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EXTRACTION OF OXIDIZED BACTERIOCHLOROPHYLL FROM ILLUMINATED PHOTOSYNTHETIC REACTION CENTER PARTICLES

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SUMMARY

Illuminated and dark-adapted reaction center particles from *Rhodopseudomonas spheroides* were extracted with methanol, and the spectra of the extracts were compared. Spectra of extracts of illuminated reaction center particles showed less absorption due to bacteriochlorophyll than did spectra of extracts of dark-adapted reaction center particles. The lost absorption in extracts of illuminated reaction center particles was restored by the addition of ascorbate. This showed that these extracts contained oxidized bacteriochlorophyll which could be rereduced *in vitro*. The spectrum of the restored absorption was different from that of the original bacteriochlorophyll, indicating that an alteration of the oxidized bacteriochlorophyll had taken place. This apparent alteration may have been due to the presence of the detergent lauryldimethylamine oxide in the extracts. However, equilibration of the protein with this detergent may have been a requirement for the extraction of photooxidized bacteriochlorophyll.

INTRODUCTION

In bacterial photosynthesis, the energy of absorbed light is transferred to the photochemical reaction centers where it drives the primary photochemistry, an oxidoreduction in which an electron is transferred from the primary electron donor, a special component of bacteriochlorophyll[1–16], to the primary electron acceptor. The detailed natures of these primary reactants are current subjects of active investigation.

No studies have yet been made with isolated primary donor bacteriochlorophyll. If this bacteriochlorophyll could be extracted and distinguished from the "nonbleachable" component of bacteriochlorophyll in the reaction center (associated with the absorption band P800), then it would be possible to study the chemical nature of this special bacteriochlorophyll directly.

A step toward this goal was made in a spectroscopic study of chromatophores which had been treated with K_2IrCl_6 (which destroyed the light-harvesting bacteriochlorophyll[9]). That study showed that preparations in which the primary donor was

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oxidized by ferricyanide yielded organic extracts showing less near-infrared absorption by bacteriochlorophyll than was present in extracts from nonoxidized preparations. The intensity of absorption by bacteriochlorophyll in the near-infrared region is a measure of the amount of nonoxidized bacteriochlorophyll present in an extract, since oxidized (bleached) bacteriochlorophyll does not absorb appreciably in this spectral region[10,17]. This result therefore suggested the interpretation that the extract of the oxidized preparation contained both oxidized and nonoxidized bacteriochlorophyll, but that the absorption in the near infrared was mainly due to the nonoxidized bacteriochlorophyll, and to bacteriopheophytin[9].

This communication reports some experiments done with reaction center particles*, a simple system containing no light-harvesting bacteriochlorophyll and no degradation products of light-harvesting bacteriochlorophyll that absorb in the visible or near-infrared spectral regions. It will be seen that oxidized bacteriochlorophyll can be extracted from reaction center particles which have been illuminated so as to photooxidize the primary electron donor: extracts of illuminated reaction center particles show less near-infrared absorption by bacteriochlorophyll than do extracts of dark-adapted reaction center particles; the lost absorption in extracts of illuminated reaction center particles can be recovered by the addition of the weak reductant ascorbate to the extracts. This finding represents an additional bridge between bacteriochlorophyll chemistry *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reaction center particles were isolated from carotenoidless mutant *Rhodopseudomonas spheroides*, strain 2.4.1/R-26 according to the method of Clayton and Wang[20]. After the final step in the isolation procedure, the reaction center particles were precipitated with $(NH_4)_2SO_4$ and resuspended in buffer containing 0.3% (v/v) of the detergent lauryldimethylamine oxide.

All absorption spectra were measured with a Cary 14R spectrophotometer (Applied Physics Corp., Monrovia, Calif.). The intensity of the exciting light was measured with a YSI radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Extraction experiments were performed at room temperature, in the dark except for the deliberate illumination program. A glass centrifuge tube containing 0.2 ml of reaction center particles was clamped into a shield but could be illuminated from the bottom through a shutter and a Wratten 87C filter. The wavelengths passed by this filter would be absorbed by reaction center particles but not by bacteriochlorophyll in methanol. The exciting light from a Sylvania 1000 W tungsten–iodine (Sun Gun) lamp was passed through water and focussed on the filter to form an illuminated area larger than that occupied by the reaction center particles in the tube. After the sample had been illuminated for 1.5 min, 3.8 ml of methanol were vigorously injected. The shutter was closed $\leqslant 2$ s after the beginning of the injection. It was hoped that this method would achieve rapid, thorough mixing, extracting the pigments while the

* The term "reaction center particle' is used rather than "reaction center" because the particles used in this study may not be the smallest morphological entities satisfying the definition of reaction center[18,19].

reaction center particles were still in a "light steady state". The tube was vigorously inverted several times and then was centrifuged to sediment aggregated protein.

For the extraction of dark-adapted reaction center particles, 0.2 ml of a suspension of reaction center particles was allowed to stand in the dark for 3 min in a tube covered with alluminum foil and clamped in the shield. Then the procedure described above was carried out (the reaction center particles were exposed for 1.5 min to the heat of the exciting light impinging on the aluminum foil).

RESULTS AND DISCUSSION

The solid line in Fig. 1 shows the spectrum of an extract of dark-adapted reaction center particles. It contains two bands in the visible spectral region, at 528 nm (due primarily to bacteriopheophytin) and at 607 nm (due primarily to bacteriochlorophyll). In the near infrared, there is a single band at 760 nm with a shoulder on the short-wavelength side. This band and shoulder are formed by the sum of peaks and shoulders in the spectra of bacteriochlorophyll and bacteriopheophytin. The addition of ascorbate had no effect on the spectrum of the extract of dark-adapted reaction center particles (aside from dilution). The spectrum of the extract of illuminated reaction center particles (dashed line in Fig. 1) shows that the magnitudes of the band and shoulder in the near-infrared region and of the bacteriochlorophyll peak in the visible region decreased as a result of illumination. The increase in absorption at wavelengths greater than 815 nm and less than 585 nm was probably due to oxidized bacteriochlorophyll (see ref. 10, Fig. 1). The dotted line in Fig. 1 shows that the addition of ascorbate to the extract of illuminated reaction center particles caused the absorption (presumably due to bacteriochlorophyll) to increase almost to the level

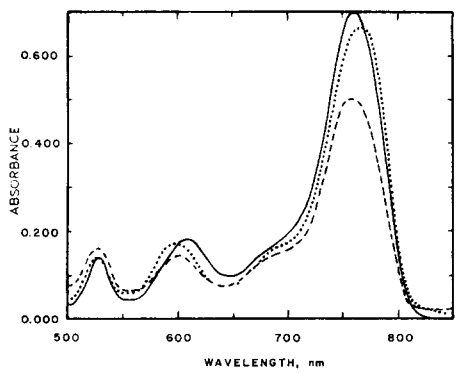

Fig. 1. Spectra of methanolic extracts of illuminated and dark-adapted reaction center particles. ———, spectrum of an extract of dark-adapted reaction center particles. The reaction center particles stood in the dark for 3 min and were exposed for 1.5 min to heat from the exciting light, through a barrier of aluminum foil; then methanol was injected. – – – –, spectrum of an extract of illuminated reaction center particles. Reaction center particles were illuminated for 1.5 min by the exciting light before methanol was injected. . . . ., spectrum recorded of an extract of illuminated reaction center particles after ascorbate had been added to the extract (to give a concentration of 1 mM). The spectrum has been corrected for dilution by the added liquid. Reaction center particles were suspended in 0.01 M Tris–HCl (pH 7.5)+0.3% lauryldimethylamine oxide before being extracted with methanol. The exciting light was of wavelengths greater than approx. 820 nm and of intensity approx. 6 mW cm^{-2}.

shown by the extract of dark-adapted reaction center particles, although the spectrum of the restored absorption was different from that of the original bacteriochlorophyll. The band in the visible region due primarily to bacteriopheophytin showed little net change from its appearance in the extract of dark-adapted reaction center particles.

The recovery of absorption presumably due to bacteriochlorophyll upon the addition of ascorbate to an extract of illuminated reaction center particles shows that the decreased absorption seen in that extract was not caused by destruction of bacteriochlorophyll and that the species responsible for the decrease in absorption was an oxidized compound. However the alteration of the recovered absorption indicates that the presumed oxidized bacteriochlorophyll had been chemically altered.

The spectra were analyzed as follows. First, the spectrum of an extract of dark-adapted reaction center particles was resolved into component spectra of bacteriopheophytin and bacteriochlorophyll*. Separate spectra of bacteriochlorophyll and bacteriopheophytin were obtained, respectively, from a methanolic extract of cells (corrected for minor contamination by bacteriopheophytin) and from an acidified methanolic extract of dark-adapted reaction center particles. These separate spectra were weighted and combined (by a "least squares" procedure) so as to yield that constructed spectrum which best fitted the measured spectrum (see Fig. 2).

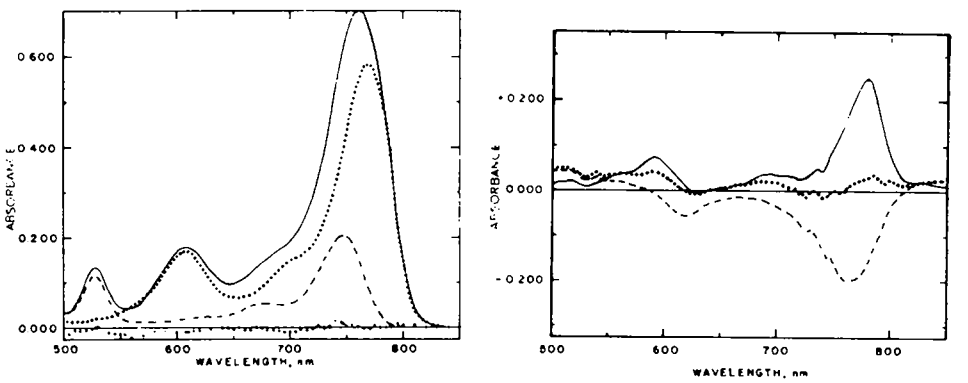

Fig. 2. Spectra of pigments in methanol. – ——, spectrum of an extract of dark-adapted reaction center particles (same as solid line in Fig. 1). . . . . , the absorption in the spectrum of the extract of reaction center particles due to bacteriochlorophyll, estimated by "least squares" fit (see text). – – – –, the bacteriopheophytin component of the spectrum of the extract of reaction center particles, estimated by "least squares" fit. – · · · –, the differences between the spectrum of the extract of dark-adapted reaction center particles and the spectrum constructed by "least squares" fit.

Fig. 3. Difference spectrum for methanolic extracts of illuminated and dark-adapted reaction center particles, and spectra of presumed altered oxidized bacteriochlorophyll and altered rereduced bacteriochlorophyll in methanol. – – – –, a light-*minus*-dark difference spectrum obtained by subtracting the solid curve of Fig. 1 from the dashed curve. . . . . , spectrum of presumed altered oxidized bacteriochlorophyll, obtained from the light-*minus*-dark difference spectrum by removing the contribution presumably due to the loss of nonoxidized bacteriochlorophyll in its photoconversion. – · – · –, spectrum of presumed altered rereduced bacteriochlorophyll, obtained as described in the text.

* Reed and Peters[21], and also Beugeling *et al.*[22], using thin-layer chromatography, have found only bacteriochlorophyll and bacteriopheophytin in reaction center particles isolated by a different procedure from the same organism.

Next, the difference was taken between the dashed and solid curves in Fig. 1, representing extracts of illuminated and dark-adapted reaction center particles. This difference spectrum, shown as the dashed curve in Fig. 3, should reflect the presence of some oxidized bacteriochlorophyll and the loss of a corresponding amount of nonoxidized bacteriochlorophyll. The oxidized bacteriochlorophyll was probably altered, as indicated by the changed spectrum of the restored material (dotted curve, Fig. 1).

Then the component of the difference spectrum attributable to (altered) oxidized bacteriochlorophyll was abstracted as follows. We computed the fraction of bacteriochlorophyll converted to the oxidized state, from the obvious loss of absorption due to nonoxidized bacteriochlorophyll and from knowledge of the absorption due to the total bacteriochlorophyll component in an extract of dark-adapted reaction center particles (see Fig. 2). This computation included a small correction, introduced after a preliminary analysis, for absorption due to the oxidized bacteriochlorophyll. We assumed that the spectrum due to bacteriopheophytin was the same in each extract. Having decided what fraction of the bacteriochlorophyll was converted, we added the absorption due to that amount of (nonoxidized) bacteriochlorophyll to the difference spectrum shown by the dashed curve in Fig. 3, thus cancelling the component that corresponded to loss of (nonoxidized) bacteriochlorophyll. The residual difference spectrum was attributed to the presumed altered, oxidized bacteriochlorophyll. It is shown by the dotted curve in Fig. 3.

In a similar way, from the difference between the dotted and solid curves in Fig. 1, we determined the spectrum of the altered and rereduced component of bacteriochlorophyll. This is shown by the solid curve in Fig. 3.

The spectrum of the presumed altered oxidized bacteriochlorophyll is roughly similar to that of bacteriochlorophyll oxidized with I_2 (ref. 10, Fig. 1). The spectrum of the presumed altered rereduced bacteriochlorophyll has a band in the visible region at 590 nm, shifted to shorter wavelengths relative to the visible band of bacteriochlorophyll (see Fig. 2), and a near-infrared band peaking at 780 nm, shifted toward longer wavelengths relative to the near-infrared band of bacteriochlorophyll. The near-infrared band has a shoulder on the short-wavelength side, which has not shifted much relative to the position of this band in the spectrum of (unaltered) bacteriochlorophyll. Similar differences in locations of bands are found between bacteriochlorophyll and bacteriochlorophyllin[23], but a positive identification of the bacteriochlorophyllous compound awaits a careful chemical study.

The apparent chemical alteration of the presumed oxidized bacteriochlorophyll may have been due to the presence of lauryldimethylamine oxide in the extracts; it took place when the combination of lauryldimethylamine oxide, oxidized bacteriochlorophyll and methanol was present. The nonoxidized bacteriochlorophyll apparently was unaffected by the low concentration of lauryldimethylamine oxide present in the extracts: pigments in methanolic extracts of cells, chromatophores, or reaction center particles (initially in the presence of 0.05% of the detergent Triton X-100) were unaffected, judging by the spectra, by the addition of the appropriate amount of lauryldimethylamine oxide.

The brief illumination while methanol was being injected had no effect in these experiments: the shutter could be closed just prior to the injection, without affecting the results.

If the experiment of Fig. 1 was repeated with lauryldimethylamine oxide replaced by an equal concentration of Triton X-100, only tiny amounts of presumed altered oxidized bacteriochlorophyll were found in the extracts of illuminated reaction center particles. The spectra of such extracts closely resembled those of extracts of dark-adapted reaction center particles. If the injected methanol contained lauryldimethylamine oxide, then spectra like those of Fig. 1 were usually obtained; however, occasionally such extractions failed to yield a significant amount of presumed oxidized bacteriochlorophyll in an extract of illuminated reaction center particles. It may be that equilibration of the detergent with the protein (possibly as well as the type of detergent) was important for the isolation of oxidized bacteriochlorophyll in these experiments. The detergent might affect the rate of rereduction of oxidized bacteriochlorophyll by affecting the way the protein is denatured when methanol is added.

If reaction center particles with lauryldimethylamine oxide were extracted with a mixture of 6 vol. of methanol and 4 vol. of acetone which contained HCl to convert the bacteriochlorophyll immediately to bacteriopheophytin, there was no noticeable difference between extracts of illuminated and dark-adapted reaction center particles. Evidently oxidized bacteriochlorophyll could not be extracted as stable oxidized bacteriopheophytin under these conditions. Perhaps any oxidized bacteriochlorophyll became re-reduced spontaneously (by electrons from the solvent?) when it was converted to bacteriopheophytin.

We conclude from these experiments that the methanolic extracts of illuminated reaction center particles contained (altered) oxidized bacteriochlorophyll. This was shown by the fact that the spectra of these extracts contained less absorption due to nonoxidized bacteriochlorophyll than did the spectra of extracts of dark-adapted reaction center particles; the addition of ascorbate regenerated the absorption of a bacteriochlorophyllous compound, thus eliminating destruction as the cause of the decreased absorption and showing that the decrease in absorption by bacteriochlorophyll was due to the oxidation of some of the bacteriochlorophyll.

These experiments support and extend the results of the work with K_2IrCl_6-treated chromatophores which were oxidized chemically[9]. In those experiments, oxidized preparations yielded less absorption by nonoxidized bacteriochlorophyll than did preparations which were not oxidized. The present work, in showing that light could be used to cause the oxidation, provides an additional link between bacteriochlorophyll chemistry *in vitro* and *in vivo*. Extracts of illuminated reaction center particles potentially can be used for the isolation of the natural oxidized photoproduct for chemical study. Is this bacteriochlorophyll chemically different from "nonbleachable" bacteriochlorophyll (associated with the aborption band P800) or from light-harvesting bacteriochlorophyl?

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