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REACTIVITY OF CHLOROPHYLL *a*/*b*-PROTEINS AND MICELLAR TRITON X-100 COMPLEXES OF CHLOROPHYLLS *a* OR *b* WITH BOROHYDRIDE

HUGO SCHEER*†, ROBERT J. PORRA and JAN M. ANDERSON
Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, GPO Box 1600, Canberra, ACT 2601, Australia

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Abstract—The reaction of several plant chlorophyll–protein complexes with $NaBH_4$ has been studied by absorption spectroscopy. In all the complexes studied, chlorophyll *b* is more reactive than Chl *a*, due to preferential reaction of its formyl substituent at C-7. The complexes also show large variations in reactivity towards $NaBH_4$ and the order of reactivity is: LHCI > PSII complex > LHCII > PSI > P700 (investigated as a component of PSI). Differential pools of the same type of chlorophyll have been observed in several complexes.

Parallel work was undertaken on the reactivity of micellar complexes of chlorophyll *a* and of chlorophyll *b* with $NaBH_4$ to study the effect of aggregation state on this reactivity. In these complexes, both chlorophyll *a* and *b* show large variations in reactivity in the order monomer > oligomer > polymer with chlorophyll *b* generally being more reactive than chlorophyll *a*. It is concluded that aggregation decreases the reactivity of chlorophylls towards $NaBH_4$ *in vitro*, and may similarly decrease reactivity in naturally-occurring chlorophyll–protein complexes.

INTRODUCTION

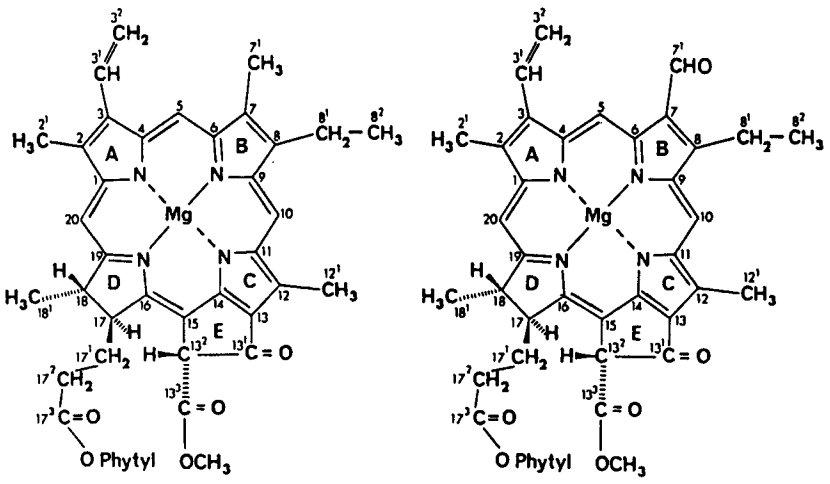
The addition of sodium borohydride to bacteriochlorophyll (BChl)‡ *a*-proteins residing in either bacterial photosynthetic reaction centres or antenna complexes has yielded interesting results which have been helpful in elucidating the arrangement, environment and function of these pigment-proteins in the bacterial photosynthetic process.

Studies with borohydride have shown that not all the BChl *a* molecules were equally accessible to or reactive with the reducing agent, presumably due to the different interactions existing either between the pigment and its various apoproteins or between the pigment-protein and the neighboring environment. For example, when Ditson *et al.* (1984) treated bacterial reaction centres with borohydride they found that it reacted with the 'extra' $BChl_M$ molecules, so designated because they belong to the 'inactive' M branch located on the M polyleptide (cf. Michel and Deisenhofer, 1986). Similarly, a selective reaction of borohydride has also been found with BChl *a*-containing antenna pigment proteins (Scheer *et al.*, 1988; Chadwick *et al.*, 1989): in every case studied, the pigments absorbing near 800 nm react preferentially with the reagent whereas those absorbing at longer wavelengths react more slowly if at all.

Studies with borohydride have produced spectroscopically homogeneous and chemically-modified reaction centre preparations from *Rhodobacter sphaeroides* R26 devoid of $BChl_M$ (Maroti *et al.*, 1985; Beese *et al.*, 1987) which have greatly assisted our understanding of the spectroscopy and function of this complex (Maroti *et al.*, 1985; Holten *et al.*, 1987; Scherer and Fischer, 1987; Shuvalov and Duysens, 1986). Two reactions have been observed when bacterial reaction centres are treated with borohydride. Firstly, the 3-acetyl substituent is reduced to a 3-(α-hydroxy)ethyl group and this modified pigment was subsequently removed from the complex due to its weaker interaction with neighbouring protein, lipid or pigment components of its environment (Maroti *et al.*, 1985). Secondly, with bacterial reaction centres there is cleavage of the M-polypeptide of 23 kDa forming a 15.5 kDa polypeptide (Beese *et al.*, 1987).

In view of the potential of borohydride to modify bacterial pigment–protein complexes, we decided to follow the reaction of a number of plant complexes with borohydride by absorption spectroscopy. The complexes of plant origin differ in several respects from the bacterial complexes. Firstly, they contain larger and often a greater variety of membrane-spanning polypeptides. Secondly, many contain two different chlorophylls, Chl *a* and *b*, which both possess a borohydride-reactive carbonyl group incorporated into the isocyclic ring at C-13; Chl *b*, in addition, contains a more reactive formyl group as a free β-pyrrole substituent at C-7. The IUB-approved numbering system for chlorophylls is shown below.

* To whom correspondence should be addressed (at the München address).
† Permanent address: Botanisches Institut der Universität, Menzinger, Str. 67, D-8000 München 19, W. Germany.
‡ *Abbreviations*: BChl, bacteriochlorophyll; Chl, chlorophyll; EDTA, ethylenediamine-tetraacetate; LHCP, light-harvesting chlorophyll-protein (the Roman numerals refer to the photosystem in which it is incorporated, and the subscripts to the fractions on non-denaturing gels); PAGE, polyacrylamide gel electrophoresis: PS, photosystem (roman numerals again refer to the photosystem involved); Q_{RS} is the ratio of absorption of the major red peak to that of the Soret peak.



Chlorophyll a Chlorophyll b

Scheme 1.

In this paper we report that there are considerable variations in reactivity to borohydride amongst the plant complexes: these variations in reactivity may include contributions from differences in stability (thermodynamics) and accessibility (kinetics), and also changes in components other than the pigments. Further, model studies on micellar complexes of Chl *a* and *b* reported here show that aggregation of the pigments depresses reactivity with borohydride and may similarly influence reactivity in naturally-occurring chlorophyll–protein complexes from plants.

MATERIALS AND METHODS

Pigment–protein complexes. Thylakoids were isolated from hydroponically-grown *Spinacia oleracea* plants (Anderson, 1980). The 'native' PSI complex and LHCII were isolated by Triton X-100 solubilization of the spinach thylakoids followed by sucrose gradient centrifugation (Burke *et al.*, 1978). LHCII was further purified by Mg^{2+}-induced aggregation according to Ryrie *et al.* (1980). An enriched PSI complex and LHCI were isolated from the Triton X-100 PSI complex by solubilization with Zwittergent-316 and dodecyl-β-D-maltoside followed by sucrose gradient centrifugation (Haworth *et al.*, 1983). A PSII complex, which contained the core PSII reaction centre complex together with some tightly-bound Chl *a*/*b*-proteins, was isolated by the procedure of Bricker *et al.* (1985). The purity of the pigment–protein complexes was checked by sodium dodecyl sulphate PAGE (Laemmli, 1970); such gels confirmed that the complexes were not contaminated with other Chl–protein complexes. Chlorophyll concentrations were determined spectrophotometrically in 80% aqueous acetone (Arnon, 1949).

Free pigments and micellar complexes. The chlorophylls were isolated using standard procedures and purified by column chromatography on sucrose (Strain *et al.*, 1965) after precipitation with dioxane in aqueous acetone (Omata and Murata, 1980) as described by Porra *et al.*, (1983). Micellar complexes were prepared in Tris-buffer (10 m*M*, pH 7.5) containing 0.1% Triton X-100 by sonication using the procedure applied to BChl complexes (Gottstein and Scheer, 1983). Large aggregates in aqueous acetone were obtained by injection of an acetone solution of the pigment into water (Gottstein and Scheer, 1983). A full report on the preparation of plant pigment complexes will be published separately (Schmidt *et al.*, in preparation).

Borohydride treatment. Solid $NaBH_4$ (0.5 mg) (Sigma Chemical Co., St. Louis, MO or E. Merck, Darmstadt, W. Germany) was added to the samples (2 mℓ) in a cuvette. Spectra were recorded at appropriate time intervals to permit H_2 evolution (bubbling) to cease sufficiently to avoid distortion. When the spectra were stable, increasingly large portions of solid $NaBH_4$ were added, until no further spectroscopic changes occurred. The effect of increasing pH (caused by adding $NaBH_4$) on the spectra was accounted for by using a sample at pH 10.2 (the pH attained in the samples after addition of 2–5 mg $NaBH_4$) as secondary reference. The instability of $NaBH_4$ in water, and the release of concomitant H_2-bubbles in the cuvette makes a strictly quantitative evaluation of its reaction with pigments difficult. To allow a consistent semi-quantitative comparison, the notation (mg/h/min) is used throughout to indicate sample history: mg denotes the total amount of $NaBH_4$ added; h is the time elapsed from the start of the experiment to the last addition and, min is the time elapsed since the last addition. During the experiment, sample and references were kept at ambient temperatures, and exposure to light was minimized.

Absorption spectra. Spectra were obtained with a model 557 (Perkin-Elmer, Überlingen) spectrophotometer in split beam mode. All data were fed into a RX02 computer (DEC) for data handling. Primary difference spectra were obtained directly with the untreated sample in the reference beam, all further spectra manipulations were done by computer coupled to a 7221B plotter (Hewlett-Packard).

P700 determinations. P700 was assayed by absorbance changes at 702 nm induced by flashes according to Haehnel *et al.* (1980) using a flash photometer constructed by Professor Wolfgang Haehnel. PSI complexes were suspended in 400 m*M* sucrose, 50 m*M* TES buffer (pH 7.5), 10 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* sodium ascorbate, 0.1 m*M* methyl viologen, 5 μ*M* dichlorophenolindophenol, 0.05% Nonidet and 30 μ*M* Chl.

Gel electrophoresis. The pigment-proteins were resolved by the non-denaturing PAGE method of Ander-

son (1980). The purity of the apoproteins of individual complexes was checked by sodium dodecyl sulphate PAGE (Laemmli, 1970).

Mg^{2+}-induced precipitation of $NaBH_4$-treated LHCII. A sample of the LHCII complex was reduced exhaustively with $NaBH_4$ (see Fig. 4, spectrum c) and was then dialysed against sorbitol (50 m*M*) to remove $NaBH_4$. The sample was precipitated with $MgCl_2$ (20 m*M*) and the washed precipitate redissolved in standard Tricine buffer (5 m*M*, pH 7.5) containing sucrose (50 m*M*) and EDTA (0.5 m*M*) and then centrifuged as described by Ryrie *et al.* (1980): the pellet and supernatant were retained for further manipulations (see Fig. 5).

RESULTS

The reaction of monomeric chlorophylls in methanol with borohydride

The reactions were carried out with isolated and purified chlorophylls in methanolic solution under conditions similar to those used by Holt (1959). Significantly, the amount of reagent required is much less than for the pigment–protein complexes (cf. legends of Figs. 1 and 2 with those of Figs 3–7).

Reduction of Chl a *in methanol.* When Chl *a* and related pigments with only one carbonyl group which is situated at C-13 in the isocyclic ring are dissolved, for example, in methanol, this group is selectively reduced by borohydride (Holt, 1959; Wolf and Scheer, 1973; Hynninen, 1979; Porra, 1986). The spectra of Chl *a* (containing a minute amount of pheophytin *a* absorbing near 520 nm) treated with borohydride show decreased absorption at 667 nm (Fig. 1): a number of isosbestic points are clearly visible up to about 60% conversion. As reduction proceeds the main Chl *a* bands at 667 and 434 nm decrease and the two main bands of the product, 13^1-deoxo-13^1-hydroxychlorophyll *a*, arise at 635 and 411 nm. The Q_{RS} ratio is decreased from 1.04 in the untreated pigment to 0.29 in the product. Whereas the absorption bands of Chl *a* in methanol differ both in position and intensity from those of the pigment–protein complexes (see later Figs.), the product spectrum in methanol is rather similar to the product spectrum of most of the pigment–protein complexes which, however, consistently absorb at slightly longer wavelengths than the reaction product in methanol.

Interestingly, there is also an increase in absorption at 519 nm during reaction with borohydride which has also been observed by Holt (1959) and Porra (1986); also, as shown by the same authors, a similar increase at 522 nm occurs when Chl *b* reacts with borohydride (Fig. 2). Such an increase suggests pheophytinization or the oxidative formation of Mg-'unstable'-chlorin diester (Porra *et al.*, 1983); however, both reactions are unlikely because they generally require acidic and oxidizing conditions whereas the reaction mixture is alkaline and reducing. A pheophytin has been isolated, however, from the reaction mixtures of reaction centres of

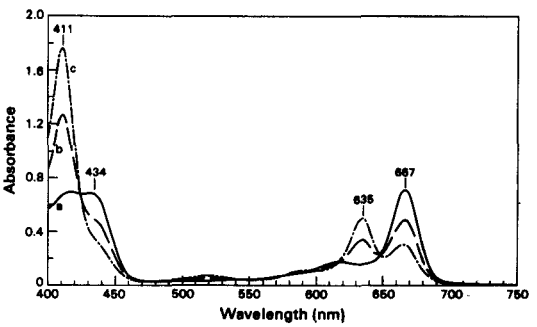


Figure 1. Absorption spectra of the reaction of Chl *a* (containing some pheophytin a) in methanol with $NaBH_4$. Original solution (a), and after addition of $NaBH_4$ 9.6/0.9/5.0 (b), and 29.6/3.5/305 (c). See Materials and Methods section for notation of history of sample.

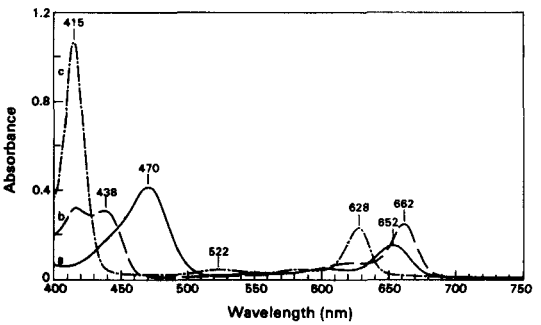


Figure 2. Absorption spectra of the reaction of Chl *b* in methanol with $NaBH_4$. Original solution (a), and after addition of $NaBH_4$ 0.5/0.02/1.0 (b), and 28.5/2/1.0 (c). See Materials and Methods section for notation of history of sample.

Rhodobacter sphaeroides R26 with borohydride (Maroti *et al.*, 1985): it was shown to be derived from the 'extra' $BChl_M$ molecules. Consequently, pheophytinization may still occur with plant chlorophylls which are more easily demetallated than BChl *a*: a further study is required to resolve this matter.

Reduction of Chl b *in methanol.* The spectra of Chl *b* reacting with borohydride (Fig. 2) clearly reveal a biphasic reaction. Chlorophyll *b*, like Chl *a*, has a borohydride-reactive carbonyl group incorporated into the isocyclic ring at C-13. In addition, Chl *b* has a formyl group as a free β-pyrrole substituent at C-7 and this group (Holt, 1959), like the acetyl group which is a free β-pyrrole substituent at C-3 of BChl *a* (Ditson *et al.*, 1984; Porra, 1986), is more reactive with borohydride than the carbonyl group incorporated into the isocyclic ring at C-13.

Although it is difficult under these experimental conditions to obtain a spectrum of the pure intermediate (7-deformyl-7-hydroxymethylchlorophyll *b*), spectrum b (Fig. 2) reveals little contamination by either the initial reactant (Chl *b*) or the final product. The spectrum of the intermediate

resembles that of Chl *a* with bands at 662 and 438 nm and a Q_{RS} = 0.8: Chl *a* has bands at 667 and 434 nm and a Q_{RS} = 1.04. The second step in Chl *b* reduction, like the reduction of Chl *a* which involved the reduction of the carbonyl group at C-13, is spectroscopically similar to the reduction of Chl *a*: it results in the formation of 7-deformyl-7-hydroxymethyl-13^1-deoxo-13^1-hydroxychlorophyll *b* which, like the reduction product of Chl *a*, has a spectrum with peaks at 628 and 415 nm and a Q_{RS} = 0.21.

The reaction of plant chlorophyll–protein complexes with borohydride

Because the addition of borohydride leads to increased pH (≈ 10.2), difference spectra of all samples treated with borohydride were read against samples titrated to pH 10.2 in the absence of borohydride. In all the complexes examined, the effect of pH was minor. For instance, in the PSII complex a slight (≈ 1 nm) blue shift of the long wavelength absorption band and some slight intensification and sharpening of bands was observed: the sharpening was partly reversed on standing. No systematic SDS-PAGE controls have been performed at this stage of the experiments. Preliminary studies indicate, however, considerable differences in proteolysis among the samples, e.g. LHCII is proteolytically more stable than CPa: A separate analysis is required to clarify this in detail.

Reaction of the PSII complex with borohydride. The reaction of PSII complex is slow but eventually complete as judged from the loss of the absorption band at 677 nm. Absorption decreases occur near 674, 466 and 439 nm and the final product absorbs near 639 and 415 nm: the Q_{RS} ratio is strongly decreased (Fig. 3A). Distinct differences in reactivity are observed for the different spectral forms and pigments. In the early stages, the decrease of absorption is faster at 466 than at 439 nm, which is interpreted as a preferential reduction of Chl *b*. Also, the difference band around 675 nm is structured (Fig. 3B): early, there is a preferential decrease at 681 nm, later followed by a decrease at 673 nm which indicates different reactivity of two separate pools of Chl *a* within the PSII complex.

Reaction of the LHCII complex with borohydride. This complex also reacts very slowly and the reaction is only about 65% complete as judged from the decreased absorption at 679 nm even after the addition of as much as 100 mg (≈ 1.3 *M*) $NaBH_4$ to the 2 mℓ sample (Fig. 4A): experiments with diluted LHCII furthermore revealed that this slowness of borohydride reduction is independent of LHCII concentration (data not shown). The absorption difference spectrum (Fig. 4B), showing decreases at 679, 652, 487, and 474 nm and increases at 638 and 416 nm, appears (within the limits of experimental error and allowing for the enriched Chl *b* content of the LHCII) identical to the spectrum of the product from the PSII complex. A pronounced difference, however, is observed in the intensity changes: the absorption increase in the blue spectral region is much less for LHCII than for the PSII complex. During the course of the reaction, there is again a distinct succession of steps. Very early, there are decreases at 487 and 644 nm and increases at 440 and 662 nm, which are interpreted as a preferential reduction of at least a part of the Chl *b* at the formyl substituent at C-7 (see Discussion). This is rapidly followed by further decreases at 487 and 644 nm and increases at 638 and 418 nm corresponding to the formation of the final product from part of the Chl *b*. Next, there is a decrease at 472 and 652 nm, corresponding also to part of (and possibly another pool of) Chl *b*, followed eventually by decreases at 440 and 679 nm due to reaction of Chl *a*.

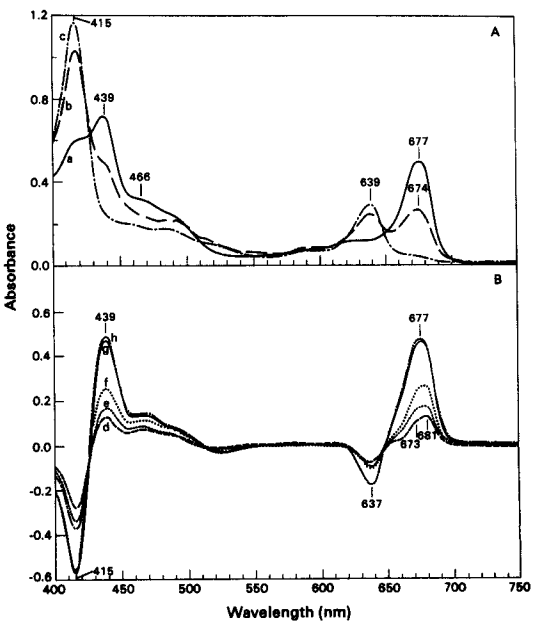


Figure 3. Absorption (A) and absorption difference (B) spectra of the PSII complex treated with $NaBH_4$. Absorption spectra of the PSII complex in the standard Tricine buffer (5 m*M*, pH 7.5) containing sucrose (50 m*M*) and EDTA (0.5 m*M*) were recorded against the same buffer at the beginning of the experiment (a), and after the addition of $NaBH_4$ 5.5/18.2/0 (b), and 5.5/18.2/130 (c). Difference absorption spectra (d) 0.5/0/40, (e) 0.5/0/100, (f) 0.5/0/1070, (g) 5.5/18.2/128, and (h) 5.5/18.2/220, were recorded against an untreated sample which was titrated to pH 10.2 at the beginning of the experiment (0/0/0). See Materials and Methods section for notation of the history of the sample. Evolution of H_2 from $NaBH_4$ in H_2O caused the discontinuity (bumps) in some spectra in all of the following Figures.

LHCII is known to be aggregated by $MgCl_2$ and completely redissolved with sufficient EDTA. Treatment with borohydride has little effect on this characteristic: at the end of the reaction period, the complex can still be precipitated completely with $MgCl_2$ but the pellet was only partly redissolved with buffer containing EDTA. A comparison of

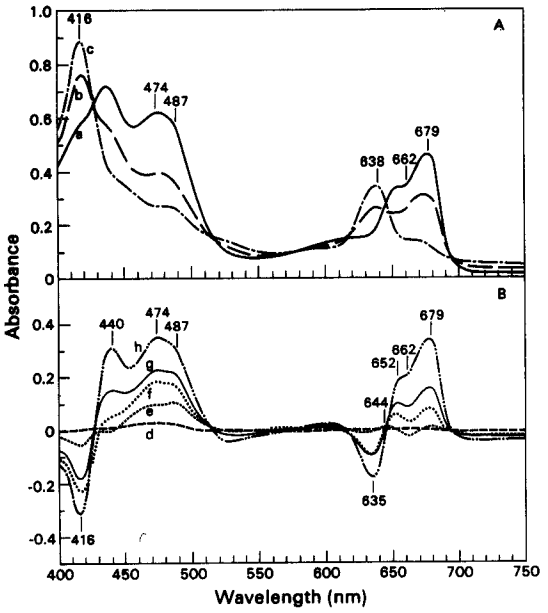


Figure 4. Absorption (A) and absorption difference spectra (B) of LHCII complex treated with $NaBH_4$. Absorption spectra of the LHCII complex in the standard Tricine buffer (see Fig. 1) were recorded against the same buffer at the beginning of the experiment (a), and after the addition of $NaBH_4$, 20.5/16.75/0 (b), and 20.5/16.75/225 (c). Difference absorption spectra (d) 0.5/0/0, (e) 5.5/0.8/5), (f) 5.5/0.8/890, (g) 20.5/16.75/0, and (h) 20.5/16.75/225, were recorded against an untreated sample which was titrated to pH 10.2 at the beginning of the experiment (0/0/0). See Materials and Methods section for the notation of the history of the sample.

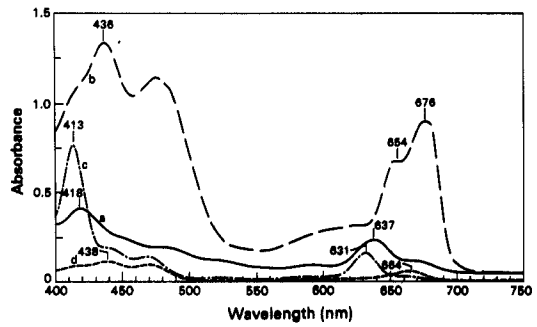


Figure 5. Mg^{2+} pelletability of LHCII after treatment with $NaBH_4$ (a,c) and with base only (b,d). A sample treated exhaustively with $NaBH_4$ (see text and Fig. 2c) was dialysed against sorbitol (50 m*M*) to remove $NaBH_4$. It was then precipitated with $MgCl_2$ (20 m*M*) according to Ryrie *et al.* (1980) and then redissolved in standard Tricine buffer (see Fig. 1) and centrifuged; the spectra of the supernatant (a) and the methanolic extract of the pellet (c) were recorded against the standard Tricine buffer or methanol, respectively. The reference sample with base only was kept for the duration of the $NaBH_4$ treatment at pH 10.2 and then received the same treatment as above: spectra of the supernatant (b) and the methanolic extract of the pellet (d) were recorded as above.

the redissolved pellet (Fig. 5, spectrum a) with the methanolic extract of the insoluble residue (Fig. 5, spectrum c) shows that both contain a large but unspecified proportion of modified pigment with, as yet, an unknown extinction coefficient. The general blue-shift of the residue (Fig. 5, spectrum c) is a solvent effect due to the methanol used as extractant. Comparison of Q_{RS} values, which are much smaller for the chlorophylls dissolved in methanol (Q_{RS} = 0.21) than for the redissolved modified complex (Q_{RS} = 0.61), indicates that there is still considerable protein-pigment interaction present in the latter.

The effect of high pH on Mg^{2+}-induced pelletability and subsequent redissolving with EDTA was minor (Fig. 5, spectra b and d). Because LHCII has a tendency to precipitate, all material was resuspended before each measurement.

Reaction of the LHCI complex with borohydride. Only a few experiments were performed with this complex because the concentrations employed ($A_{670} \approx 0.1$) were lower than those used with the other complexes. This complex reacts very rapidly with $NaBH_4$: the reaction sequence and high reactivity of this complex is similar to that of a Chl *a*/*b* mixture solubilized in the monomeric state in buffer

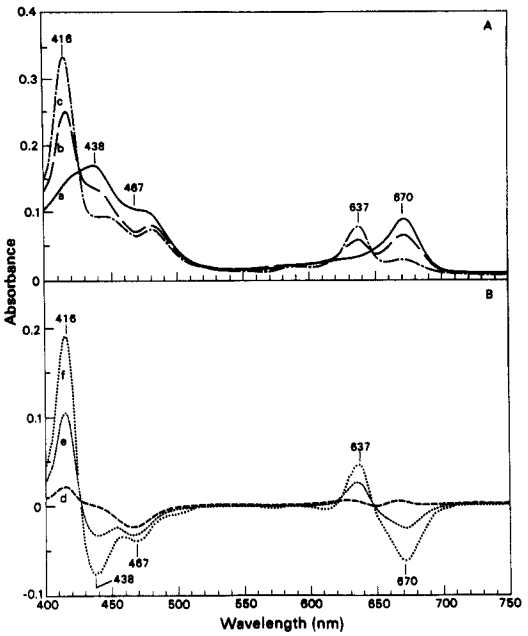


Figure 6. Absorption (A) and absorption difference (B) spectra of LHCI treated with $NaBH_4$. The absorption spectra of LHCI in Tricine buffer (20 m*M*, pH 7.8) containing sucrose (0.1 m*M*), dodecyl maltoside (1%) and Zwittergent-16 (0.2%) were recorded against the same buffer at the beginning of the experiment (a) and after the addition of $NaBH_4$ 1.7/1.1/40 (b), and 4.2/2.4/75 (c). Difference absorption spectra (d) 0.5/0/50, (e) 1.7/1.1/40, and (f) 4.2/2.4/75, were recorded against an untreated sample which was titrated to pH 10.2 at the beginning of the experiment (0/0/0). See Materials and Methods section for the notation of the history of the sample.

containing Triton X-100 (see below). After about 3.5 h and with about 4.2 mg $NaBH_4$/2 mℓ, when the reaction of LHCI is already 75% complete (Fig. 6A) the reaction with LHCII is only commencing (Fig. 4A). The first spectral change in this complex is an increase at 665 nm which is characteristic for the reduction of the C-7 formyl substituent of Chl *b*. The difference spectra show decreases at 670, 467 and 438 nm, and increases at 637 and 416 nm (Fig. 6B). The product spectrum shows a Q_{RS} ratio = 0.2 which is again very similar to that obtained from the products of all the other pigment–protein complexes.

The reaction of the PSI complex with borohydride. Two PSI complexes were investigated. The first, designated PSI, was the 'native' PSI complex isolated by Triton X-100 solubilization of spinach thylakoids (Burke *et al.*, 1978): it contained 154 Chl *a*/P700. The other, designated PSI-e (for PSI-enriched), was the heavy band obtained during preparation of LHCI after dissociation of native PSI with dodecyl-maltoside and Zwittergent-16: it contained 81 Chl *a*/P700.

PSI was the slowest reacting pigment–protein complex investigated (Fig. 7). It readily formed a fluffy precipitate in the presence or absence of $MgCl_2$ which was carefully resuspended before recording spectra. The spectra revealed progressive decreases at 682, 495, 470 and 439 nm, and increases at 638 and 417 nm. The succession of steps is less pronounced than in other complexes, but the early reduction of the formyl substituent at C-7 of Chl *b* is clearly observed; that is, a decrease at 470 nm and an increase firstly at 665 and 439 nm and then later at 638 and 417 nm. The early but small increase at 497 nm may be due to precipitate-induced baseline drift or to the presence of carotenoids. During later stages, the decrease of Chl *a*-related bands at 682 and 439 nm becomes more pronounced. A noteworthy feature is the comparably small increase at 417 nm, which is similar to the spectral changes in LHCII. In this case the absorption 'increase' at 417 nm is zero, and the only true increase in the spectral range from 400–750 nm is around 637 nm.

The PSI reaction centre, P700, seems unaffected or even slightly activated by the $NaBH_4$ treatment, as judged by flash-induced absorbance changes at 702 nm because the Chl *a*/P700 ratio decreases during the reaction from 154 to 89.

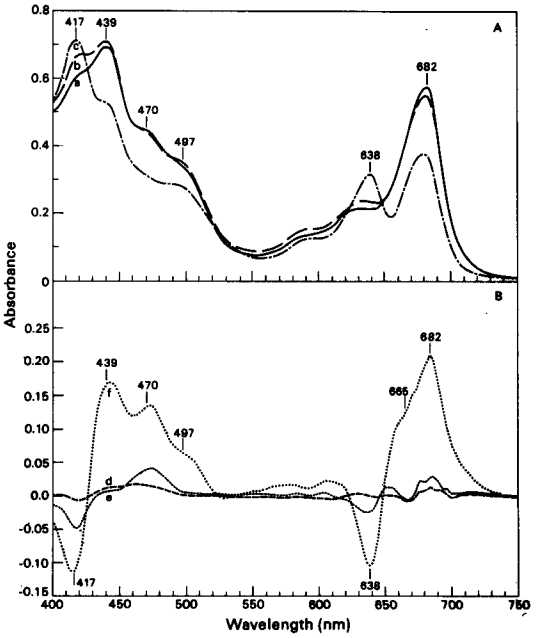


Figure 7. Absorption (A) and absorption difference (B) spectra of the PSI complex treated with $NaBH_4$. Absorption spectra of the PSI complex in standard Tricine buffer but containing no EDTA (cf. Fig. 1) were recorded against the same buffer at the beginning of the experiments (a) and after the addition of $NaBH_4$ 1.8/0/30 (b), and 15.8/1.75/1065 (c). Samples shown in (a) and (c) were used in P700 determinations. Difference absorption spectra (d) 1.8/0/3, (e) 1.8/0/80, and (f) 15.8/1.75/1065 were recorded against an untreated sample titrated to pH 10.2 at the beginning of the experiment (0/0/0). See Materials and Methods section for the notation of the history of the sample.

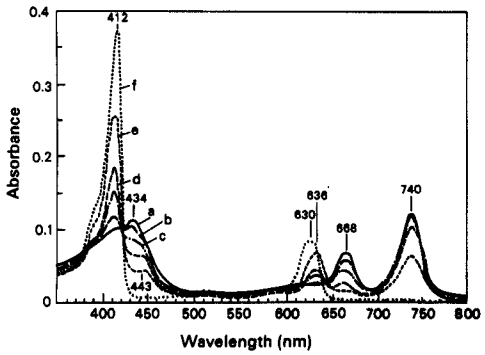


Figure 8. Absorption spectra of the reaction of Chl *a* in micellar solution (0.5% Triton X-100 in water) treated with $NaBH_4$. Original solution (a), and reaction mixture after addition of $NaBH_4$ 1.6/0/65 (b), 3.2/1.3/60 (c), 5.3/2.6/65 (d), 12.6/6.9/25 (e), and 29.3/28/180 (f). See Materials and Methods section for notation of the history of sample.

The effect of aggregation of chlorophylls on reactivity with borohydride

Chlorophylls in micellar solution. Chlorophylls are insoluble in water, but can be solubilized with detergents. Depending on the temperature, the structures and concentrations of pigment and detergent, the chlorophylls are solubilized in monomeric or aggregated states. Spectra of the latter (Figs. 8 and 9), compared to those of the monomeric pigments (Figs. 1 and 2), usually have red-shifted absorption bands, are hyperchromic, and resemble the spectra of chlorophyll–protein complexes; they have been used, therefore, as models (Gottstein

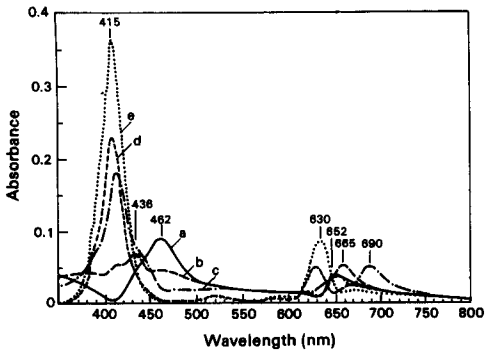


Figure 9. Absorption spectra of the reaction of Chl *b* in micellar solution (0.1% Triton X-100 in water) treated with $NaBH_4$. Original solution (a), and reaction mixture after addition of $NaBH_4$ 2.0/2.6/130 (b), 6.5/6.9/960 (c), 18.1/24/120 (d), and 24.3/28/195 (e). See Materials and Methods section for notation of history of sample.

and Scheer, 1983, Scheer *et al.*, 1985; Scherz and Parson, 1984, 1986). Thus, it seemed relevant to the study of the borohydride reactivity of natural pigment complexes to study the effect of aggregation on the borohydride reactivity of pure chlorophylls.

Aggregated Chl a *in micellar solution.* We have used Triton X-100 at concentrations of 0.05–0.1% to study the reaction of detergent-solubilized chlorophylls with $NaBH_4$. Under these conditions, Chl *a* is present in two forms (Schmidt, 1987): the monomeric form which absorbs at 668 and 434 nm, and an oligomeric form absorbing at 740 and 443 nm (Fig. 8 spectrum a). Due to strong hyperchromism of the Q_Y-band of the latter, its Soret band shows only as a shoulder in the original solution, but becomes a pronounced band around 443 nm (Fig. 8 spectrum b) during the reaction (see below). Addition of borohydride leads first to a predominant reaction of the monomeric pigment (Fig. 8 spectra b and d), with the product absorbing at 636 and 412 nm. When 63% of the monomer has reacted, as judged by reduced absorption at 668 nm, the oligomer absorption at 740 nm has decreased by only 14% (spectrum d). Under these conditions, the Soret band of the oligomer at 443 nm becomes better resolved. Later (Fig. 8, spectra e and f), the oligomeric pigment is also reduced and its product, like that of monomeric Chl *a*, absorbs at 636 and 412 nm. As in methanolic solution, there is a distinct absorption increase at 518 nm indicative of pheophytinization.

As shown with bacteriochlorophylls, a 3-(α-hydroxy)ethyl substituent prevents aggregation in micellar solution (Scheer *et al.*, 1985): the same seems to be true of a reduced Chl *a* with a 13^1-hydroxy substituent as judged by the similarity of the spectra of the reduction-product obtained from either the monomeric or oligomeric pigment.

Aggregated Chl b *in micellar solution.* The reaction of Chl *b* in micellar solution is shown in Fig. 9. The aggregation of this pigment in Triton X-100, to our knowledge, has not yet been thoroughly investigated. The spectrum of the original solution (Fig. 9, spectrum a) is unusual and the two distinct bands at 652 and 463 nm show broad and unstructured long-wavelength tails. Consistent with the general red-shift observed in oligomeric Triton X-100 complexes of chlorophylls, these tails are probably due to (a mixture of) Chl *b* aggregates of only poorly defined structures. Treatment with $NaBH_4$ leads to a loss of the narrow bands at 652 and 462 nm, with the rise of well defined bands at 665 and 436 nm (Fig. 9, spectrum b) which together with the increased Q_{RS} ratio again indicates reduction of the formyl substituent at C-7 yielding a monomeric Chl *a*-type pigment (Holt, 1959) which does not aggregate because of the hydroxymethyl-substituent at C-7. Subsequently, the common product with absorption bands at 630 and 415 nm is formed (Fig. 9, spectrum e). During this reaction, a smaller but broad and unstructured band centred at ≈ 665 nm becomes clearly visible (Fig. 9, spectrum d). The related pigment reacts only very slowly, but also eventually yields the product absorbing at 630 and 415 nm. This suggests, as did the results obtained with Chl *a* in Triton X-100-micelles, that monomeric Chl *b* is also more reactive than its oligomeric forms. Since there are two successive steps possible for each of the original pigment forms present, this reaction needs further study.

To further characterize the effect of aggregation on reactivity, very highly aggregated Chl *b* preparations were made by injection of an acetonic solution of chlorophylls into excess water (Gottstein and Scheer, 1983; Scheer *et al.*, 1985). The spectra of these aggregates (not shown) with an absorption maximum at 660 nm are exceedingly stable: the reduction of the C-7 formyl group requires about 0.25 *M* $NaBH_4$, and the second reduction step occurs only when $NaBH_4$ concentrations approaching 2.5 *M* are reached and proceeds to less than 50% completion.

DISCUSSION

As in the case of the various BChl *a*-proteins of bacterial reaction centre and antenna complexes, the susceptibility of different plant Chl *a*/*b*-protein complexes to reaction with $NaBH_4$ varies markedly.

In all the plant chlorophyll–protein complexes investigated and in the chlorophyll–Triton X-100 micellar complexes, the same course of reactions appears to be followed and the spectra of the products are very similar irrespective of the plant complex treated. The first reaction is the reduction of the formyl at C-7 of Chl *b*. The product always shows the typical Chl *a*-type spectrum with an increased Q_{RS} value, a bathochromically-shifted

Q_Y-band and a hypsochromically-shifted Soret band. This reaction is always followed by the reduction of the C-13 carbonyl groups of both chlorophylls.

Nonetheless, there are two major differences amongst the plant complexes. The first concerns the different reactivity of these complexes to reduction by borohydride. The second concerns certain spectral differences observed between the complexes when treated with borohydride.

Differences between borohydride reactivity of various plant chlorophyll–protein complexes

The reactivity of the complexes, with due regard to the earlier mentioned limitations on quantitative evaluation, differs in the order LHCI > PSII > LHCII > PSI > P700: the latter was only investigated as a component of PSI and, thus, may have been protected by associated antenna pigments. This difference in reactivity may indicate different interactions of the chlorophyll-proteins with the neighbouring protein, lipid and pigment molecules in the different environments provided by the complexes. In addition, the current study showing that as aggregation of purified Chl *a* and *b* increases that reactivity with borohydride decreases suggests that differences in aggregation of pigments may also contribute to variation in reactivities amongst the plant complexes.

The extreme sensitivity of the Chl *a*/*b*-proteins of LHCI to borohydride correlates with the ready loss of these pigments during detergent solubilization and subsequent electrophoresis (Green, 1988). Consistent with the relative slowness of LHCII to react with borohydride, the modified and unmodified pigments of this complex are precipitated with $MgCl_2$ and, at least partly, redissolved with EDTA suggesting that these pigments are more tightly bound within the complex. In this regard, it is noteworthy that the borohydride-reactive B-800 pigments of the bacterial antenna complex (Scheer *et al.*, 1985) are believed to be less tightly bound than the non-reactive pigments absorbing at longer wavelengths (Kramer *et al.*, 1984; Scherz and Parson, 1986). Also, it is only the 'extra' $BChl_M$ molecules of the bacterial reaction centre which react with borohydride and the modified pigments were also readily removed from the complex by chromatography on DEAE-cellulose (Ditson *et al.*, 1984; Maroti *et al.*, 1985) to produce reaction centre particles devoid of $BChl_M$ which were useful in further structure and function studies. Thus borohydride may also be useful in the preparation of experimentally-useful plant complexes lacking specific chlorophyll-proteins in much the same way as Ford *et al.* (1987) used sodium dodecyl sulphate to prepare PSI particles.

In contrast to our studies showing that borohydride reacts more readily with LHCII than with the PSII complex, Siefermann-Harms and Ninnemann (1982) elegantly demonstrated that isolated PSII and PSI complexes were more sensitive to lower pH with some, but not all, Chl *a* being converted to phaeophytin *a* while, on the other hand, the Chl *a*, Chl *b* and xanthophylls of isolated LHCII were remarkably insensitive to acidic treatment. Thus, the network of undefined, non-covalent bonding interactions which anchor the pigments to different amino acids within the membrane-spanning α-helices of their specific apoproteins, allows precise orientation of the pigments with respect to each other and gives rise to different environments for Chl *a* and Chl *b*. It is possible that differences in electrostatic environments might be responsible for the reversed activity of the LHCII and PSII complexes to the negative borohydride and positive hydrogen ions by influencing local concentrations of these ions. Also, it has been suggested from experiments with intact thylakoids, where proteolytic treatments (Andersson *et al.*, 1982) and treatments with acid or base (Brody and Gregory, 1981) failed to release chlorophylls or carotenoids, that these pigments are mainly buried in a hydrophobic core of the membranes. However, location within the hydrophobic core of the membranes may not necessarily be synonymous with inaccessibility to enzymes or other reagents since it was recently shown that chlorophyllase not only dephytylated chlorophylls in Triton X-100-solubilized chlorophyll-proteins from *Phaeodactylum tricornutum* (phaeophyta), but also up to 60% of the chlorophylls in broken chloroplasts (Schoch and Brown, 1987). Thus the failure to release pigments in proteolytic treatments where the proteolytic reagents varied considerably in size (*cf.* Andersson *et al.*, 1982; Brody and Gregory, 1981) may not be associated with the inaccessibility of the chlorophyll proteins: even if the reagents reach the appropriate reaction sites the prevailing environment may not favour hydrolysis.

Spectral differences between plant complexes treated with borohydride

The difference spectra in this paper show small differences in peak positions for the intermediate- and product-proteins arising from borohydride treatment of the various plant complexes. This may be due to the different interaction of the chromaphore with different apoproteins of the various complexes. Also, it may be due to different interactions of the chlorophyll-proteins with components of the various environments provided by the different complexes. Differences in environment may, in addition, affect the aggregation state of the chlorophylls and hence their absorption spectra (*cf.* Figs. 1 and 2 with 8 and 9). The observed spectral differences between the Chl *a*-like intermediate from Chl *b* could also be partly due to aggregation effects arising from the presence of the 7-hydroxymethyl

group and differences in the polarity of the environments provided by the various plant complexes. While the replacement of an alkyl- by an α-hydroxyalkyl substituent does not greatly alter the spectrum of monomers in solution (Scheer *et al.*, 1989), it may drastically increase aggregation in polar solvents as shown in studies of aggregation of BChl c and d (Olson and Pedersen, 1988; Smith *et al.*, 1983; Brune *et al.*, 1988).

The difference spectra also show large variations in Q_{RS} ratios arising from differences in the magnitudes of decreased absorption in the red relative to the increased absorption in the blue spectral region. While it is large in LHCII and PSI, it is very small in PSII complex and LHCI, and in the sample designated PSI-e, there is even no increase at all at short wavelengths. This effect may be due both to a difference in the spectra of the initial chlorophylls (Chl *a* and *b*) or product pigments due to their interactions with different environmental components offered by the various plant complexes. The Q_{RS} ratios are determined by several factors including the asymmetry of the tetrapyrrole chromophore: the ratio is small (≈ 0.1) in highly symmetrical metalloporphyrins of, or near, D4h-symmetry, but is increased to about 1.0 in the more unsymmetrical Chl *a* (Smith, 1975). The protein environment can also contribute to the asymmetry of the naturally-occurring pigment-proteins. Aggregation also influences Q_{RS} ratios: hyperchromism of the Q_Y band at the expense of the Q_X and Soret bands has been investigated in detail in bacteriochlorophylls and can be substantial (Scherz and Parson, 1984, 1986). Consequently, aggregation may not only influence reactivity but also the nature of the spectral changes. Yet another relevant factor affecting the Q_{RS} value is the presence or absence of the central magnesium: it decreases from about 1.0 in Chl *a* to about 0.4 in pheophytin *a* (Katz *et al.*, 1966). Because experiments with bacterial reaction centre (Maroti *et al.*, 1985) clearly show that demetallation may take place upon treatment with $NaBH_4$ even at the high pH attained in these experiments some of the observed changes in Q_{RS} ratios may be attributable to pheophytin formation; clearly, more data are needed to resolve this problem.

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