EVIDENCE FOR TWO LIGHT-DRIVEN REACTIONS IN THE PURPLE PHOTOSYNTHETIC BACTERIUM, RHODOSPIRILLUM RUBRUM*

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Based mainly on negative arguments, the present consensus is that bacterial photosynthesis is driven by a single light reaction analogous to the photochemical reaction of system I of higher plants and algae (cf. ref. 1). Both Goedheer[2] and Clayton[3] indicated the possibility of two light reactions in photosynthetic bacteria without presenting convincing evidence. Recently, however, Morita[4] reported action spectra for the light-induced oxidation of the cytochromes C553 and C555 in *Chromatium* D. His results suggest that these cytochromes are involved in two different light reactions. In most photosynthetic bacteria, at least two cytochromes (which are probably of the *c* type) seem to participate in the light reaction,[5-7] one of these in a cyclic back reaction mediating cyclic photophosphorylation.[8] Cytochromes of the *b*-type have been found in various photosynthetic bacteria.[9, 10] Light-induced reduction *in vivo* of a *b*-type cytochrome has been reported in *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* by Nishimura and Chance,[11] and more recently in *R. rubrum* by Ke and Ngo.[12]

A complete kinetic separation of the light-induced reactions of the two *c*-type cytochromes can be observed in the green photosynthetic bacterium *Chloropseudomonas ethylicum*, by using anaerobic cells suspended in a medium depleted of substrate.[7] Duysens[6] has reported a partial kinetic separation of the light-induced oxidation of a cytochrome-like pigment, C428, from the light-induced oxidation of a *c*-type cytochrome (cytochrome c_2, refs. 9 and 13) in *R. rubrum*.

The present paper reports kinetic and spectral separation of the light-induced oxidation of C428 and cytochrome c_2 in *R. rubrum*, using moderate intensities of the actinic light, under strict anaerobic conditions with cell suspension depleted of substrate. This separation allowed a measurement of the action spectra of light-induced oxidation of each of the two cytochromes. The apparent difference between the action spectra resulting from such measurements indicated that each of the cytochromes is involved in a separate light reaction.

Materials and Methods.—*R. rubrum* was grown anaerobically in the light in the malate-containing medium described by Ormerod, Ormerod, and Gest.[14] One-day-old cultures were harvested by centrifugation and resuspended in the growth medium without substrates. To re-establish the anaerobic state, the suspensions were bubbled with dry nitrogen for about half an hour, transferred to the 0.4-ml, 1-mm light path cuvettes, and kept in the dark for another half hour. The optical density in the main far-red absorption band at 883 nm was about 0.6.

Measurements of light-induced absorbance changes were made with a "split-beam" absorption difference spectrophotometer, based on a design of Duysens.[15] Homogeneous illumination of the sample with actinic light was provided by a 500-watt, d-c-fed filament lamp and a lens system. The wavelength of the actinic light was selected by Farrand

interference filters which were blocked at both sides of the main transmission bands. The half width of the transmission bands ranged between 15 and 20 nm.

The intensity of the actinic light was measured by placing in the sample position a photocell previously calibrated against an Eppley thermopile. All measurements were done at approximately 3°C. The absorption spectrum in Figure 3 was measured with a Cary 14 R recording spectrophotometer.

Results and Interpretation.—The kinetic pattern of light-induced absorbance changes in *R. rubrum* in the spectral region between 400 and 600 nm is largely dependent on the intensity of the actinic light.[6] At very low intensity (about 0.1 nEinstein/cm² sec) simple monophasic kinetics were observed. In a substrate-depleted medium, the light-minus-dark difference spectrum of these changes showed a perfectly symmetric trough with minimum at 428 nm (see Fig. 1a, *solid line*). The shape of the difference spectrum suggests the oxidation of a cytochrome and confirms the findings of Duysens,[6] who called the cytochrome C428. At higher intensities of the actinic light (about 1 nEinstein/cm² sec), the kinetics of the light-induced absorbance changes started to show a biphasic character; the "light-off" transition also had a rapid and slow phase. The light-minus-dark spectrum of the slow "light-on" transition (Fig. 1b, *dashed line*) had a minimum at 422 nm, a shoulder at about 430 nm, and minima at 513 and 551 nm. The light-minus-dark spectrum of the rapid "light-on" transition (Fig. 1b, *solid line*) had a minimum at 428 nm, a smaller peak at about 422 nm, and minima at 513, 518, and 553 nm. In cases of a biphasic decay in the "light-off" transition, the rapid phase always had a dark-minus-light spectrum with maxima at 422, 512, and 551 nm. At still higher intensities of the actinic light (about 2.5 nEinstein/cm² sec), the slow phase of the "light-on" transition became smaller (Fig. 1c, *dashed line*), and the light-minus-dark difference spectrum of the rapid phase, although skewed toward longer wavelengths in the Soret region, showed definite minima at 422, 513, and 551 nm (Fig. 1c, *solid line*). Duysens[6] reported similar spectral changes with anaerobic *R. rubrum* cells suspended in water. Light-induced absorbance changes measured with actinic light on top of a low-intensity background light showed a perfectly symmetric light-minus-dark spectrum with a minimum at 422 nm (Fig. 1a, *dashed line*).

These experiments clearly show that the observed kinetic phenomena include contributions from two cytochromes, Duysens' C428 (ref. 6) and, as suggested by the minima in the light-minus-dark spectra, cytochrome c_2 (refs. 9, 10, and 13). Very dim light (intensities <0.3 nEinstein/cm² sec) oxidized only C428 (see the spectrum in Fig. 1a, *solid line*). The light-minus-dark spectra observed at higher intensities suggest that the initial slow increment of the light-induced absorbance change observed on top of a rapid phase at these intensities is due to the oxidation of cytochrome c_2. The "light-off" transitions observed also suggest that this cytochrome is reduced rapidly in the dark. The oxidation of only cytochrome c_2 by actinic light on top of a (weak) background light (Fig. 1a, *dashed line*) suggests that the oxidation of C428 is saturated at relatively low intensity of the actinic light.

Figure 2 shows the rapid and the slow phase of the time courses observed at 425 nm as a function of the intensity of the actinic light at 881 nm. Similar

light curves were observed at different wavelengths of the actinic light. A comparison of the light curves with the spectral data, such as given in Figure 1, makes it reasonable to assume that in the intensity range up to about 0.6 nEin-
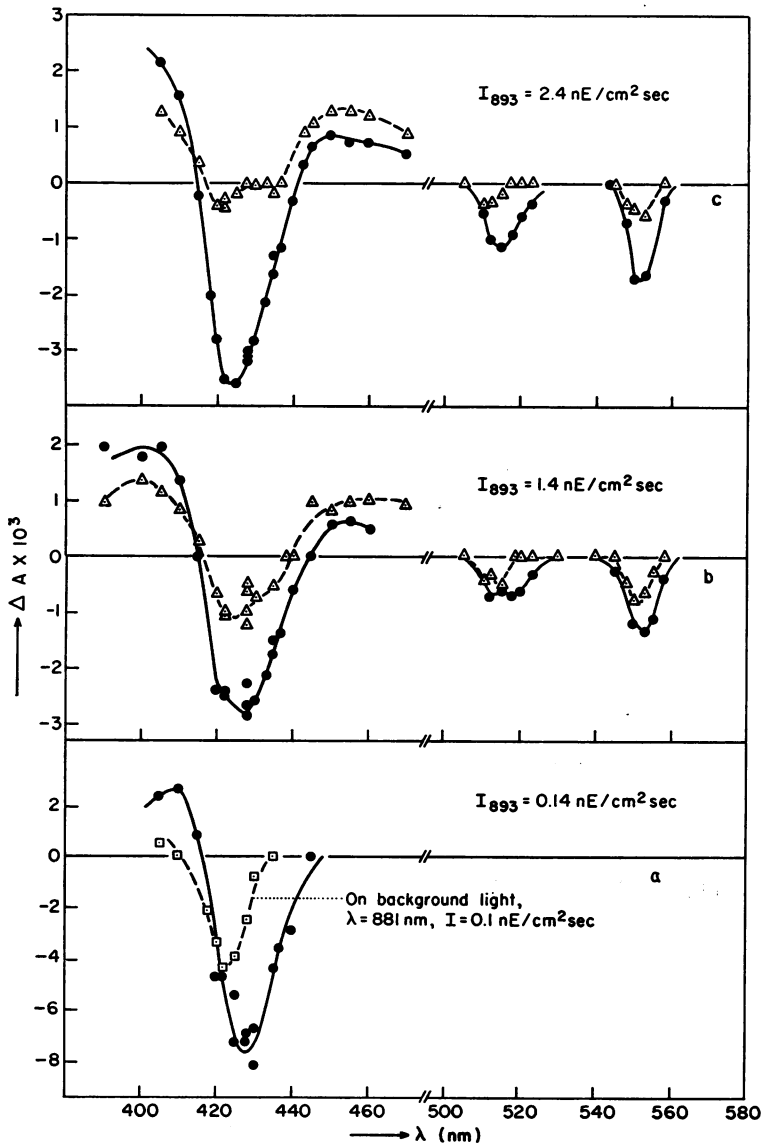

FIG. 1.—Light-minus-dark difference spectra of absorbance changes in substrate-depleted *R. rubrum*.

(*a*) *Solid curve:*   monophasic absorbance changes induced by low-intensity light of 893 nm; *dashed curve:*   monophasic absorbance changes induced by low-intensity light of 892 nm on top of low-intensity background light of 881 nm.

(*b, c*) *Solid curve:*   rapid initial absorbance changes induced by intermediate and high-intensity light of 881 nm; *dashed curve:*   slow absorbance changes induced by intermediate and high-intensity light of 881 nm.
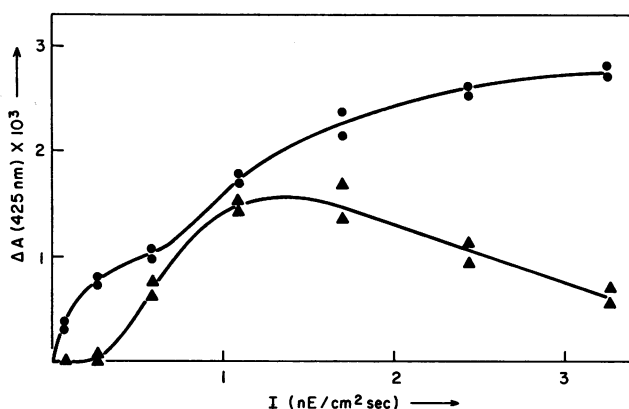
FIG. 2.—Rapid initial (●) and slow (▲) absorbance changes at
425 nm induced by light of 881 nm in substrate-depleted *R.
rubrum*, as a function of the intensity of the actinic light.

stein/cm² sec the "rapid phase," which is the only transition observed at very
low intensity, is largely due to the oxidation of C428, while the "slow phase"
observed at intensities higher than about 0.3 nEinstein/cm² is largely due to the
oxidation of cytochrome c_2. Based on this assumption, an action spectrum de-
termined from the light curves for a number of wavelengths of the actinic light at
intensities lower than about 0.3 nEinstein/cm² sec reflects the action spectrum of
C428 oxidation, while an action spectrum of the "slow phase" determined from
the light curves in the intensity range up to about 0.6 nEinstein/cm² sec reflects
the one for cytochrome c_2 oxidation. In Figure 3, such action spectra, corrected
by taking the statistical average of the light intensity in the light path of the
actinic beam, are shown and compared with the near infrared absorption spec-
trum of the suspension. Three series of measurements with three different
samples all show substantially the same result, indicating that while C428 is
oxidized by light mainly absorbed in the major absorption band of the suspension
at 883 nm, cytochrome c_2 is oxidized by light absorbed in both the major absorp-
tion band at 883 nm and the minor absorption band at 804 nm.

Discussion.—Although the action spectra given in Figure 3 still allow a trivial
interpretation in which the photosynthetic pigment system contains two regions
in which the light-harvesting pigments B890 (bulk of bacteriochlorophyll) and
P800 (the minor component responsible for the absorption band at 804 nm) are
present in different ratios, the simplest explanation of the present data involves
two different light-driven reactions. One light reaction involves the oxidation of
a pigment, C428, presumably a cytochrome.[6] The rapid light-induced oxidation
and subsequent slow reduction in the dark in the substrate-depleted medium
indicate that this reaction is directly linked to the oxidation of the substrate.
The second light reaction involves the oxidation of cytochrome c_2. The slow
and retarded oxidation of this cytochrome by light of moderate intensity sug-
gests a cyclic reaction. Such a cycle could provide for a site of photophosphoryl-
ation,[8] which may be indicated by the close correlation between oxidation-
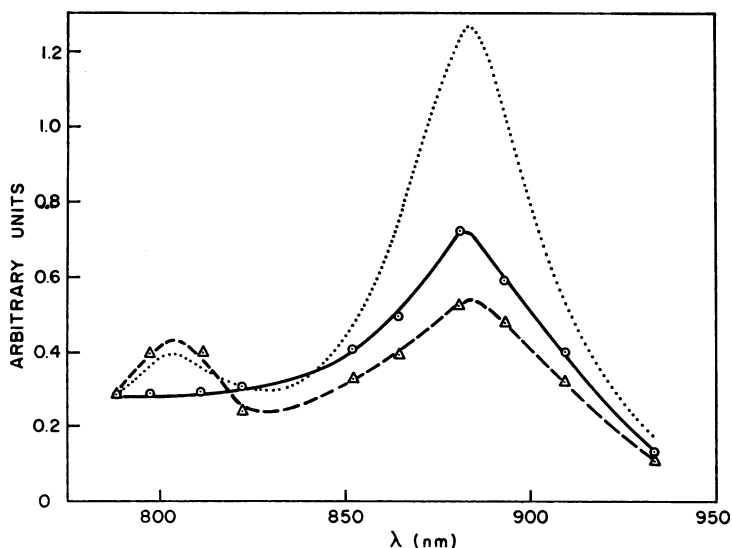
Fig. 3.—Action spectra of the rapid (*solid curve*) and the slow (*dashed curve*) light-induced decrease in absorbance at 425 nm in substrate-depleted *R. rubrum*. The action spectra are normalized at 788 nm. *Dotted curve:* far-red absorption spectrum of the suspension.

reduction reactions involving cytochrome c_2 and a functional phosphorylation system, demonstrated by Smith and Baltscheffsky.[16] Another component of the cycle possibly is a substance responsible in part for the light-induced increase of absorbance at 435 nm,[3, 5, 6] as suggested by a comparison of this light-induced absorbance change and the cytochrome reactions and near-infrared absorbance changes.[17] A chemical linkage between the two light reactions could occur via a pool probably containing ubiquinone that has been demonstrated to participate in light-induced reactions in purple bacteria, including *R. rubrum*,[18] and is present in relatively large amounts.[19] At low intensities of the actinic light, the cycle is operating at a low rate and oxidation of C428 can proceed efficiently, thereby reducing the pool. At higher intensities of the actinic light, when this reaction becomes saturated, the light-induced oxidation of cytochrome c_2 can compete effectively with the back reaction in the cycle.

Convincing evidence exists that the efficient light-induced decrease in absorbance at about 883 nm in *R. rubrum* (and similar light-induced changes in the near-infrared absorbance in other purple bacteria) is due to the oxidation of a reaction center, P890, the site at which excitation energy is converted to chemical energy.[20, 21] This light-induced absorbance change always seemed to be accompanied by a blue shift of an absorption band at about 805 nm.[20, 22] Most probably, these two kinds of light-induced absorbance changes involve reactions of two different entities,[23] both of which resist treatment, leading to the destruction of the light-harvesting bulk of bacteriochlorophyll.[23, 24] Furthermore, Clayton and Sistrom[25] have demonstrated that light absorbed at about 800 nm is transferred to P890, causing its bleaching. This seems to argue against the

possibility that the substance responsible for the blue shift at 805 nm (P800) is the trap for the cyclic light reaction; the fact that the cyclic reaction, involving the oxidation of cytochrome c_2, is driven by light absorbed by both P800 and B890 (Fig. 3) also seems to indicate that P890 rather than P800 is the reaction center. The substrate oxidation then could be mediated by an entirely different reaction center, the nature of which remains unknown. However, the possibility that this reaction center consists of P890 molecules which are not connected with P800 remains open; the fact that in dim light quanta absorbed by B890, the bulk of bacteriochlorophyll, are transferred to P890 argues in favor of such a reaction center.

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