BBA 45659

INTERACTION OF A SPINACH PROTEIN FACTOR WITH BACTERIOCHLOROPHYLL

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(Received October 30th, 1967)

SUMMARY

1. The absorption spectrum of colloidal bacteriochlorophyll gradually changes from one with two absorption maxima in the near infrared part of the spectrum into one with a single maximum in this region. This conversion is accelerated by a spinach protein factor. Spinach protein factor also causes a shift of the single remaining absorption maximum to a shorter wavelength.

2. The change in the absorption spectrum, together with results of experiments on dilution of aqueous colloidal bacteriochlorophyll with methanol suggest that colloidal bacteriochlorophyll may exist in two different forms, one with two maxima in its near infrared absorption spectrum (about 790 and 847 nm) and one with a single maximum in this region (775 nm).

3. Light sensitivity of the bacteriochlorophyll–spinach protein factor complex is highest if the colloidal bacteriochlorophyll is completely converted into the form with a single absorption maximum in the far-red part of the spectrum.

4. The light sensitivity of the bacteriochlorophyll–spinach protein factor complex depends on ionic strength and pH. The experimental results indicate that a positively charged site of bacteriochlorophyll combines with negatively charged spinach protein factor.

5. The elution pattern obtained on filtration of spinach protein factor through Sephadex G-100 is dependent on the buffer used.

INTRODUCTION

In a previous paper[1] a factor from spinach leaves was described which interacts with chlorophylls. In an aqueous medium this factor causes an increased light sensitivity and fluorescence capacity of the pigment. With bacteriochlorophyll in aqueous medium it was shown to cause a change in the absorption spectrum as well. Therefore, in the present work the interaction of the spinach factor with bacteriochlorophyll was investigated further.

From the results some conclusions about the properties of both colloidal bacteriochlorophyll and the spinach factor are suggested.

MATERIAL AND METHODS

These were the same as described in previous papers[1,2] with some slight alterations:

a. In part of the experiments spinach factor preparations were made and gel-filtered in 0.05 M tris(hydroxymethyl)-methylamine buffer, pH as indicated, instead of 0.1 M phosphate buffer. Unless mentioned otherwise, gel-filtered spinach protein preparations were used.

b. Protein was determined according to the method of LOWRY *et al.*[3].

c. The sample was irradiated at a distance of 20 cm from a 100-W incandescent lamp.

RESULTS AND CONCLUSIONS

Absorption spectra of colloidal bacteriochlorophyll with and without spinach protein factor

On rapid mixing of a methanolic solution of bacteriochlorophyll with a relatively large quantity of buffer (final methanol concentration 4%), a colloidal solution of bacteriochlorophyll is formed. The absorption spectrum between 1000 and 560 nm shows three clear maxima, at 847, about 785 and 589 nm, respectively (*cf.* refs. 4–7). During storage at room temperature in the dark the spectrum very slowly (2–10 h) changes (Fig. 1): The 847-nm absorption band disappears, the second band shifts to a shorter wavelength, the 589-nm band broadens and also shows some shifting to a shorter wavelength and a band of oxidized bacteriochlorophyll appears at about 690 nm (*cf.* ref. 8). Moreover, a maximum (generally low) at about 900 nm may show up, especially in phosphate buffer. The kinetics in the change in the spectrum appears

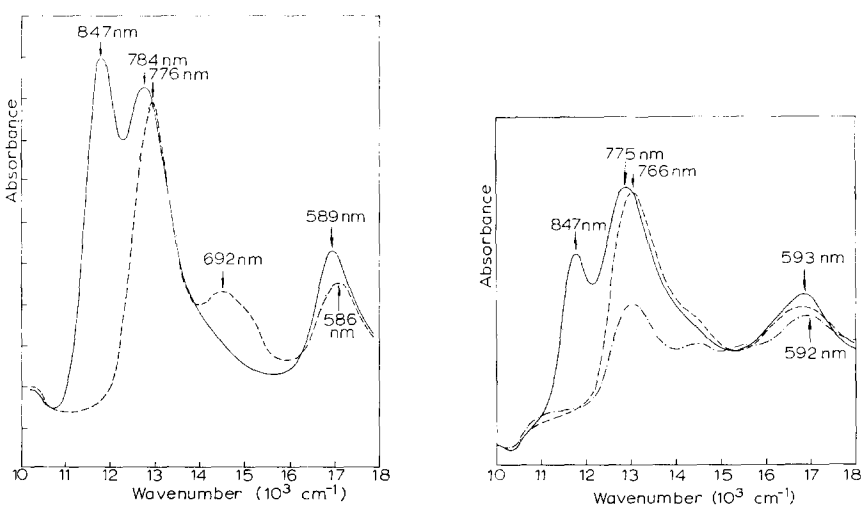

Fig. 1. Absorption spectra of colloidal bacteriochlorophyll. ———, immediately after preparation; – – – –, after 18 h at room temperature. Reaction mixture: 0.3 ml 0.1 M phosphate buffer, pH 6.9; 0.9 ml 0.1 M Tris buffer, pH 8.9; 0.05 ml methanolic bacteriochlorophyll solution.

Fig. 2. Absorption spectra of colloidal bacteriochlorophyll in the presence of spinach protein factor. ———, immediately after preparation; – – – –, after 10 min at room temperature; –·—·–, after illumination for 1 min. Reaction mixture: 1.2 ml 0.1 M phosphate buffer, pH 7.0, containing spinach protein factor; 0.05 ml methanolic bacteriochlorophyll solution.

to depend on various factors, such as pH and the kind of buffer used. Also, the age of the methanolic bacteriochlorophyll solution is important; solutions that have been kept for some days at about 4° in the dark show a less pronounced 847-nm absorption band on mixing with buffer than freshly prepared ones.

Centrifugation of the colloidal solution for 1 h at 144000 × *g* results in elimination of about half of the bacteriochlorophyll from the supernatant, but there is no significant preferential sedimentation of one of the two principal absorption maxima (847 and about 785 nm). This indicates that these two maxima do not correspond to large and small particles.

Addition of spinach factor[1] to a colloidal bacteriochlorophyll solution in buffer results in (a) an accelerated vanishing of the 847-nm absorption band and (b) an additional shift of the second red absorption band to a shorter wavelength (Fig. 2).

The resulting bacteriochlorophyll–spinach factor complex has become much more light sensitive than colloidal bacteriochlorophyll (*cf.* ref. 1). Sedimentation of bacteriochlorophyll upon centrifugation (1 h at 144000 × *g*) is not influenced by the addition of the spinach factor. In order to be able to explain the action of the spinach factor, an attempt was made to obtain an insight into the effects mentioned.

If the 847-nm absorption band were due to particles of aggregated chlorophyll (*cf.* refs. 4, 7) that are converted into a monomeric form absorbing at about 780 nm, then a rise in the latter absorption band might be expected to be accompanied by a decrease in the 847-nm band. However, on disappearance of the 847-nm absorption band only a slight change, increase or decrease, in the second red absorption maximum occurs. The latter effect might be due to some dark-oxidation of bacteriochlorophyll, as indicated by an absorption increase at 690 nm. The effect of this oxidation on the height of the 780-nm absorption band can be estimated from the data of SMITH AND CALVIN[8]. If this effect is taken into account and if the absorption coefficients of both bands are assumed to be about equal, then the increase at the shorter wavelength maximum is still much less than that which might be expected from the decrease at 847 nm (*cf.* Fig. 2).

The possibility was considered that during the 847-nm band conversion, part of the bacteriochlorophyll is gradually destroyed in some way. This was tested as follows: Equal amounts of bacteriochlorophyll were added to 0.1 M phosphate buffer and to the same buffer containing the spinach factor. The first colloidal bacteriochlorophyll solution showed a near infrared absorption spectrum with two bands (848 and 782 nm) (Fig. 3A), the latter one, after standing for 10 min, a spectrum with one band (770 nm, Fig. 3B). Both solutions were diluted with three times their volume of methanol (final methanol concentration 76 %).

The resulting spectra showed one absorption band at 770 nm in both cases. The height of the band resulting from the two-peaked spectrum was only slightly more than that from the one-peaked spectrum (Fig. 3A, 3B). The difference can be readily explained from the oxidation of some of the bacteriochlorophyll in the latter case[8].

From these experiments it was concluded that apart from this slight oxidation no bacteriochlorophyll destruction occurs during disappearance of the 847-nm absorption band.

Conversion of the 847 nm-absorption band in buffer is accompanied by a shift of the second absorption maximum (approx. 780 nm) of about 4–10 nm to a shorter

wavelength, depending on the initial relative height of the 847-nm absorption peak. The exact ultimate site of the maximum depends somewhat on the pH of the medium. No shift of the 847-nm band is measured during its disappearance. In the presence of spinach protein factor the resulting single absorption peak may be shifted 10 nm more to the shorter wavelength side than that of a comparable bacteriochlorophyll solution without added spinach protein factor. Location of the ultimate absorption band at a short wavelength corresponds to a high light sensitivity of the bacteriochlorophyll–spinach protein factor complex and *vice versa*.

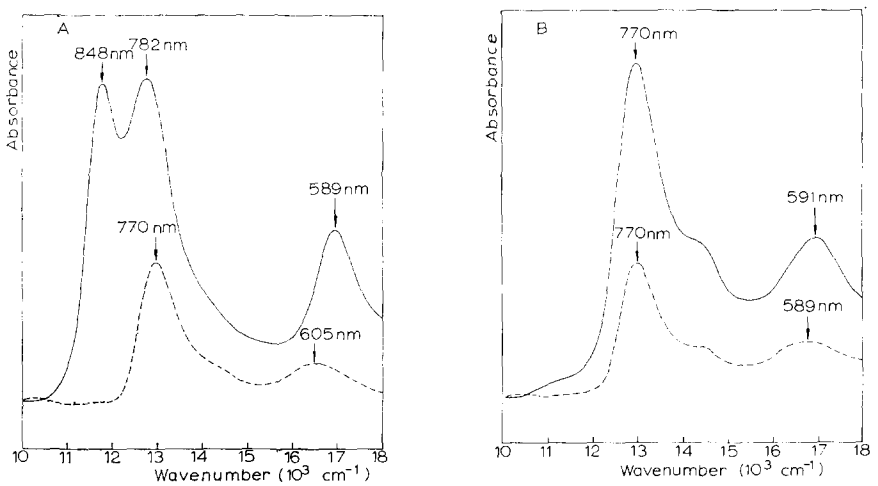

Fig. 3A. Absorption spectra of colloidal bacteriochlorophyll in phosphate buffer, 0.1 M, pH 7.0 (———) and the same solution diluted 4 times with methanol (– – – –). 3B. Absorption spectra of colloidal bacteriochlorophyll in phosphate buffer, 0.1 M, pH 7.0, containing spinach protein factor, after 10 min at room temperature (———) and the same solution diluted 4 times with methanol (– – – –).

Effect of some reagents

Comparison of absorption spectra of equal amounts of bacteriochlorophyll in buffer containing spinach factor, buffer containing the detergent Triton X-100, 0.25 %, and in methanol shows that the height of the red absorption maximum is nearly equal in methanol and Triton X-100, but is considerably smaller in buffer with the spinach factor (Fig. 4, *cf.* also Fig. 3B).

The absorption maximum located at 769 nm in methanol and in spinach factor solution occurs about 10 nm towards the longer wavelength in Triton solution. The band situated at 589 nm in spinach factor solution virtually coincides with that in Triton solution (586 nm, the difference may be due to some oxidized bacteriochlorophyll[8]), while this band in methanol is located at 607 nm (*cf.* ref. 9).

Chlorophylls in colloidal aqueous solution become light sensitive if detergents or spinach factor are added. In the latter case, photobleaching of bacteriochlorophyll is completely inhibited by 5 % urea, while urea at this concentration does not significantly influence photobleaching in Triton X-100 solution.

The activity of the spinach factor, as measured by its capacity to render bacteriochlorophyll in aqueous colloidal solution light sensitive, is inhibited to a large extent upon incubation with protease (from *Streptomyces griseus*), 40 min at 30°.

Protease also inhibits the acceleration of dark-conversion of the 847-nm absorption band. It was checked that the spinach factor is not perceptibly inactivated either by incubation without protease or by protease with very short incubation time. From these results, together with arguments mentioned in a previous paper[1], it is concluded that the spinach factor is proteinaceous. Henceforth it will be called spinach protein factor.

Some results of experiments with Triton X-100, urea and protease are collected in Table I. As the bleaching depends on several factors (concentrations of reagents, activity of spinach protein factor, bacteriochlorophyll concentration) that are not equal in the different experiments, these cannot be compared.

Photobleaching of bacteriochlorophyll with spinach protein factor

The rate of photobleaching of bacteriochlorophyll in the presence of spinach protein factor depends on the form of bacteriochlorophyll, as judged from its absorption spectrum. Generally, an active preparation of spinach protein factor induces a rapid conversion in the dark of bacteriochlorophyll into the form with one far-red absorption band (Fig. 2). The conversion occurs in 1–10 min, while in a comparable solution without spinach protein factor the same conversion takes 2–10 h. Photobleaching in the presence of spinach protein factor of the resulting band at about 770 nm is greatest when the 847-nm band has completely disappeared. However, some photobleaching of the 770-nm band occurs already if the 847-nm band is present. The effect remains the same until about 50 % of the 847-nm band has disappeared and increases upon further conversion of this band (Fig. 5). There is no measurable difference in the very slight capacity of photobleaching of the approx. 780-nm absorption band of colloidal bacteriochlorophyll in buffer without spinach protein factor before and after conversion of the 847-nm absorption band.

Influence of pH and salts

In order to get some insight into the way in which bacteriochlorophyll is bound to spinach protein factor, the influence of pH and salts on the photobleaching reaction was determined. pH and ions do not significantly influence light sensitivity of colloidal bacteriochlorophyll in buffer without spinach protein factor. Spinach protein factor preparations made and gel-filtered in 0.1 M phosphate buffer, pH 7.0, induce an increasing light sensitivity of bacteriochlorophyll if the pH of the reaction medium is raised from pH 7 to pH 9 (Fig. 6). This means that activity of spinach protein factor is greatest at high pH. The high pH is obtained by adding Tris buffer.

The rate of disappearance in the dark of the 847-nm maximum under the conditions used in these experiments decreases with increasing pH. Increasing the ionic strength (0.05 M NaCl) does not significantly influence the activity of spinach protein factor in the reaction medium used (0.1 M phosphate buffer, pH 7); lowering of the ionic strength results in a decreased activity (Table I).

For investigation of the influence of bivalent cations, a preparation of spinach protein factor in 0.05 M Tris, pH 8.0, was made. After gel filtration, such a preparation shows only a very low activity. It was checked that addition of Tris does not inhibit activity of preparations in phosphate buffer. The activity in Tris buffer is increased somewhat upon addition of NaCl, while bivalent cations, especially Ca^{2+}, cause a very conspicuous increase (Table I). Dark-conversion of the 847-nm absorption maximum is likewise accelerated by Na^{+} and Ca^{2+}.

In the presence of Ca^{2+} at a sufficiently high concentration ($5 \cdot 10^{-3}$ M) the activity of spinach protein factor increases with increasing pH (from about 7 to 9) (Fig. 6). Without Ca^{2+} there is a decrease of the (low) activity with increasing pH.

TABLE I

INFLUENCE OF SOME REAGENTS ON BLEACHING OF COLLOIDAL BACTERIOCHLOROPHYLL

Different experiments cannot be compared.

Expt.	*Compound(s) added to colloidal bacteriochlorophyll solution*	*Medium*	*Bleaching of the 770–780-nm band upon illumination 1 min (arbitrary units)*
A	Triton X-100 1.66%	0.1 M phosphate buffer, pH 7.0	148
	The same + 5 M urea	0.1 M phosphate buffer, pH 7.0	164
B	Spinach protein factor, not gel-filtered	0.1 M phosphate buffer, pH 7.0	168
	The same + 5 M urea	0.1 M phosphate buffer, pH 7.0	16
C	Spinach protein factor, not gel-filtered	Tris buffer 0.05 M, pH 8.1	156
	The same + protease 0.07%, 40′, 30°	Tris buffer 0.05 M, pH 8.1	25
D	Spinach protein factor, gel-filtered	Tris buffer 0.05 M, pH 8.0	13
	The same + $2 \cdot 10^{-3}$ M NaCl	Tris buffer 0.05 M, pH 8.0	16
	The same + 10^{-2} M NaCl	Tris buffer 0.05 M, pH 8.0	28
	The same + 10^{-4} M $CaCl_2$	Tris buffer 0.05 M, pH 8.0	21
	The same + 10^{-3} M $CaCl_2$	Tris buffer 0.05 M, pH 8.0	57
E	Spinach protein factor, gel-filtered	Phosphate buffer 0.1 M, pH 7.0	56
	The same	Phosphate buffer 0.05 M, pH 7.0	27
	The same + $5 \cdot 10^{-2}$ M NaCl	Phosphate buffer 0.1 M, pH 7.0	54

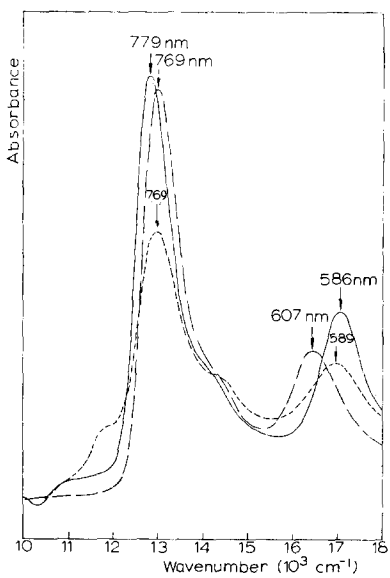

Fig. 4. Absorption spectra of bacteriochlorophyll in buffer containing spinach protein factor (------), methanol (– – – –) and Triton X-100, 0.25%, in buffer (——). Buffer solution: 3 parts Tris, 0.1 M, pH 8.75; 1 part phosphate, 0.1 M, pH 7.0.

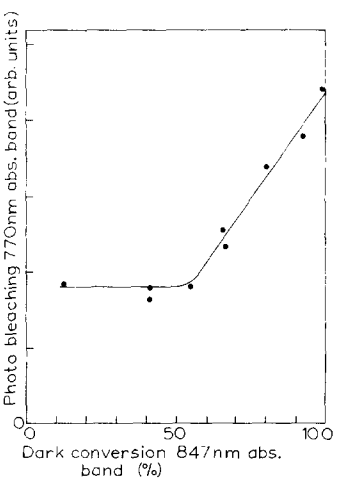

Fig. 5. "Activity" of spinach protein factor as releated to the intensity of the 847-nm absorption band. The activity of spinach protein factor is measured as its ability to render the approx. 770-nm band of colloidal bacteriochlorophyll light sensitive.

In the reaction medium, used for these experiments, with and without added Ca^{2+}, the dark-conversion of the 847-nm absorption maximum was retarded at increased pH.

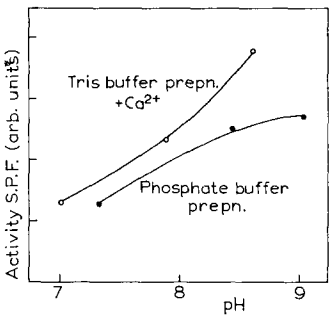

Fig. 6. Influence of pH on the "activity" of spinach protein factor (S.P.F.). For measurement of activity *cf.* Fig. 5. ●, spinach protein factor prepared in phosphate buffer, 0.1 M, pH 7.0; ○, spinach protein factor prepared in Tris buffer, 0.05 M, pH 8.0. The reaction medium contains Ca^{2+}, $5 \cdot 10^{-3}$ M.

Gel filtration on Sephadex G-100 in 0.1 M phosphate buffer, pH 7, of spinach protein factor prepared in the same buffer results in an active protein fraction reasonably well separated from the bulk of inactive proteins filtering through the column in the first fractions[1] (Fig. 7A). If the same is done in 0.05 M Tris buffer, pH 7.2 or 8.0, the result is different; the greatest activity is found in the fractions filtering out together with, or just after the bulk of the proteins (Fig. 7B).

This indicates that in this case the active protein is aggregated or bound to some other protein. Filtration of a preparation made in Tris buffer through a column

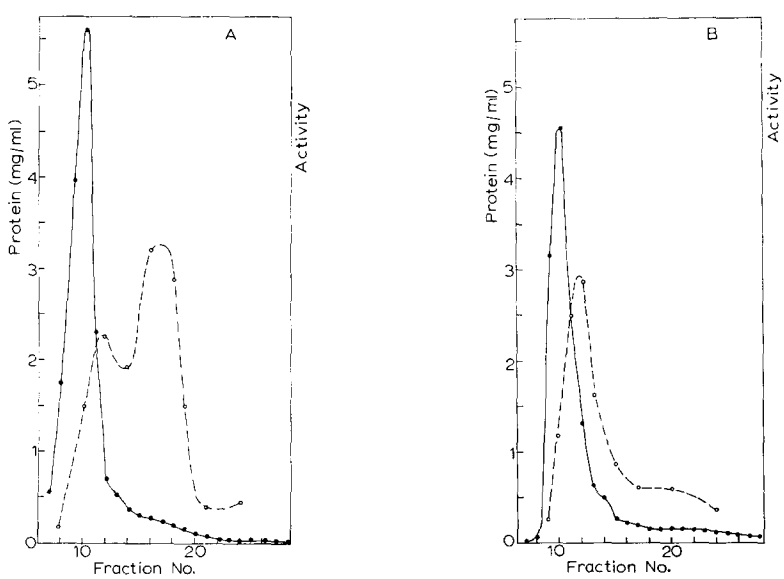

Fig. 7. Elution pattern of spinach protein factor upon filtration over Sephadex G-100. A, in 0.1 M phosphate buffer, pH 7.0; B, in 0.05 M Tris buffer, pH 7.2. Measurement of activity: *cf.* Fig. 5. ●—●, protein; ○---○, spinach protein factor activity.

with 0.1 M phosphate buffer yields the same results as a preparation made in phosphate buffer. Presumably, the ions of the phosphate medium cause a dissociation of the aggregated or bound active molecules. It may be recalled here that the measured activity of a gel-filtered preparation in Tris buffer is very low if no Ca^{2+} is added to the reaction medium.

The filtration pattern of a spinach protein factor preparation in Tris buffer shows that purification of the most active fraction is poor compared to that in phosphate buffer (Fig. 7).

DISCUSSION

KRASNOVSKY *et al.*[4] measured two absorption maxima in the near infrared in colloidal bacteriochlorophyll solutions. These maxima were ascribed to different aggregation types: absorption at 790–800 nm is supposed to belong to the less aggregated form, while absorption at 850–890 nm belongs to a more highly aggregated form. RABINOWITCH[10], considering the results of JACOBS *et al.*[11,12], suggests that the 840–850-nm band belongs to a crystalline phase and the 790–800-nm band to a non-crystalline "colloidal" phase.

SAUER, SMITH AND SCHULTZ[13] show spectra of a monomer and dimer of bacteriochlorophyll in carbon tetrachloride. The monomer has an absorption maximum at 780 nm, while the dimer shows two maxima in the near infrared region, at 785 and about 820 nm. Considering these data the possibility that in colloidal solutions of bacteriochlorophyll an aggregated form with two absorption maxima shows up cannot be rejected *a priori*.

In the experiments described in the previous section it was found that disappearance of the 847-nm absorption band is not accompanied by a comparable increase of the shorter wavelength band. This might be expected to occur if the 847-nm band belongs to a higher aggregated form of bacteriochlorophyll with a single absorption maximum in the infrared that is converted into a lower aggregated form, with a single maximum at a shorter wavelength. As it was shown that practically no pigment is lost during disappearance of the 847-nm absorption band, it is concluded that a transition of an 847-nm absorbing form into a 780-nm absorbing form is not likely to occur.

An alternative is that the 847-nm absorption band belongs to a bacteriochlorophyll form with two peaks in the near infrared; this form then is gradually converted into a one-peaked form. This would agree with the shift measured in the absorption band at about 780 nm. The initial site of this band is variable, during disappearance of the 847-nm band there is a considerable shift to shorter wavelength. This could readily be explained by assuming that the 780-nm band is composed of two absorption bands, one at about 790 nm, disappearing together with the 847-nm band and one at 775–776 nm, increasing at the same time. The 847-nm absorption band would belong then to a form of colloidal bacteriochlorophyll with two absorption maxima in the far-red (847 and about 790 nm), the 776-nm band would belong to a form with a single absorption maximum in this region.

There is no shift in the 847-nm band during its disappearance. This indicates that no appreciable absorption of the 776-nm band occurs in this region. This is confirmed by the absorption spectrum of the totally converted colloidal bacteriochlorophyll (Fig. 1).

The view just presented would agree with the experiments on the time course of the light sensitivity of the bacteriochlorophyll–spinach protein factor complex. The rate of photobleaching of this complex is maximum if the 847-nm band has completely disappeared, while some bleaching still occurs if the 847-nm band is present. This may be explained by assuming that the 847-nm band functions as an energy acceptor for energy absorbed by the 780-nm band. In colloidal particles the bleaching of the 790–770-nm band will be inhibited as long as the 847-nm band is present. The slight bleaching in the presence of the 847-nm band may be ascribed to some bacteriochlorophyll particles completely in the one-peaked form or to an energy transfer efficiency less than 100%.

These experiments thus favour the view that a form of colloidal bacteriochlorophyll with a two-peaked far-red spectrum is gradually converted into a form with a single maximum in this region. This conversion is accelerated by the spinach protein factor.

It was shown that the action of spinach protein factor differs from that of detergents in several respects. It may be noted that the height of the absorption maximum at 780 nm is about the same in methanol and in detergent (Triton X-100) solution, while in the presence of spinach protein factor the band is considerably lower, and of about the same height as in the absence of spinach protein factor after prolonged storage in the dark. This, together with the results from centrifugation experiments indicates that the colloidal bacteriochlorophyll is not solubilized in the presence of spinach protein factor.

In dimers, the C-9 carbonyl of one bacteriochlorophyll molecule is supposed to interact with the Mg of a second molecule[14–16]. It seems reasonable to assume that such an interaction also occurs in colloidal particles. From the results of former experiments it was decided that spinach protein factor probably acts *via* the Mg in the bacteriochlorophyll. This would agree with the finding that spinach protein factor interferes with the conversion of one bacteriochlorophyll form into the other.

Experiments on the influence of pH show that the activity of the spinach protein factor, measured as its ability to induce an increased light sensitivity in bacteriochlorophyll, increases at rising pH. This may indicate that negative charge of the protein favours binding with the bacteriochlorophyll. As Mg in chlorophylls is still able to bind electron donors[17], these experiments also agree with our previous suggestion that binding between spinach protein factor and (bacterio)chlorophyll occurs *via* the Mg atom.

The influence on pH and ions suggests that this bond is of an ionic character.

The strong influence of Ca^{2+} upon activity of spinach protein factor in Tris buffer cannot be explained only by an influence of Ca^{2+} on the bacteriochlorophyll–spinach protein factor interaction. Preliminary experiments indicate that in preparations in Tris buffer spinach protein factor is bound to another protein that inhibits the light sensitivity-increasing action of spinach protein factor upon bacteriochlorophyll; Ca^{2+} probably acts by inactivating this protein. This problem will be dealt with in our future experiments.

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