gradient across the membrane plays a key role in biological energy transduction [1]. In phototrophic and aerobic bacteria, membrane-linked electron transfer drives the extrusion of protons, thus generating the gradient which subsequently drives ADP phosphorylation by the BF_0F_1-ATPase, several types of secondary solute transport, and other physiological processes. Since those bacteria may retain their viability for extended periods of time in the absence of photosynthesis and aerobic respiration, even when culture conditions do not allow dark anaerobic growth, it appears that the cell must be able to sustain a certain level of protonmotive force in order to carry out some work functions, such as motility and the uphill transport of some low M_r solutes to prevent their diffusive equilibration between the external and the internal medium. The need for protonmotive force buffering has been discussed by Skulachev [2], who proposed that the Na^+/K^+ gradient

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; TCS, 3,3',4,5'-tetrachlorosalicylanilide.

might be the physiological buffer.

In order to investigate the existence of the residual protonmotive force, phototrophic bacteria appear to be a convenient working material because the pigment-protein complexes of the photosynthetic membrane can be used as sensitive endogenous probes of the electric component of the proton gradient [3]. Lolkema et al. [4] observed that dark anaerobic suspensions of intact cells of *Rhodopseudomonas sphaeroides,* a facultative phototroph, had significant levels of transmembrane electrical potential. In contrast, the membrane potential of *Rhodopseudomonas capsulata* cells under similar conditions was attributed to the presence of low levels of oxygen which were difficult to remove from the suspension [5]. In the present work, we have investigated the membrane potential of *Rhodospirillum rubrum* cells when photosynthesis and aerobic respiration are prevented and dark anaerobic growth cannot occur. It has been found that the potential of those cells amounts to about 65–75% of the value reached when respiration takes place. It appears that a weak electrogenic pump may sustain such relatively high levels of membrane potential in dark anaerobic cells, since the intrinsic ionic conductance of the membrane decreases sharply as the membrane potential is lowered [6]. ATP hydrolysis by the proton translocating ATPase seems to be the electrogenic system that operates in the absence of light- and oxygen-dependent electron transfer. A preliminary report of this work has been presented elsewhere [7].

Materials and Methods

The wild-type strain of *R. rubrum* (S1), the reaction-center-less mutant, strain T102, and the cytochrome-oxidase-deficient mutant, strain CAF10, have been described before [8–10]. Usually, cultures were carried out in the dark under limiting oxygen [11] in the medium of Lascelles [12], which contained 20 mM each malic and glutamic acids and was supplemented with 2 g/l yeast extract. For some experiments (see Results), the wild-type strain was cultured in the light and in the absence of oxygen [8], using the same growth medium. *Escherichia coli* K12 was grown under intense aeration in Nutrient Broth no. 2 (Oxoid) at 37°C. Bacterial cells were collected by centrifugation at room temperature, suspended in the medium of Lascelles [12], and kept at 0°C till further use.

Absorbance changes were followed with a Hitachi spectrophotometer (model 356) in the double-wavelength mode. The *R. rubrum* cell suspension was placed in a 1 cm optical cell and brought to room temperature. Oxygenation was achieved by bubbling air through the suspension. Oxygenation by addition of controlled amounts of H_2O_2 was also attempted. However, the peroxide elicited large absorbance changes which were not reproducible in the same sample and whose recovery was highly dependent on the level of added catalase (in contrast to Ref. 5). Anaerobiosis resulted from the respiratory activity of the cells. When desired, a mixture of glucose, glucose oxidase and catalase [13] or *E. coli* cells (which took up oxygen at a rate double than that of *R. rubrum* cells) were included in the suspension. In some experiments, the *R. rubrum* suspension was bubbled for 2 h with Ar (less than 3 ppm O_2) in a rubber-stoppered optical cell. Prior to absorbance recording, the gas inlet was raised to about 1 mm over the suspension surface and gassing was continued throughout the measurement. When required, uncouplers were added in μl amounts of methanol. It was checked that the solvent alone did not elicit significant absorbance changes. After additions, the suspensions were gently stirred with a glass rod, taking special care not to alter the position of the optical cell. Alternatively, air or Ar bubbling were used to achieve mixing.

The bacteriochlorophyll content of intact cells was estimated in vivo from the difference in absorbance between 879 and 930 nm, using an extinction coefficient of 144 $mM^{-1} \cdot cm^{-1}$ [10].

Results

When subjected to intense electric fields, the pigment-protein complexes of the photosynthetic membrane undergo spectral alterations (electrochromic changes), which consist mainly in bandshifts. Those changes can be used as sensitive intrinsic indicators of the electrical component of the protonmotive force [3]. Both the carotenoid and the chlorophyll bands of the membrane-bound

complexes exhibit electrochromic changes. Since, in the present work, respiration-linked changes of membrane potential were measured, we preferred to follow the absorption changes of the major long-wavelength bacteriochlorophyll band, because none of the electron-transfer constituents of the respiratory chain displays interfering bands in the near infrared range of the spectrum. However, parallel results were obtained when the carotenoid changes were followed. The dependence of the bacteriochlorophyll changes on a membrane potential generated by pyrophosphate hydrolysis [9,14] or by K^+ diffusion [14] has been previously demonstrated in *R. rubrum* chromatophores. In isolated membrane vesicles, the electrochromic changes have been calibrated in millivolts with artificially imposed diffusion potentials, and have been found to be linear over a wide potential range [5,15]. However, such calibration is hardly practicable in intact cells, because it is difficult to set well-defined diffusion potentials across the plasma membrane [5]. Thus, no attempts were made here to calibrate the absorption changes.

When suspensions of *R. rubrum* cells in culture medium were aerated and then allowed to exhaust oxygen by respiration, changes of optical absorbance at 895.5 − 865 nm were observed as the membrane potential switched between the aerobic and the anaerobic steady-state values (Fig. 1). These wavelengths correspond to the peak and the trough, respectively, of the bacteriochlorophyll red shift. The extent of the changes was not significantly different in the presence of high NH_4^+ levels (not shown), which indicated that the contribution of a proton gradient to the protonmotive force was small [16]. That was expected because the capacitance of the membrane is very low [2]. A fraction of the optical changes was suppressed by 10 μM CCCP (Fig. 1), what suggested that at least a part of them was associated to a delocalized membrane potential. The absorbance changes that took place in the presence of 10 μM CCCP (Fig. 1) could be due to an intramembrane field associated to the formation of charged species during respiratory electron transfer, or to changes in the membrane surface charge. Alternatively, it seems also possible that the increase of protonic conductance elicited by the uncoupler was not sufficient to bring the steady-state value of the delocalized membrane potential of respiring cells to zero. In any case, these possibilities were not investigated further.

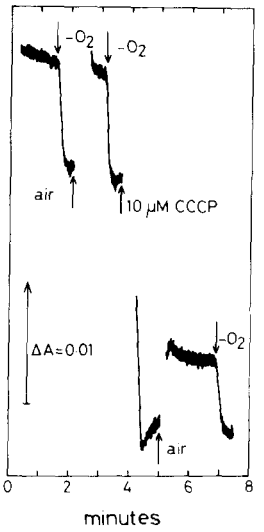

Fig. 1. Bacteriochlorophyll absorption changes induced by aeration and by CCCP at 895.5 minus 865 nm. The suspension contained 25 nmol bacteriochlorophyll (as intact dark-grown cells of the wild-type strain) in 2.5 ml growth medium.

The addition of 10 μM CCCP to the cell suspension after oxygen exhaustion induced a decrease of membrane potential which was about 3-fold larger than the decrease that went along with the aerobic to anaerobic transition in the absence of the uncoupler (Fig. 1). TCS, another uncoupler, induced also a decrease of membrane potential in dark anaerobic cells (Table I). A similar but lesser change had been observed in *Rps. capsulata* cells and was attributed to the dissipation of a membrane potential caused by residual respiration at low oxygen levels [5]. However, that does not seem to be the case here because the uncoupler-elicited decrease was not lower when the cell suspension has been gassed previously with oxygen-free Ar. In fact, the uncoupler-elicited change could still be observed in cells which had been kept dark under Ar for 2 h. Moreover, CCCP induced a similar membrane potential decrease in cell suspensions to which an oxygen-trapping system had been added. Either actively respiring *E. coli* cells or a mixture of glucose, glucose oxidase and catalase were used. Such results, which are summarized in Table I, would not be expected if

TABLE I

UNCOUPLER-ELICITED CHANGES OF BACTERIOCHLOROPHYLL ABSORPTION IN *R. RUBRUM* CELL SUSPENSIONS

The standard cell suspension was as in Fig. 1. The extent of the absorbance decrease induced by the uncoupler (10 μM CCCP, except where indicated) is given relative to that of the decrease which followed oxygen exhaustion in the absence of uncouplers and CO (see Fig. 1 for the absolute value of this latter change). Cells of wild type (S1), cytochrome-oxidase-defective (CAF10), and reaction-center-defective (T102) strains were used.

Strain	Incubation conditions	$\Delta A_{895.5-865}$ (relative units)
S1	standard	2.5
	standard [a]	2.6
	+ Ar gas (2 h)	2.9
	light-grown *R. rubrum* + *E. coli* cells	4.0
	+ glucose oxidase system	3.2
	+ 55 mM K_2SO_4	2.3
	+ 0.5 mM DCCD [b]	0.25
CAF10	+ CO	3.1
T102	standard	2.2

[a] Change induced by 10 μM TCS.

[b] DCCD increased the change due to respiration by a factor 2.2.

the uncoupler-elicited membrane potential decrease were dependent on residual respiration. If that were the case, the rate of respiration would be limited by oxygen and any reduction of the oxygen levels should bring about a decrease of the membrane potential before uncoupler addition and, consequently, a reduction of the uncoupler-elicited change. The observation that an uncoupler-dependent decrease of membrane potential was also observed in a mutant that lacks cytochrome oxidase activity [10] when the alternate oxidase was inhibited by CO (Table I) strengthens our interpretation that the uncoupler-sensitive potential does not depend on respiration.

The experiments of this work were usually carried out with cells grown in the dark under low oxygen tensions, which exhibited simultaneously high levels of respiratory activities and photosynthetic pigments [17]. However, similar results were obtained with cells from phototrophic cultures (Table I). Since the cell suspension was irradiated by the weak measuring light of the spectrophotometer, it seemed possible that the membrane potential that was dissipated by uncouplers in anaerobic cells could be generated by photosynthetic electron transfer. That possibility was discarded, though, because the membrane potential decrease was shown also by cells of a reaction-center-less mutant (Table I), unable to perform any photosynthetic reaction [9]. Therefore, it appears that *R. rubrum* cells are able to maintain a considerable level of membrane potential even when light-driven and oxygen-dependent electron flows do not take place. It should be mentioned here that, although some *R. subrum* strains have been reported to grow anaerobically in the dark under certain culture conditions [18], no growth could be detected for 48 h in dark anaerobic cultures of our strain (not shown).

The K^+ gradient that is built up across the bacterial membrane during respiration or photosynthesis has been proposed to act as an energy buffer that can sustain the membrane potential when, transiently, the rate of primary ion transport falls below that of the processes that depend on the protonmotive force [2]. However, a K^+ diffusion potential does not seem to be responsible for the membrane potential of dark anaerobic *R. rubrum* cells because the addition of 110 mM K^+ to the suspension did not suppress the uncoupler-elicited change (Table I). That was not surprising, since, given the small size of the bacterial cell, the dissipation of ionic gradients should be relatively fast even in the absence of specific solute carriers. In contrast, the ATPase inhibitor DCCD [19] brought about a clear reduction of the uncoupler-elicited potential decrease (Table I). It should be noted that DCCD induced also a significant stimulation of the oxygen-elicited change of membrane potential in the absence of uncouplers, as expected from a typical inhibitor of energy transfer.

While CCCP concentrations around 1 μM dissipated most of the membrane potential of dark anaerobic cells, they reduced only slightly the membrane potential of respiring cells. Therefore, a larger membrane potential decrease, as judged by the change in bacteriochlorophyll absorbance, was observed upon the transition from aerobic to anaerobic conditions (Fig. 2). A similar enhancement was elicited by low concentrations of TCS and was also observed when the carotenoid electrochromic changes were monitored (Fig. 2). Thus,

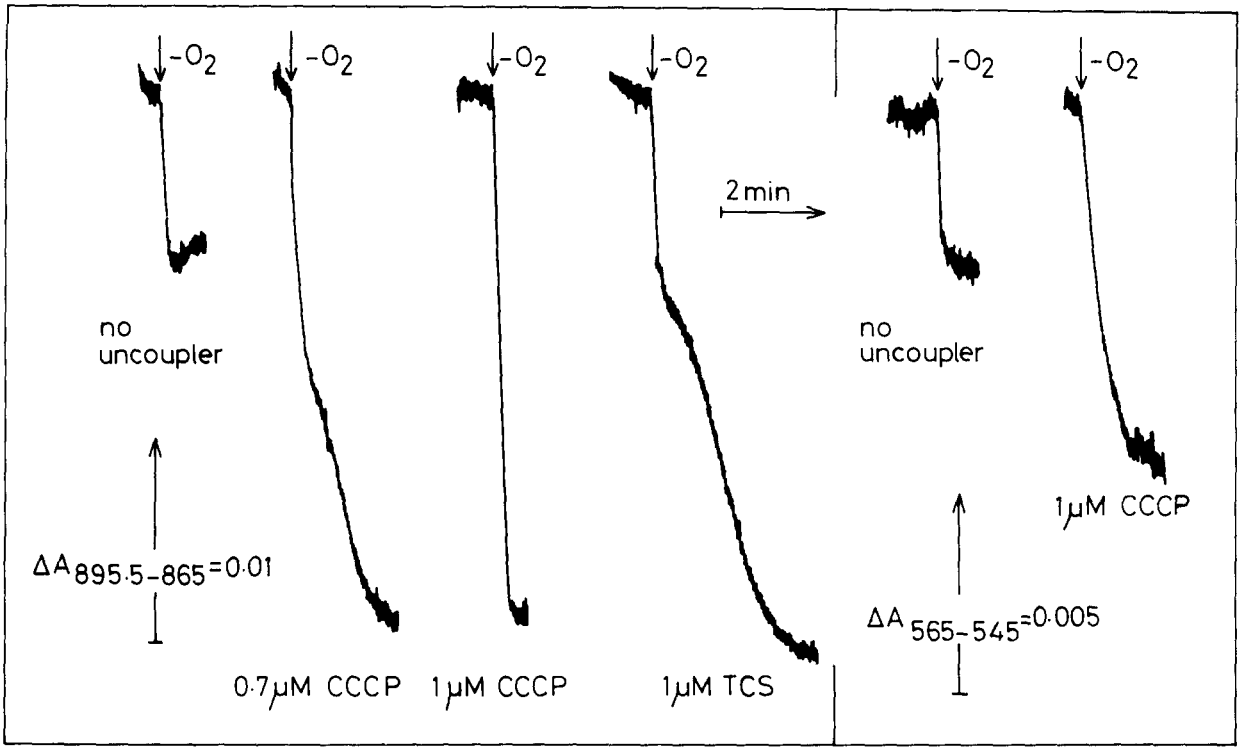

Fig. 2. Effect of low concentrations of uncouplers on the electrochromic changes associated to oxygen exhaustion. The cell suspension was as in Fig. 1. The cell batches used for bacteriochlorophyll changes (at 895.5 minus 865 nm) and for carotenoid changes (at 565 minus 545 nm) were different.

it seems that the uncoupler-elicited stimulation corresponded to an enhancement of the membrane potential change and not to a secondary effect of CCCP on the optical absorption of bacteriochlorophyll. As it can also be observed in Fig. 2, the time-course of the membrane potential decrease was clearly biphasic in the presence of 0.7 μM CCCP or 1 μM TCS. That was possibly due to the fact that the membrane potential was dissipated by two different pathways: the first one was provided by the intrinsic ion channels of the membrane, whose conductance decreases with the membrane potential [6]; the second one was due to the artificial proton channels created by the uncoupler, that are voltage-independent and become predominant when, at lower membrane potentials, the intrinsic conductance reaches low values. The observation that the last part of the trace became steeper at higher uncoupler concentrations (Fig. 2) is consistent with such an interpretation.

Discussion

The experimental data of this work demonstrate that intact cells of *R. rubrum* may have a relatively high level of membrane potential when neither light-driven nor oxygen-linked electron transfer can take place. The physiological significance of the membrane potential of dark anaerobic cells is not difficult to understand, since many processes, including several types of solute transport, are energized by the components of the protonmotive force [20]. If nongrowing cells were unable to retain a certain capability to energize active transport, ordinary or facilitated diffusion or both would equalize in a short period of time the concentrations of mineral ions and of low M_r metabolites in the internal and external cell spaces. The steady-state membrane potential of *Rps. sphaeroides* cells in the dark and in the absence of oxygen, as measured by the distribution of a per-

meant ion, was also found to be close to that of dark aerated cells [4]. In contrast, a similar dark anaerobic potential was not detected in *Rps. capsulata* [5].

It may seem strange that the membrane potential decrease which is induced by high concentrations of uncouplers in dark anaerobic cells is significantly larger than the changes associated to respiration in the absence of uncouplers (Fig. 1). It should be realized, though, that such an observation does not mean that the electrogenic pump responsible for the dark anaerobic membrane potential is more active than respiration. In fact, the finding that much lower levels of uncouplers are required to dissipate the membrane potential of dark anaerobic cells (Fig. 2) indicates the opposite. In a variety of respiratory systems it has been shown that the steady-state value of the membrane potential does not increase linearly with the rate of electron transfer [6,21–24]. Although it has been proposed that such nonlinear dependence is due to a decrease of the H^+/e^- ratio, work from Jackson and co-workers [6,25] strongly suggests that, at least in phototrophic bacteria, there exists a fixed stoichiometry between transferred electrons and translocated protons, and that the nonlinearity is due to the properties of the intrinsic ionic conductance of the membrane, which decreases sharply as the membrane potential is lowered. Then, it is possible to maintain a relatively high steady-state value of membrane potential with a weak electrogenic pump if the intrinsic conductance of the membrane is low, i.e., if the activities of the secondary ion transporting systems are restricted to minimum values by regulatory mechanisms.

The observation that the membrane potential of dark anaerobic *R. rubrum* cells is largely suppressed by DCCD (Table I) suggests that at least a considerable fraction of that potential is generated by the proton-translocating ATPase of the membrane [19]. Although *R. rubrum* does not grow fermentatively in the culture medium used here, and the hydrolytic reaction of the ATPase under physiological conditions has been shown only in fermentative anaerobes [26], it seems reasonable to admit that the slow breakdown of poly-β-hydroxybutyrate, polyphosphate or other metabolic reserves [27] may provide ATP at a rate high enough to support a weak primary transport of protons across the membrane. The observation that this microorganism maintains relatively high levels of ATP after some time of dark anaerobic incubation [28] is consistent with such an interpretation.

Since DCCD had to be used at high concentrations with intact cells, this inhibitor may have altered other catalytic or transport systems and our interpretation of its effect on the membrane potential of dark anaerobic *R. rubrum* might be uncorrect. Therefore, the possible contribution of other electrogenic pumps to such potential should also be considered. Thus, *R. rubrum* contains a membrane-bound, proton-translocating pyrophosphatase which is DCCD-insensitive [29] and which has been reported to be more active than the ATPase [2]. Anaerobic respiration seems also possible. The membrane of *R. rubrum* has been shown to contain a rhodoquinone-dependent fumarate reductase activity [30] and, although the presumable electrogenic function of that catalytic system in *R. rubrum* has not been investigated in detail, it is well documented in a variety of prokaryotes and in some eukaryotes [31].

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