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LIGHT-INDUCED REACTIONS OF PHOTOSYNTHETIC BACTERIA

I. REACTIONS IN WHOLE CELLS AND IN CELL-FREE EXTRACTS AT LIQUID NITROGEN TEMPERATURES

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SUMMARY

The light and dark reactions at liquid nitrogen temperatures of *c*-type cytochromes and associated spectral components of three species of photosynthetic bacteria have been studied with respect to the differential responses of the components in the whole cells or cell-free extracts of these species to the conditions of anaerobiosis and oxygenation prior to freezing. In light-induced reactions at 77°K irreversibility is induced under anaerobic conditions and reversibility by oxygenation. The half-times of laser-induced reactions measured at different wavelengths have been effectively used in differentiating and identifying various spectral components undergoing concurrent absorbance changes.

INTRODUCTION

During the course of investigations on the photooxidation of *c*-type cytochromes in many different species of photosynthetic bacteria[1,2], it was observed that not only the amplitude and the reversibility of absorbance changes of certain spectral components, but in some cases the disappearance or emergence of light-induced reactions, depended upon the degree of aerobiosis and anaerobiosis in the sample. In some species of bacteria, slight aeration prior to freezing to 77°K resulted in the complete disappearance of photooxidation of *c*-type cytochromes; in others, it induced the reaction to become partly reversible in the dark. It was also observed that the reversible changes at 77°K due to P435 and bacteriochlorophyll can be greatly enhanced upon aeration or totally eliminated under anaerobic conditions. In addition, under native, unperturbed conditions, both quantitative and qualitative differences in light-induced changes at 77°K exist among different species of bacteria investigated.

Some observations on the effects of anaerobic and oxygen-saturating conditions on the light and dark reactions at liquid nitrogen temperatures of *c*-type cytochromes and other spectral components in three species of photosynthetic bacteria are presented in this communication.

MATERIALS AND METHODS

Cultures of *Chromatium* D (American Type Culture Collection), *Rhodopseudomonas palustris* (an isolate from ATH 2.1.2) and *Rhodopseudomonas gelatinosa* Strain I (isolated by E. F. Haskins and T. Kihara) were used in this study. They were grown and harvested as described eleswhere[3]. Cell-free extracts were prepared by ultrasonic disruption (Branson Sonifier type S75; output 3.5 d.c. A; 2 min at 4°) of whole cells washed once and resuspended in 0.1 M Tris-HCl buffer at pH 7.9. Cell debris was removed by centrifuging 3 times at 30000 × *g* for 20 min, and the resultant supernatant fraction, designated cell-free extracts (crude or unwashed "chromatophores"), was kept under nitrogen gas at 4°. Samples described as "anaerobic" were maintained under nitrogen prior to use. "Oxygenated" samples were obtained by passing a steady stream of oxygen through sample suspensions for 5 min at room temperatures immediately before freezing to liquid nitrogen temperatures.

Absorbance changes induced by continuous illumination with red light and during a subsequent dark period were measured as previously described[1] with a recording dual wavelength spectrophotometer equipped for cross illumination of the sample which was maintained at 77°K in a clear Dewar flask.

Since some of the reactions at 77°K were irreversible, it was necessary to use a fresh new sample for each point shown in the difference spectra (Figs. 1A, 1B, 2A, 2B, 3A and 3C). When reversible reactions were being studied (Figs. 1C, 2C, 3B and 3D) a single sample could be used throughout the spectrum and the sample maintained at 77°K by a continuous flow of liquid nitrogen into the Dewar flask. When scanning the spectrum for absorbance changes from 360 to 640 nm in anaerobic samples, as many as 120 individual samples were used to construct a light-induced difference spectrum at liquid nitrogen temperatures to ensure greater accuracy.

The reaction rates of some spectral components at 77°K were determined in a single beam spectrophotometer with a Q-switched ruby laser[5]. The instrument was equipped with a new fast photomultiplier preamplifier developed recently by D. DeVault of this laboratory. Its rise time is approx. 50 nsec.

RESULTS

The photooxidation of a *c*-type cytochrome at liquid nitrogen temperatures in the anaerobic whole cells of *Chromatium* D is apparent in the well defined light-induced difference spectrum (Fig. 1A) which shows absorbance changes of the α (550 nm), β (520 nm) and γ (421 and 408 nm) bands. The cytochrome change does not undergo dark reduction when actinic light is turned off, and further on and off periods induce no additional changes; this agrees with the earlier report of CHANCE AND NISHIMURA[6]. Such changes which are "frozen in" will be described as irreversible. The relatively small changes at about 435 nm and at 610–615 nm are completely reversible in the dark. Cell-free extracts of *Chromatium* D under the same conditions exhibit absorbance changes (Fig. 1B) which are indicative of the photooxidation of the same *c*-type cytochrome showing the α-band at 550 nm and the Soret band at 421 and 408 nm as in the whole cells of the same species (Fig. 1A); however, the absence of the β-band in the difference spectrum, which has been observed repeatedly, is unexplained at present.

Oxygenation of the cell-free extracts of *Chromatium* D results in the elimination of the absorbance changes in the Soret region due to the cytochrome *c* and in the emergence of complex reversible changes throughout the visible spectrum (Fig. 1C). The broad band peaking at 430–440 nm is attributed to P435. The changes at 610–615 nm (assigned to a spectral component tentatively designated as P610) and about 385 nm are assumed to be caused by the photochanges of small fractions of the bacteriochlorophyll bands located at about 590 and 375 nm, respectively. The anomalous absorbance decrease at 553 nm has not been identified with any known component of the photosynthetic bacterial system.

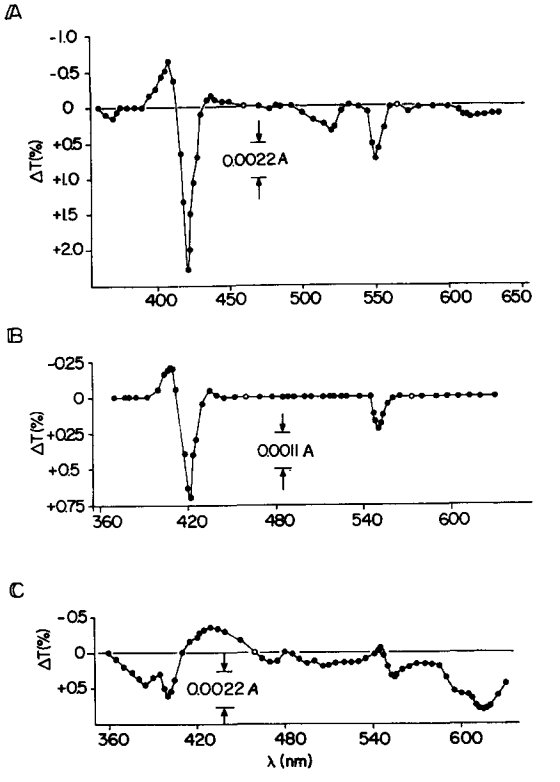

Fig. 1. Light-induced absorbance changes at 77°K in *Chromatium* D. A. Anaerobic whole cells; irreversible oxidation of a *c*-type cytochrome showing absorbance changes of the α (550 nm), β (520 nm) and γ (421/408 nm) bands. 0.31 mM bacteriochlorophyll. B. Anaerobic cell-free extracts; irreversible oxidation of a *c*-type cytochrome without the β-band. 0.55 mM bacteriochlorophyll. C. Oxygenated cell-free extracts; reversible changes. 0.55 mM bacteriochlorophyll.

Anaerobic whole cells of *Rps. palustris* undergo mixed irreversible and reversible reactions. The graph of the irreversible changes (Fig. 2A; upper curve) shows the photooxidation of a *c*-type cytochome with absorption minima at 550, 523 and 421 nm, and a maximum at 410 nm. The reversible absorbance changes which accompany the irreversible changes present a multi-component difference spectrum (Fig. 2A; lower curve). The spectrum exhibits absorbance changes in the Soret region due to a *c*-type cytochrome with peaks at 421 and 410 nm. The prominent minima at 557 and 520–523 nm have been attributed to the α- and β-bands of this cytochrome which is spectrally

similar to the irreversible species[1]. Preliminary work on the laser-induced reaction rates at these wavelengths indicates that they are in the msec range which is further evidence that these minima belong to the cytochrome since the absorbance changes of other components such as carotenoids, P435 and bacteriochlorophyll take place in less than 0.5 μsec at 77°K; no fast changes attributable to carotenoid shift were detected in the visible region of the spectrum. The large absorbance minimum centered around 610 nm is due to P610 and a broad P435 change is seen between 430 and 450 nm.

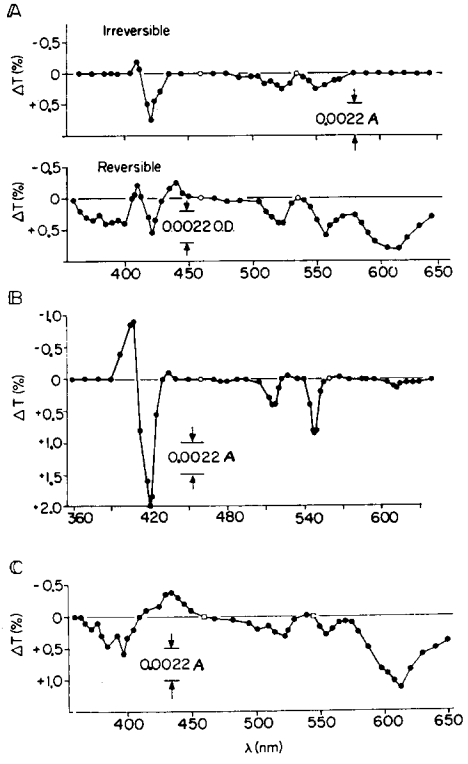

Fig. 2. Light-induced absorbance changes at 77°K in *Rps. palustris*. A. Anaerobic whole cells; upper curve, irreversible changes; lower curve, reversible changes. 0.24 mM bacteriochlorophyll. B. Anaerobic cell-free extracts; irreversible oxidation of a *c*-type cytochrome peaking at 548, 517 and 420/408 nm. 0.44 mM bacteriochlorophyll. C. Oxygenated cell-free extracts; reversible changes. 0.33 mM bacteriochlorophyll.

The oxidation of a *c*-type cytochrome in anaerobic cell-free extracts of *Rps. palustris* is evidenced by the α-, β- and γ-bands at 548, 517 and 420/408 nm, respectively (Fig. 2B). These absorbance changes are irreversible. The amplitude of the small reversible changes at 612 and 435 nm can be further diminished under more reducing conditions or it can be amplified greatly and the extent of cytochrome oxidation correspondingly diminished if the sample is aerated before freezing.

The absorbance changes of oxygenated cell-free extracts (Fig. 2C) of *Rps. palustris* are similar to those of oxygenated *Chromatium* D cell-free extracts (Fig. 1C) with the exception of a well-defined minimum at 522 nm which is absent in the latter.

These changes are also completely reversible in the dark and the amplitude remains unchanged during repetitive light and dark periods.

Anaerobic whole cells of *Rps. gelatinosa* undergo mixed reversible and irreversible absorbance changes due mainly to a *c*-type cytochrome and carotenoids (Fig. 3A). The Soret region of the cytochrome shows a minimum at 421.5 nm and a maximum at 408 nm. The shape of the curve (dotted line) for the reversible changes in the same region is similar to that of the total initial changes (solid line) peaking at 408 and 421.5 nm. The pronounced absorbance changes between 440 and 540 nm are caused

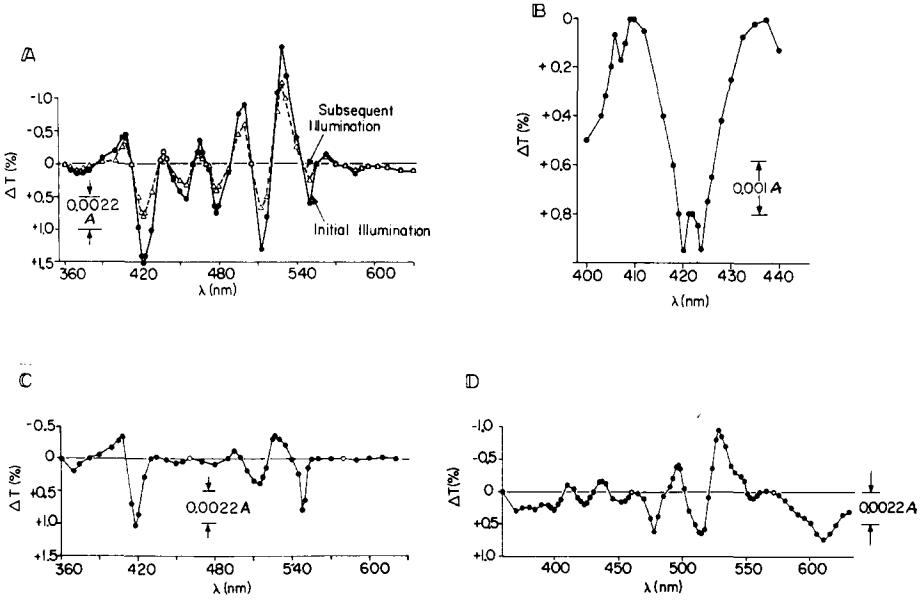

Fig. 3. Light-induced absorbance changes at 77°K in *Rps. gelatinosa*. A. Anaerobic whole cells; solid curve, total changes in absorbance during initial illumination; broken curve, reversible changes during subsequent illumination. 0.22 mM bacteriochlorophyll. B. Oxygenated whole cells; reversible changes. 0.43 mM bacteriochlorophyll. C. Anaerobic cell-free extracts; largely irreversible changes. 0.46 mM bacteriochlorophyll. D. Oxygenated cell-free extracts; reversible changes. 0.42 mM bacteriochlorophyll.

by the shifting to the longer wavelengths of the absorption bands of carotenoids at about 462, 484 and 512 nm. At 300°K the shifting of these bands gives rise to maxima at about 466, 500 and 535 nm, and minima at 453, 482 and 518 nm in light-induced difference spectra of whole cells and cell-free extracts. At 77°K these maxima and minima are shifted slightly to the shorter wavelengths and appear at about 460, 497, 528, 450, 478 and 515 nm. The peak positions of the reversible carotenoid changes during subsequent illumination at 77°K are the same as those of the irreversible changes of carotenoids. A relatively small change at 550 nm is attributed partly, if not entirely, to the α-band of the *c*-type cytochrome since it is apparently outside of the visible spectral region where carotenoid shifts occur in this species. In comparison with the fast photooxidation rate, the slow dark reduction of the *c*-type cytochrome in the whole cells proceeds with a half-time of 2–4 sec. The energy of activation for the reverse reaction has been estimated to be 100–500 cal/mole which is comparable with

the value reported for *Rps. palustris*[4]. It was observed earlier[1] that cytochrome photooxidation in whole cells of *Rps. gelatinosa* was not sensitive to mild aeration. Detailed analysis of light-induced absorbance changes in oxygenated whole cells of this species reveals that reversible changes in the Soret region exhibit splitting of the peaks into absorption minima at 420 and 423.5 nm and maxima at 406 and 410 nm (Fig. 3B).

The absorbance changes in anaerobic cell-free extracts of *Rps. gelatinosa* (Fig. 3C) are similar to those of the whole cells (Fig. 3A) with respect to the position of various maxima and minima. The changes attributed to a *c*-type cytochrome designated as C419 (77°K) (minima at 419 and 548 nm, and maximum at 408 nm), unlike those in the whole cells, exhibit little reversibility.

The oxygenation of the cell-free extracts of *Rps. gelatinosa* (Fig. 3D) results in the enhancement of carotenoid shifts and of absorbance changes centered around 435 and 610 nm as in the previous cases. The elimination by oxygenation of the irreversible *c*-type cytochrome observed in the anaerobic sample (Fig. 3C) is accompanied by the appearance of new minima at 553–555 and 422–423 nm and a maximum at 410 nm due to a new completely reversible *c*-type cytochrome, designated as C422 (77°K). It has been noted that more prolonged oxygenation greatly diminishes the magnitude of the changes at these wavelengths while the other spectral components are comparatively unaffected; the amplitude of the reversible cytochrome changes appears to become maximal under semi-aerobic conditions[7]. The rate of dark reduction of C422 (77°K) and the activation energy of the dark reaction are the same as observed in the anaerobic whole cells.

The kinetics of absorbance changes in anaerobic cell-free extracts of *Rps. gelatinosa* at 548, 528, and 419 nm were studied with a pulsed laser at 77°K. The half-time determined at 548 and 419 nm gave a range of 10–20 μsec which is the same as that for the cytochrome oxidation at 421 nm in the whole cells of this species as previously determined[1]. These laser studies show that the disproportionately large band peaking at 548 nm can be identified with the α-band of the *c*-type cytochrome which gives rise to an absorption minimum at 419 nm and a maximum at 408 nm. The rate of laser-induced reaction indicates a half-time of about 60 μsec for the new *c*-type cytochrome C422 (77°K) at 422 nm. The reaction at 528 nm was found to be monophasic and the half-time was estimated to be less than 0.5 μsec, identifying it as a carotenoid shift.

Additional studies on the kinetics of laser-induced cytochrome oxidation in anaerobic cell-free extracts of *Chromatium* D and *Rps. palustris* show half-times at 421 nm of about 2.4 and 12 msec, respectively, at 77°K and half-times at 423 nm of about 2 and 1.8 μsec, respectively, at 300°K; these values are comparable to those of whole cells[1].

DISCUSSION

In the anaerobic cell-free extracts of all three species reversible absorbance changes observed in whole cells are virtually eliminated; P435 and P610 changes are scarcely detectable, and the reversible part of the carotenoid changes in *Rps. gelatinosa* is greatly diminished. The minimum in the Soret region of the photooxidized *c*-type cytochrome of *Rps. palustris* and *Rps. gelatinosa* appears to have shifted slightly

to the shorter wavelengths to about 419–420 nm; however, the light-*minus*-dark difference spectra of the *c*-type cytochromes observed under anaerobic conditions in both the whole cells and cell-free extracts of the three species are similar and will be referred to as anaerobic *c*-type cytochromes. In the whole cells of *Rps. palustris* and *Rps. gelatinosa* anaerobic *c*-type cytochromes undergo reversible reaction; however, reversible photooxidation of this type of cytochrome has not yet been demonstrated in the cell-free extracts of any of the three species investigated. Whether sonic treatment has resulted in the loss of the reversible anaerobic *c*-type cytochromes cannot be known at present.

All of the spectral components in the light-induced difference spectra of the oxygenated materials undergo completely reversible absorbance changes; some of these changes are not present in anaerobic samples. These results suggest that an increase in the ambient redox potential brought about by oxygenation places the components in the range where they become spectrally apparent and indicate the redox dependence of light-induced absorbance changes. This view is consistent with observations at room temperatures, and also with earlier reports by other investigators[9–11] that photo-induced changes of bacteriochlorophyll types in the near-infrared showed redox dependence and appeared over a characteristic range of redox potentials.

The identity of the anomalous absorption minima appearing at 553–555 nm of all three oxygenated species (Figs. 1C, 2C and 3D) and of a trough at 522 nm in *Rps. palustris* (Fig. 2C) are as yet to be determined. They can not be attributed to any known components, however, they could be due to the photooxidation of an unusual form of *c*-type cytochrome with its γ-band not participating in "frozen in" conformation at these low temperatures. In this connection the absence of absorbance change due to the β-band in the difference spectrum of anaerobic *Chromatium* D cell-free extracts (Fig. 1B) is of interest since this phenomenon might be related to the above observation; a similar observation has been made with *Chromatium vinosum* (T. KIHARA, unpublished). The disproportionately large α- and β-bands relative to the small changes of the γ-band observed in whole cells of *Rps. palustris* (Fig. 2A; lower curve) also constitute a marked deviation from the known oxidized-*minus*-reduced difference spectrum for the *c*-type cytochrome in this species at room remperatures[1]. Similarly, the α-band at 548 nm of the photooxidized anaerobic *c*-type cytochrome C419 (77°K) of *Rps. gelatinosa* cell-free extracts has been found to be disproportionately large with respect to the γ-band. A shift to slightly shorter wavelengths of the photooxidized cytochrome bands at 77°K compared with those at 300°K is known to be an effect of low temperature upon absorption bands of proteins[12]. The identification of photooxidizable *c*-type cytochromes at 77°K may be based on the shape and the location of the α-, β- and γ-bands irrespective of the observed disproportionality of these bands although, at present, little is known about the cause of the effect.

Photooxidation of anaerobic *c*-type cytochromes apparent under anaerobic conditions is no longer detected in any of the oxygenated samples of the three species. The simplest interpretation is that the anaerobic *c*-type cytochromes being low potential cytochromes are oxidized under the oxygen-saturating conditions[13]. Since cytochrome photooxidation at 77°K in *Chromatium* D is apparent only under anaerobic conditions it would appear that the cytochrome in question is the low potential, autooxidizable C552. Whether *Rps. palustris* possesses reversible *c*-type cytochromes (high potential) spectrally distinct from the low potential anaerobic *c*-type cytochromes

and similar to C422 (77°K) of *Rps. gelatinosa* is not known at present. However, it is possible that *Rps. palustris* cell-free extracts contain, as do whole cells (Fig. 2A), such a cytochrome which becomes spectrally apparent in an intermediate redox potential range between the anaerobic and oxygenated conditions attained by the methods employed.

The difference between a half-time of 60 μsec for C422 (77°K) oxidation and that of 10–20 μsec for C419 (77°K) suggests a different spacial arrangement for each of these two cytochrome species and the electron acceptor system. Applying the quantum mechanical electron tunnelling theory[5], the difference in half-time of oxidation between the two cytochromes would indicate only a slight increase in tunnelling distance for C422 (77°K) photooxidation if one assumes no significant increase in barrier height, or *vice versa*. The slow rate and the small activation energy obtained for C422 (77°K) dark reduction would also suggest a tunnelling mechanism for the reverse electron flow. Although the source and the path of the electrons for C422 (77°K) reduction are unknown, a direct electron donation from the reduced primary acceptor, or a reverse flow by way of fully dark-reduced bacteriochlorophyll may be considered possible.

The photooxidation of two spectrally distinguishable *c*-type cytochromes, C422 (77°K) and C419 (77°K) in *Rps. gelatinosa* poses an intriguing question concerning their origin, identity and interrelationship. One interpretation is that there are two spectrally distinct *c*-type cytochromes, each of which undergoes photooxidation over a separate range of oxidation–reduction potentials, and the two participate in separate paths for light-activated electrons. An entirely different view proposes a mechanism of transformation of one spectral species to another following a rise or fall in the ambient redox potentials[14]. An indirect line of evidence for the interconversion of the two *c*-type cytochromes (high [C422 (77°K)] and low [C419 (77°K)] potential forms) may be found in the observation that the light-induced absorbance changes of the oxygenated whole cells (Fig. 3B) exhibited splitting in the Soret region. It can be speculated that the splitting is brought about by the conversion of part of C419 (77°K) into C422 (77°K) upon oxygenation and that both cytochromes are concurrently and reversibly photooxidized. Alternatively, the splitting of the γ-band evident in the whole cells may be a low-temperature phenomenon and may not necessarily indicate the presence of two cytochrome species.

Comparison of laser-induced cytochrome oxidation rates at 77 and 300°K has shown close similarity in half-times between whole cells and cell-free extracts of *Chromatium* D, *Rps. palustris* and *Rps. gelatinosa*, and several other species[2] suggesting that the macromolecular structure required for the interaction of these cytochromes and their electron acceptor remains intact after ultrasonic disruption of whole cells. The failure in the past[11,15] to demonstrate this reaction in *Chromatium* D is attributable not to destruction of structural integrity but most probably to the anaerobic *c*-type cytochrome being in an oxidized state at the point of freezing.

Irreversible photo-reactions at liquid nitrogen temperatures are observed when anaerobic conditions (lower redox potential range) are established, whereas only reversible reactions occur under oyxgen-saturating conditions (higher redox potential range). Both irreversible and reversible reactions may be observed in certain cases (Figs. 2A and 3A) when the range of prevailing ambient potentials is thought to be intermediate. Carotenoid absorbance changes observed in *Rps. gelatinosa* exhibit both

reversibility and irreversibility, the extent of which is determined by the degree of anaerobiosis. The light-induced reactions of P435 and P610 are always completely reversible whenever they are spectrally detected. These steady-state changes are optimal under oxygen-saturating conditions but minimal or absent under anaerobic conditions when the extent of cytochrome photooxidation is maximal.

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