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ABSORBANCE AND FLUORESCENCE PROPERTIES OF THE BACTERIOCHLOROPHYLL *a* REACTION CENTER COMPLEX AND BACTERIOCHLOROPHYLL *a* PROTEIN IN GREEN BACTERIA

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SUMMARY

Absorbance, emission and excitation spectra were measured at both room and liquid-nitrogen temperatures for a photochemically active bacteriochlorophyll *a* reaction center complex and a bacteriochlorophyll *a* protein isolated from *Chlorobium limicola* and *Chlorobium thiosulfatophilum*. The low-temperature absorbance spectrum for the complex has a band centered at 833 nm, which is not seen in the spectrum of the bacteriochlorophyll *a* protein. We attribute this difference to a modification of the bacteriochlorophyll *a* protein in the active complex. The room-temperature fluorescence spectra for the bacteriochlorophyll *a* protein and the complex are similar, as are those measured at low temperatures. The 833-nm component of the low-temperature absorbance spectrum of the complex is relatively nonfluorescent.

INTRODUCTION

A photochemically active bacteriochlorophyll *a* reaction center complex has been isolated recently from green photosynthetic bacteria[1]. The complex is enriched in bacteriochlorophyll *a* and contains carotenoids and cytochrome and exhibits a light-dependent reversible oxidation at the reaction center (P840 bleaching), as well as a cytochrome oxidation. The complex is apparently a polymeric association of similar subunits with a minimum size of $1.5 \cdot 10^6$ daltons and has been estimated to contain one P840 per 80 bacteriochlorophyll *a* and one P840 per 1000–1500 total chlorophylls (including *Chlorobium* chlorophyll). A highly purified protein that has a mol. wt of 152000 and contains 20 bacteriochlorophyll *a* molecules has been isolated from the green bacteria *Chloropseudomonas ethylica* strain 2-K and *Chlorobium thiosulfatophilum* PM. This protein is thought to be made up of four subunits, each containing five bacteriochlorophyll *a* molecules. It does not, however, exhibit any photochemical activity[2].

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This paper is concerned primarily with a comparison of the P840–bacteriochlorophyll *a* complex and the corresponding bacteriochlorophyll *a* protein from both *Chlorobium limicola* and *C. thiosulfatophilum* PM on the basis of fluorescence and absorbance techniques. The nature of some of the differences and their role in photosynthesis are discussed in detail.

All cultures previously designated *C. ethylica* that have been examined contain at least two organisms; a colorless motile rod and a green nonmotile photosynthetic organism[3,4]. Morphologically and physiologically the green organism more clearly resembles *C. limicola.* Some of the data presented in this paper suggest that the work reported here for the purified green organism (*C. limicola*) corresponds to that published earlier for *C. ethylica*[1,2,5].

MATERIALS AND METHODS

The green bacteria *C. limicola* and *C. thiosulfatophilum* PM were used in all experiments described in this paper. The organisms were grown as previously described, except that *C. limicola* was grown autotrophically with increased $[S^{2-}]$ of 0.2%[1,6,7]. 20-l quantities of 4-day-old cultures were harvested by centrifugation and resuspended in a 0.01 M sodium ascorbate–0.01 M sodium phosphate buffer (pH 7.4).

Bacteriochlorophyll *a* protein was prepared as previously described from both broken cells and the bacteriochlorophyll *a* reaction center complex[5,8]. The bacteriochlorophyll *a* reaction center complex was isolated as previously reported[1], with the following additional purification step. An LKB8101 electrofocusing column was used to focus isoelectrically the bacteriochlorophyll *a* reaction center complex obtained from sucrose density centrifugation. A discontinuous 20–60% sucrose gradient containing a 1.0% Ampholine carrier ampholyte (pH 3–10) was used to fill the column. The electrofocusing was done for 44 h with the anode at the bottom and set at 300 V.

Chlorophylls were quantitated by extraction with 80% methanol and by measurement of the absorbance at 773 nm for bacteriochlorophyll *a* and at 670 nm for *Chlorobium* chlorophyll[9,10].

RESULTS

Preparation of an isoelectrically focused bacteriochlorophyll a reaction center complex from C. limicola

Since initial attempts to observe the fluorescence from bacteriochlorophyll *a* reaction center complexes from both *C. limicola* and *C. thiosulfatophilum* were obscured by extraneous fluorescence, and since 90% of the cytochrome present in the complex did not undergo reversible light-dependent oxidation, further purification was undertaken[1]. The material obtained after isoelectric focusing (IEF fraction) had an isoelectric point of 5.0–5.5, contained all the photochemically active components and exhibited no loss in activities. Prior to isoelectric focusing the initial material showed a nonmigrating band and as many as 15 protein bands on a 5% acrylamide gel. Only a single nonmigrating band containing the reaction center was observed after electrofocusing. The original fraction contained nearly 50 cytochromes and 80 bacteriochlorophyll *a* molecules per P840, whereas the IEF fraction contained 5–10

cytochromes per P840 (ref. 1). The initial preparation contained one *Chlorobium* chlorophyll molecule per 2–3 molecules of bacteriochlorophyll *a* and the IEF fraction contained around one per 5 bacteriochlorophyll *a* molecules. All subsequent experiments were done with the IEF fraction, which will henceforth be called bacteriochlorophyll *a* reaction center complex.

Absorbance properties

The room-temperature absorbance spectra of the photochemically active bacteriochlorophyll *a* reaction center complexes and the corresponding bacteriochlorophyll *a* proteins from *C. limicola* and *C. thiosulfatophilum* are shown in Figs 1a and 1b. In the spectral region between 550 and 900 nm, the absorbance spectrum for the bacteriochlorophyll *a* complex is reasonably similar to that of the corresponding bacteriochlorophyll *a* protein for both organisms. The principal differences are the presence of a 672-nm maximum in the complex, which is absent in the protein and the shift in the absorbance maximum in the bacteriochlorophyll *a* protein spectrum from 809 nm to 810.5 nm for *C. limicola* and to 812 nm for *C. thiosulfatophilum*. At wavelengths shorter than 550 nm, the photochemically active complexes have absorbance maxima characteristic of carotenoids and cytochromes, in addition to the peaks due to bacteriochlorophyll *a*. The large absorbance of the P840–bacteriochlorophyll *a* complexes in the Soret spectral region compared to that of the bacteriochlorophyll *a* protein suggests that unidentified components are also present. At room temperature, therefore, the most obvious differences between the inactive bacteriochlorophyll *a* proteins and the photochemically active complex are due to the presence of pigments other than bacteriochlorophyll *a*. The room-temperature spectra for the bacteriochlorophyll *a* proteins from the two organisms are essentially identical over the entire spectral range measured.

The data presented in Figs 2a and 2b, however, show that at liquid-nitrogen temperature there are differences between the bacteriochlorophyll *a* proteins themselves, as well as between each protein and its corresponding P840–bacteriochlorophyll *a* reaction center complex. In Fig. 2a the absorbance spectra taken at liquid-nitrogen temperature for the bacteriochlorophyll *a* protein and the P840–bacteriochlorophyll *a* complex from *C. limicola* are compared. The 809-nm absorbance band that occurs in the bacteriochlorophyll *a* protein at room temperature is split into several maxima at 824, 813.5 and 805 nm, with a shoulder at 790 nm (identical to that published previously for *C. ethylica* 2-K, ref. 5). The absorbance spectrum of the P840–bacteriochlorophyll *a* reaction center complex from *C. limicola* is obviously different from the bacteriochlorophyll *a* protein, due to the presence of an 833-nm band in the P840–bacteriochlorophyll *a* reaction center complex. Otherwise, the peak positions and the relative heights are nearly the same. Fig. 2b shows the low-temperature absorbance spectra for the bacteriochlorophyll *a* protein and the P840–bacteriochlorophyll *a* reaction center complex from *C. thiosulfatophilum*. Again, as with *C. limicola*, the complex has an 833-nm band not seen in the bacteriochlorophyll *a* protein. However, there are other more striking differences in *C. thiosulfatophilum* than those seen in *C. limicola*. In particular, the relationship of the peaks at 814.5 and 806.5 nm is reversed when the purified protein is isolated. Other species differences exist, again involving the peaks at 814.5 and 806.5 nm.

There are at least three possible explanations for the occurrence of the 833-nm

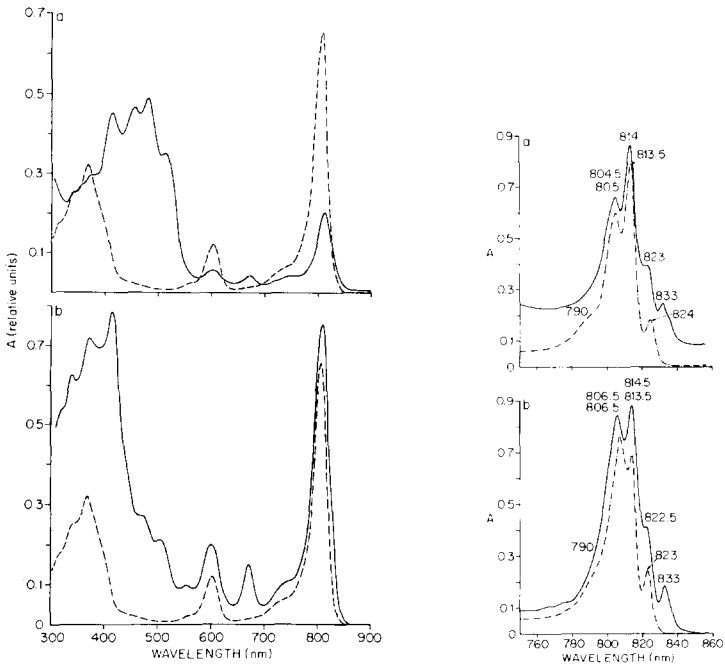


Fig. 1. (a) Room-temperature absorbance spectra for the bacteriochlorophyll *a* protein (----) and bacteriochlorophyll *a* reaction center complex (———) from *C. limicola*. In the protein spectrum, maxima occur at 809, 602 and 370 nm, with shoulders around 550, 395, 342 and 320 nm. (b) Room-temperature absorbance spectra for the bacteriochlorophyll *a* protein (----) and reaction center complex (———) from *C. thiosulfatophilum*. In the protein spectrum, maxima occur at 809, 602 and 370 nm, with shoulders near 395, 342 and 320 nm. In the spectrum for the reaction center complex, maxima occur at 812, 672, 601, 558, 501, 469, 413, 372 and 339 nm, with a shoulder near 446 nm.

Fig. 2. (a) Liquid-nitrogen-temperature absorbance spectra for the bacteriochlorophyll *a* protein (—·—) and bacteriochlorophyll *a* reaction center complex (———) from *C. limicola*. In the protein spectrum, peaks occur at 824, 813.5 and 805 nm, with a shoulder at 790 nm. In the spectrum for the reaction center complex, peaks occur at 833, 823, 814 and 804.5 nm, with shoulders at 836 and 790 nm. (b) Liquid-nitrogen-temperature spectra for the bacteriochlorophyll *a* protein (—·—) and bacteriochlorophyll *a* reaction center complex (———) from *C. thiosulfatophilum*. In the protein spectrum, peaks occur at 823, 813.5 and 806.5 nm, with a shoulder at 790 nm. In the spectrum for the reaction center complex, peaks occur at 833, 822.5, 814.5 and 806.5 nm, with a shoulder at 790 nm.

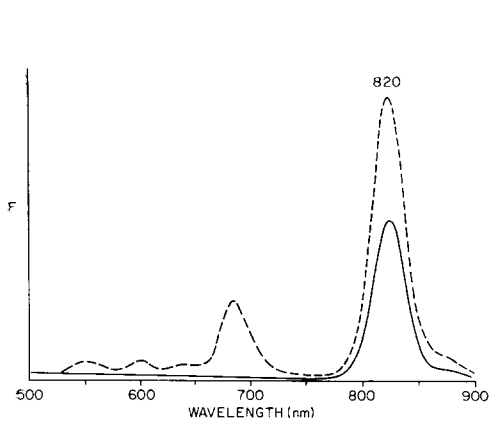
component. (1) It could be a completely separate pigment system whose pigments are unspecified. (2) It could be the chlorophyll P840 of the reaction center. (3) It could be due to a modification of the bacteriochlorophyll *a* protein when it is contained in a photochemically active preparation. None of these can be completely discounted. The position of the 833-nm peak suggests that it might be P840. It is estimated that there is around one P840 molecule per 80 bacteriochlorophyll *a* molecules in the complex[1]; however, the contribution of the 833-nm component is much greater than this. Additionally, the low-temperature spectrum for the bacteriochlorophyll *a* reaction center complex from both organisms is changed very little when a suspension of the complex is chemically oxidized; *i.e.* under conditions where

P840 is chemically bleached, the 833-nm peak is still present. These data strongly suggest that either P840 and the 833-nm component are not the same pigment or only a small amount of the 833-nm component can undergo bleaching. The relative extinction at room temperature was calculated for the 810.5-nm peak in the bacteriochlorophyll *a* reaction center complex from *C. limicola*. The value of 154 $mM^{-1} \cdot cm^{-1}$ for the 809-nm peak in the bacteriochlorophyll *a* protein was used[5]. Methanol extracts from the complex and the bacteriochlorophyll *a* protein were compared and a relative extinction was determined to be 133 $mM^{-1} \cdot cm^{-1}$. The decrease in extinction and the red shift of the near-infrared maximum in the complex compared to the bacteriochlorophyll *a* protein suggests modifications of the bacteriochlorophyll *a* protein. When the complex is incubated for 6 h in 5% Triton X-100, a concentration that is known not to affect the spectrum of bacteriochlorophyll *a* protein[11], the low-temperature spectrum is that of the bacteriochlorophyll *a* protein, with no evidence of an 833-nm spectral component. This suggests that the 833-nm component may arise from the same modification that decreases the extinction at 810.5 nm.

Fluorescence properties

The fluorescence emission and excitation spectra were measured at room temperature for both the bacteriochlorophyll *a* reaction center complex and the bacteriochlorophyll *a* protein from *C. limicola*. The emission spectra from 500 to 900 nm for the two preparations are shown in Fig. 3. These data are presented uncorrected as relative amounts of fluorescence and were measured under identical instrumental conditions. When excited at 390 or 600 nm, the bacteriochlorophyll *a* protein had a major emission band at 820 nm and a shoulder at 880 nm, in agreement with previously published results[5]. On the other hand, the bacteriochloropyhll *a* reaction center complex exhibited a much more complex spectrum when excited at 390 nm. The bands at 820 and 880 n are identical to those found for the bacteriochlorophyll *a* protein. The major emission band was narrowed considerably and shifted to 828 nm in samples measured at liquid-nitrogen temperature; but as seen in the room-temperature measurements, the spectra from the bacteriochlorophyll *a* reaction center complex and the bacteriochlorophyll *a* protein were very similar at long wavelengths. The results are not what might be expected if the 833-nm component equilibrated rapidly with the other spectral components. Calculation of the theoretical low-temperature emission bands for the bacteriochlorophyll *a* protein and the complex from *C. limicola* predicts that the maximum for the complex should be red-shifted at least 10 nm relative to the bacteriochlorophyll *a* protein[12]. Since no such shift occurs, the 833-nm component contributes relatively little to the overall fluorescence.

The uncorrected excitation spectra measured from 350 to 750 nm for the 820-nm emission in both the bacteriochlorophyll *a* reaction center complexes are shown in Figs 4a and 4b. For comparison, the absorbance spectrum for each is also presented. In the spectral region below 550 nm, the excitation spectrum for the bacteriochlorophyll *a* reaction center complex (Fig. 4b) does not resemble the absorbance spectrum of the complex but closely resembles the bacteriochlorophyll *a* protein (Fig. 4a). The relative height of the peak in the 400-nm region is much less in the complex than in the protein. This can be attributed to the high absorbance in this spectral region by pigments not transferring excitation energy to bacteriochlorophyll *a*. The only excitation peaks observed in the region between 450 and 750 nm



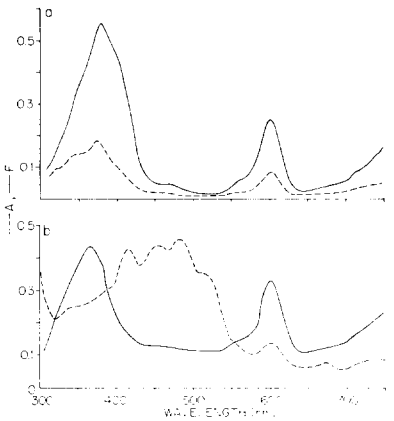


Fig. 3. Fluorescence emission spectra for the bacteriochlorophyll *a* protein (———) and bateriochlorophyll *a* reaction center complex (----) from *C. limicola*. Excitation was at 390 nm. The entrance and exit slits were 1 mm. In the spectrum for the reaction center complex, emission bands are at 550, 595, 630, 678 and 820 nm, with a shoulder at 880 nm; the sample contained 1 μM bacteriochlorophyll *a*. In the protein spectrum, the emission band is at 820 nm, with a shoulder at 880 nm; the sample contained 4.7 μM bacteriochlorophyll *a*. Presented on 1/3 scale.

Fig. 4. (a) Excitation (———) and absorbance (----) spectra for the bacteriochlorophyll *a* protein from *C. limicola*. The sample contained 4.7 μM bacteriochlorophyll *a*. See Fig. 1 for peak positions in the absorbance spectrum. In the excitation spectrum (for 820-nm fluorescence), peaks are at 380 and 602 nm, with shoulders near 470, 560 and 370 nm; 1-mm entrance and exit slits were used. (b) Excitation (———) and absorbance (----) spectra for the bacteriochlorophyll *a* reaction center complex from *C. limicola*. The sample contained 4.5 μM bacteriochlorophyll *a*. See Fig. 1 for peak positions in the absorbance spectrum. In the excitation spectrum (for 820-nm fluorescence), peaks are at 365 and 602 nm, with a shoulder at 560 nm; 1-mm entrance and exit slits were used.

are due to bacteriochlorophyll *a*. However, the peaks at 510, 480 and 446 nm in the absorbance spectrum of the bacteriochlorophyll *a* reaction center complex show that carotenoids are present. Chemical analysis shows that a γ-carotene is present in a greater than one-to-one ratio with bacteriochlorophyll *a*. The lack of any excitation maxima associated with the carotenoids suggests that these pigments do not transfer excitation to bacteriochlorophyll *a*. Also, no photochemically dependent carotenoid spectral shifts, as observed in the purple bacteria, are seen in the green bacteria[1]. Thus bacteriochlorophyll *a* seems to be the sole light-harvesting pigment in the bacteriochlorophyll *a* reaction center complex exciting bacteriochlorophyll *a* fluorescence.

The above data do not show whether the bacteriochlorophyll *a* fluorescence is in any way coupled to the bleaching of reaction center P840. When P840 was chemically oxidized by ferricyanide, or when the acceptor was reduced by dithionite, thereby inhibiting reaction center activity, the amount of 820-nm emission was approximately doubled, with equal excitation intensity. All other emission bands remained unchanged. This increase in fluorescence strongly suggests, as expected, that the bulk of bacteriochlorophyll *a* is transferring light energy to the reaction center, resulting in P840 bleaching.

The absorbance spectrum for the bacteriochlorophyll *a* reaction center complex

has a band at 672 nm, which is also inactive in energy transfer to bacteriochlorophyll *a*, raising a question about the nature of this spectral component. The position of the peak suggests that it may be either monomeric *Chlorobium* chlorophyll or *Chlorobium* pheophytin or both. An 80% methanol extract shows that there is approx. 1 *Chlorobium* chlorophyll molecule per 5 molecules of bacteriochlorophyll *a* in most preparations. If it is assumed that the extinction coefficient of the 672-nm absorbance band of the *Chlorobium* chlorophyll in the complex is about the same as that of the *Chlorobium* chlorophyll in methanol, then practically all the *Chlorobium* chlorophyll in the complex must be in the monomeric form. The excitation spectrum (not shown) for the emission maximum at 678 nm agrees very closely with that which would be expected for monomeric *Chlorobium* chlorophyll[8]. There is, however, a small absorbance and excitation peak at 550 nm, which suggests that some pheophytinized *Chlorobium* chlorophyll is present. Other fluorescing components were not examined in detail, but all excitation spectra exhibited porphyrin-like maxima in the Soret region.

CONCLUSIONS

Isoelectric focusing of sucrose gradient fractions containing the bacteriochlorophyll *a* reaction center complex from either *C. limicola* or *C. thiosulfatophilum* results in a considerably cleaner preparation. Low-temperature absorbance spectra of the complexes from both organisms have maxima at 833 nm, which are not seen in the purified bacteriochlorophyll *a* protein. The component responsible for the 833-nm maximum is not P840, since the peak persisted when P840 was known to be chemically oxidized by ferricyanide. The room-temperature extinction coefficient of the 810.5-nm band in the complex is less than that of the 809-nm band in the bacteriochlorophyll *a* protein. This decrease in extinction coefficient and the shift for the main chlorophyll absorbance band in the complex suggest that the bacteriochlorophyll *a* protein is somehow modified in the active complex. The 833-nm spectral component probably is a result of this modification.

The room-temperature emission bands at 820 and 880 nm from the bacteriochlorophyll *a* reaction center complex and the bacteriochlorophyll *a* protein are very nearly the same. The primary emission bands obtained at liquid-nitrogen temperature from both components are narrowed and shifted to 828 nm, but are also similar. The similarity of the emission bands for the bacteriochlorophyll *a* protein and complex suggests that the 833-nm component is relatively nonfluorescent.

The excitation spectra show that bacteriochlorophyll *a* alone absorbs light energy, leading to bacteriochlorophyll *a* fluorescence. This is true in spite of the presence of the carotenoid γ-carotene in quantities near to a ratio of 1 : 1 with bacteriochlorophyll *a*. *Chlorobium* chlorophyll is present in a ratio of 1 : 5 to bacteriochlorophyll *a* but is present predominantly in the monomeric form and does not transfer excitation energy to bacteriochlorophyll *a*.

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