# Spectral and photochemical properties of borohydride-treated D1-D2-cytochrome *b*-559 complex of photosystem II

Anatoli Ya. Shkuropatov[a,*], Ravil A. Khatypov[a], Tatyana S. Volshchukova[a], Valentina A. Shkuropatova[a], Thomas G. Owens[b], Vladimir A. Shuvalov[a]

[a]*Institute of Soil Science and Photosynthesis, Russian Academy of Sciences, Pushchino, Moscow Region 142292, Russian Federation*
[b]*Section of Plant Biology, Cornell University, Ithaca NY 14853, USA*

Received 6 November 1997; revised version received 24 November 1997

**Abstract The D1-D2-cytochrome *b*-559 reaction center complex of photosystem II with an altered pigment composition was prepared from the original complex by treatment with sodium borohydride ($BH_4^-$). The absorption spectra of the modified and original complexes were compared to each other and to the spectra of purified chlorophyll *a* and pheophytin *a* (Pheo *a*) treated with $BH_4^-$ in methanolic solution. The results of these comparisons are consistent with the presence in the modified complex of an irreversibly reduced Pheo *a* molecule, most likely $13^1$-deoxo-$13^1$-hydroxy-Pheo *a*, replacing one of the two native Pheo *a* molecules present in the original complex. Similar to the original preparation, the modified complex was capable of a steady-state photoaccumulation of $Pheo^-$ and $P680^+$. It is concluded that the pheophytin *a* molecule which undergoes borohydride reduction is not involved in the primary charge separation and seems to represent a previously postulated photochemically inactive Pheo *a* molecule. The $Q_y$ and $Q_x$ transitions of this molecule were determined to be located at 5°C at 679.5–680 nm and 542 nm, respectively.**



*Key words:* Photosystem II; D1-D2-cytochrome *b*-559 complex; Pheophytin *a*; Sodium borohydride; Chemical modification

## 1. Introduction

Recent studies performed on bacterial reaction centers (RCs) have shown that chemical modification of the RC chromophores by the exchange procedure [1,2] or with borohydride ($BH_4^-$) treatment [3–6] is a powerful way to investigate the contribution of particular pigments to the spectral and electron-transfer properties of RCs. It is of great interest to apply similar methods to the isolated D1-D2-cytochrome (cyt) *b*-559 reaction center complex of photosystem II (PS II) [7], the spectroscopic and functional studies of which are seriously complicated by the fact that at least six chlorophyll and two pheophytin molecules contribute to the $Q_y$ absorption band of the complex in the range of 670–680 nm [8–12]. It has been previously shown [13] that borohydride treatment of a PS II complex, containing the PS II RC together with some tightly bound Chl *a*/*b*-proteins, leads to distinct changes in the absorption spectrum of the complex. However, the absorbance changes related to the reaction center pigments have not yet been specified [13].

Here, we report on the preparation and the initial spectral and photochemical characterization of the chemically modified D1-D2-cyt *b*-559 reaction center complex obtained by the reaction of the constituent pigments with sodium borohydride.

## 2. Materials and methods

The D1-D2-cyt *b*-559 complex was isolated from sugar-beet chloroplasts as described earlier for spinach [14,15], using the method developed by Nanba and Satoh [7]. The $Q_y$ absorption band of the complex was located at 5°C at 675–675.5 nm. The absorption spectra of the samples from different isolations showed some variation of the amplitude of the carotenoid peak at 485 nm due probably to the presence of two forms of the RC complex [16]. However, that did not affect the results described in this communication. In the borohydride treatment experiments, various amounts of solid $NaBH_4$ (Sigma) were added to the complex suspended in buffer Tris-HCl (100 mM; pH 7.2)/0.05% *n*-dodecyl-β-D-maltoside (DM) (Sigma). All operations were performed at 5°C in dim green light. The titration with $NaBH_4$ was followed by absorption spectroscopy using a UV-1601PC spectrophotometer (Shimadzu). For this purpose, absorption and absorption difference spectra (treated-minus-original sample) were recorded at 5°C at appropriate time intervals after an addition of $NaBH_4$ and then new portions of $NaBH_4$ were added; the final pH of the solution was less than 9.0. When the amplitude of the absorption increase at 655 nm reached its maximum (see Section 3), the borohydride-treated complex was dialyzed extensively against Tris-HCl (50 mM; pH 7.2)/0.05% DM. Then both the borohydride-treated and original (untreated) preparations were separately loaded onto Fractogel TSK DEAE-650(S) (Merck) columns, washed with Tris-HCl (50 mM; pH 7.2)/0.05% DM/30 mM NaCl and eluted with the same buffer containing 150 mM NaCl. For the borohydride-treated complexes this purification procedure was repeated twice. The chlorophyll *a* concentration in the complexes was determined optically using an extinction coefficient of 103 $mM^{-1}$ $cm^{-1}$ at the red peak of the absorption spectra [17].

To compare the ability of the complexes to photoaccumulate $Pheo^-$ or $P680^+$, the light-minus-dark absorption difference spectra were measured point by point at 10°C with a phosphoroscopic setup [18] in a vacuum-tight quartz cuvette (optical pathlength of 1 cm) containing the sample degassed by a mild application of vacuum. Essentially the same experimental conditions (concentration of RC complexes, actinic light intensity) were used for the untreated and modified samples. The photoaccumulation of $Pheo^-$ was measured in the presence of 1 μM methyl viologen and 6 mM sodium dithionite [7]. To photoaccumulate $P680^+$, 450 mM silicomolybdate (final concentration) was added to the sample [19], and prior to the measurements the sample was preilluminated for 45 s with continuous red light ($\lambda > 630$ nm, $\sim 50$ mW/$cm^2$) in order to bleach β-carotene irreversibly [20,21].

Chlorophyll *a* and pheophytin *a* were prepared from nettle using standard procedures and purified by column chromatography on sugar powder [22]. The reactions of purified Chl *a* and Pheo *a* with $NaBH_4$ were carried out at 5°C in methanolic solution under conditions similar to those used in [13,23].

*Corresponding author. Fax: (7) (0967) 79-05-32.
E-mail: shkur@issp.serpukhov.su

*Abbreviations:* Chl *a*, chlorophyll *a*; cyt, cytochrome; DM, *n*-dodecyl-β-D-maltoside; P680, primary electron donor in PS II; Pheo *a*, pheophytin *a*; PS II, photosystem II; RC, reaction center


*PII* S0014-5793(97)01512-3

## 3. Results

Fig. 1A,B shows that titration of the D1-D2-cyt *b*-559 reaction center complex with $NaBH_4$ leads to distinct changes in the absorption spectrum of the complex. In the early stages of treatment, a preferential reaction of $BH_4^-$ with the reaction center pheophytin *a* takes place that manifests itself as a pronounced decrease in absorption of the $Q_x$ band of Pheo *a* at 542 nm, an absorption decrease in the Pheo *a* $Q_y$ region at 680 nm and in the appearance of absorption of the product near 655, 500 and 400 nm. Another, somewhat slower reaction leads to an absorption decrease of Chl *a* near 670 nm and to the appearance of the reaction product absorption near 640 and 410 nm. As expected, the absorbance changes at 559, 429 and 415 nm are observed due to the reduction of cytochrome *b*-559 [10].

In order to identify the products formed by treatment of the RC pigments with $NaBH_4$, the reactions of purified Pheo *a* and Chl *a* with $NaBH_4$ in methanolic solution were studied. In accordance with earlier reports [13,23], the borohydride reduction of Chl *a* led to a decrease of its absorption bands at 665 and 432 nm and to the appearance of the two main bands of the product at 633 and 410 nm. This product has been previously established to be $13^1$-deoxo-$13^1$-hydroxy-Chl *a* [13,23]. In the case of Pheo *a*, the decrease in its absorption bands at 665, 538, 507 and 410 nm was accompanied by the appearance of the bands of the product at 652, 500 and 395 nm. As these spectral changes are similar to those found for the selective reduction at C-$13^1$ of the closely related pigments, methyl pheophorbides [23,24], the $NaBH_4$ reduction product of Pheo *a* is most likely $13^1$-deoxo-$13^1$-hydroxy-Pheo *a*. The absorption difference spectra for the described reactions of free Pheo *a* and Chl *a* with $NaBH_4$ in methanolic solution are given in Fig. 2B (dashed and dotted line, respectively). A comparison of these spectra with the difference spectra presented in Fig. 1B indicates that both $13^1$-deoxo-$13^1$-hydroxy-Pheo *a* and $13^1$-deoxo-$13^1$-hydroxy-Chl *a* are formed in the course of $NaBH_4$ treatment of the D1-D2-cyt *b*-559 complex although their absorption maxima seems to be slightly shifted to the red as compared to methanolic solution.

Some time after the start of the reaction of the D1-D2-cyt *b*-559 complex with $NaBH_4$, the formation of $13^1$-deoxo-$13^1$-hydroxy-Pheo *a* is completed as judged from the lack of growth in the absorption at 655 nm (Fig. 1B). At this moment, we stopped the addition of $NaBH_4$, and the preparation was dialyzed followed by two-step purification on a DEAE-cellulose column (see Section 2). Fig. 2A shows the absorption spectra of the purified $NaBH_4$-treated (dashed line) and un-

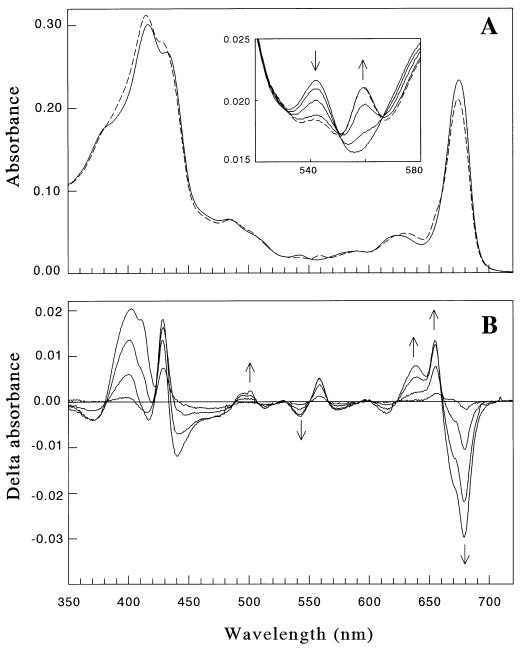


Fig. 1. Absorption (A) and absorption difference (B) spectra of the isolated D1-D2-cyt *b*-559 complex treated with $NaBH_4$. A: Absorption spectra of the complex before (solid line) and after (dashed line) 150 min treatment with $NaBH_4$ (final concentration is ~2 mg/ml). Inset: Absorption spectra of the complex in the green region before and after $NaBH_4$ treatment for 20, 50, 80 and 150 min. The arrows show the directions of the absorbance changes at 542 and 559 nm. B: Difference spectra obtained by subtraction of the original absorption spectrum of the D1-D2-cyt *b*-559 complex (A, solid line) from the absorption spectra taken at 20, 50, 80 and 150 min after the start of $NaBH_4$ treatment. The arrows show that the amplitude of the absorbance changes successively grows with the increase in the time of treatment. The spectrum with the largest amplitude is the difference between the absorption spectra shown in A.

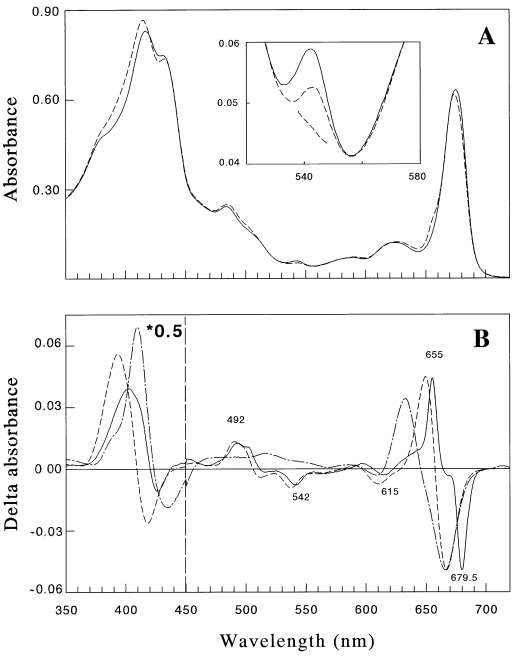


Fig. 2. A: Absorption spectra of the D1-D2-cyt *b*-559 complex before (solid line) and after $NaBH_4$ treatment and subsequent purification on DEAE-cellulose (dashed line). The spectra are normalized at 623 nm. Inset: Enlargement of the pheophytin *a* $Q_x$ region of the absorption spectra. The spectra are brought into coincidence in the region of 555–560 nm. The short dashed line shows the expected 'baseline' used to determine the absorbance of Pheo *a* at 542 nm (see Section 3). B: The difference between the absorption spectra of the D1-D2-cyt *b*-559 complexes presented in A, 'treated-minus-untreated complex' (solid line) and the absorption difference spectra for the reactions of the purified pheophytin *a* (dashed line) and chlorophyll *a* (dotted line) in methanolic solution with $NaBH_4$. The difference spectra for the pigments were obtained by subtraction of the original absorption spectra from those after treatment with $NaBH_4$. The treatment time was about 1 h for both Chl *a* and Pheo *a*. All the difference spectra are normalized at their long-wavelength minima (the delta absorbance values refer to the spectrum of the complex). The value numbers show the wavelength positions of the extrema in the difference spectrum of the complexes.

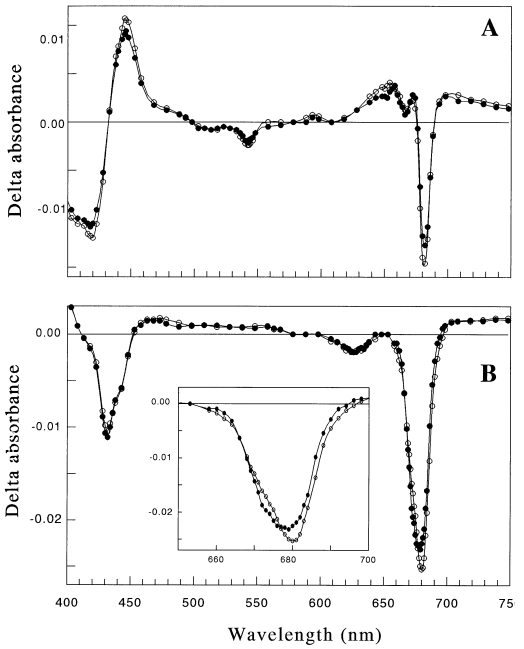


Fig. 3. A,B: Light-minus-dark difference spectra of reversible absorbance changes in the original (open circles) and $NaBH_4$-treated (closed circles) D1-D2-cyt *b*-559 complexes in the presence of 6 mM sodium dithionite and 1 μM methyl viologen (A) or 450 μM silicomolybdate (B). A: Chlorophyll concentration was about 5.1 μg/ml. Samples were excited with red actinic light ($\lambda > 630$ nm; $\sim 7$ mW/cm$^2$). B: Chlorophyll concentration was about 3.4 μg/ml. Actinic light was as in A. Samples were preilluminated with red light for 45 s (see Section 2). Inset: Enlargement of the $Q_y$ region of the light-minus-dark difference spectra.

treated (solid line) complexes normalized to the amplitude at the isosbestic point at 623 nm (see Fig. 1). Fig. 2B demonstrates that the difference between these normalized spectra (solid line) includes the spectral features related to both $13^1$-deoxo-$13^1$-hydroxy-Pheo *a* and $13^1$-deoxo-$13^1$-hydroxy-Chl *a* but the latter are much less pronounced. A mean value of the ratio of absorbance change at 640 nm to that at 655 nm was 0.17. Based on this value and on a ratio of the differential extinction coefficients for the reduced free pigments (Fig. 2B, dashed and dotted lines), the presence of 0.1 molecule of $13^1$-deoxo-$13^1$-hydroxy-Chl *a* per molecule of $13^1$-deoxo-$13^1$-hydroxy-Pheo *a* was estimated for the purified complex.

The absorbance at 542 nm in the normalized absorption spectrum of the purified complex was found to be $53 \pm 2\%$ of that in the spectrum of untreated samples (Fig. 2A, inset).

Fig. 3A,B compares the photochemical activity of the original and $NaBH_4$-treated preparations at equal concentrations of RC complex that was obtained using samples with an equal absorbance at the isosbestic point at 623 nm. Fig. 3A shows the spectra of reversible absorbance changes induced by illumination of the $NaBH_4$-treated (closed circles) and untreated (open circles) complexes in the presence of sodium dithionite and methyl viologen. The spectra are close to each other both in shape and in amplitude and show all the characteristics of Pheo *a* reduction [25]. The negative peaks at 420, 515, 543, 682 nm and positive peaks at 445, 595, 656, 672 nm are similar to those previously reported for the Pheo$^-$-minus-Pheo spectrum [7,19,26].

The spectra of light-induced reversible absorbance changes obtained for both types of complexes incubated with silicomolybdate (Fig. 3B) show the structure characteristic of P680$^+$-minus-P680 [19,20,27–29]. It includes bleaching around 432 and 680 nm, a shoulder near 670 nm and a broad absorption increase with an equal amplitude of the signals between 720 and 840 nm (not shown). As compared to the untreated complex, the maximum of the bleaching in the $Q_y$ region of the borohydride-treated preparation is blue-shifted from 680 to 678 nm, and a shoulder near 670 nm is more pronounced (Fig. 3B, inset).

## 4. Discussion

The results presented here show that purification of the $NaBH_4$-treated D1-D2-cyt *b*-559 complex by repeated chromatography on DEAE-cellulose yields a modified complex containing reduced pheophytin *a*, most likely $13^1$-deoxo-$13^1$-hydroxy-Pheo *a*, instead of the native Pheo *a* molecule absorbing near 679.5–680 and 542 nm (Fig. 2A,B).

The notable peculiarity of the reaction of the D1-D2-cyt *b*-559 complex with $NaBH_4$ is that the reduction of Pheo *a* to $13^1$-deoxo-$13^1$-hydroxy-Pheo *a* terminates after some time (Fig. 1B). This results in about 50% decrease in absorption at 542 nm as follows from a comparison of the normalized absorption spectra of the original and modified complexes after purification (Fig. 2, inset). These data are consistent with the presence in the original D1-D2-cyt *b*-559 complex of two Pheo *a* molecules which absorb equally at 542 nm but differ in their accessibility and/or reactivity to borohydride. Of these two Pheo *a* molecules, only one seems to be reduced by $BH_4^-$ at the C-$13^1$ position without a concomitant degradation of the complex. This explanation is in agreement with the now widely accepted assumption that there are two Pheo *a* molecules per reaction center complex [7].

After two-step purification, only a minor fraction of the modified complexes (about 10%) contains $13^1$-deoxo-$13^1$-hydroxy-Chl *a*. The modified complexes with the reduced Chl *a* seem to be relatively unstable and are lost during repeated chromatography on DEAE-cellulose.

The observation that the Pheo$^-$-minus-Pheo absorption difference spectra obtained for the original and borohydride-treated complexes with equal RC concentration are close to each other both in shape and in amplitude (Fig. 3A) indicates that the photochemical activity of the modified complex is comparable to that of the original preparation. It suggests that the Pheo *a* molecule which takes part in photo-induced electron transfer is not largely perturbed by $BH_4^-$ treatment and, consequently, that $13^1$-deoxo-$13^1$-hydroxy-Pheo *a* present in the modified complex originates from the modification of the Pheo *a* molecule which is not involved in the primary charge separation. Based on this consideration, we conclude that the borohydride-treated Pheo *a* seems to represent a previously postulated pheophytin *a* molecule located in the inactive branch of the PS II RC [10,30] and assign to this molecule the $Q_y$ and $Q_x$ transitions at 679.5–680 nm and 542 nm, respectively. This assignment implies that the $Q_y$ transition of photochemically inactive Pheo *a* is located at longer wavelengths than was presumed earlier (672–673 nm) [29]. The location of the $Q_x$ transition of this molecule at 542 nm corresponds to that determined at 10 K [31] but differs from that (537 nm) reported for 77 K [30].

The preserved ability of the borohydride-treated complex to photoaccumulate Pheo$^-$ suggests that the primary electron

donor remains functionally active in the modified preparation. This is confirmed by the similarity in shape and amplitude between the absorption difference spectra for accumulation of $P680^+$, measured for the untreated and modified complexes in the presence of silicomolybdate (Fig. 3B). Note, however, that the differences in the structure of these spectra in the red region (Fig. 3B, inset) seem to reflect some alterations in the shape and/or position of the bleaching $Q_y$ absorption band of the primary electron donor in the modified complex.

*Acknowledgements:* We thank Dr. M.G. Zvereva for preparation of chlorophyll *a* and pheophytin *a*. Support by CRDF (Grant RB1-281) and the Russian Found of Basic Research (Grant 97-04-49866) is gratefully acknowledged.

## References

[1] Struck, A. and Scheer, H. (1990) FEBS Lett. 261, 385–388.
[2] Scheer, H. and Struck, A. (1993) in: The Photosynthetic Reaction Center (Deisenhofer, J. and Norris, J.R., Eds.), pp. 157–192, Academic Press, San Diego, CA.
[3] Ditson, S.L., Davis, R.C. and Pearlstein, R.M. (1984) Biochim. Biophys. Acta 766, 623–629.
[4] Maroti, P., Kirmaier, C., Wraight, C., Holten, D. and Pearlstein, R.M. (1985) Biochim. Biophys. Acta 810, 132–139.
[5] Shuvalov, V.A., Shkuropatov, A.Ya., Kulakova, S.M., Ismailov, M.A. and Shkuropatova, V.A. (1986) Biochim. Biophys. Acta 849, 337–346.
[6] Struck, A., Muller, A. and Scheer, H. (1991) Biochim. Biophys. Acta 1060, 262–270.
[7] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[8] Kobayashi, M., Maeda, H., Watanabe, T., Nakane, H. and Satoh, K. (1990) FEBS Lett. 260, 138–140.
[9] Gounaris, K., Chapman, D.J., Booth, P.J., Crystall, B., Giorgi, L.B., Klug, D.R., Porter, G. and Barber, J. (1990) FEBS Lett. 265, 88–92.
[10] Satoh, K. (1993) in: The Photosynthetic Reaction Center (Deisenhofer, J. and Norris, J.R., Eds.), pp. 289–318, Academic Press, San Diego, CA.
[11] Seibert, M. (1993) in: The Photosynthetic Reaction Center (Deisenhofer, J. and Norris, J.R., Eds.), pp. 319–356, Academic Press, San Diego, CA.
[12] Konermann, L. and Holzwarth, A.R. (1996) Biochemistry 35, 829–842.
[13] Scheer, H., Porra, R.J. and Anderson, J.M. (1989) Photochem. Photobiol. 50, 403–412.
[14] Shuvalov, V.A., Heber, U. and Schreiber, U. (1989) FEBS Lett. 258, 27–31.
[15] Kaminskaya, O.P. and Shuvalov, V.A. (1994) FEBS Lett. 355, 301–3004.
[16] Montoya, G., Yruela, I. and Picorel, R. (1991) FEBS Lett. 283, 255–258.
[17] De Las Rivas, J., Telfer, A. and Barber, J. (1993) Biochim. Biophys. Acta 1142, 155–164.
[18] Shuvalov, V.A., Klimov, V.V., Krahmaleva, I.N., Moskalenko, A.A. and Krasnovskii, A.A. (1976) Dokl. Acad. Nauk. USSR 227, 984–987.
[19] Barber, J., Chapman, D.J. and Telfer, A. (1987) FEBS Lett. 220, 67–73.
[20] Telfer, A., He, W.-Z. and Barber, J. (1990) Biochim. Biophys. Acta 1017, 143–151.
[21] Telfer, A., De Las Rivas, J. and Barber, J. (1990) Biochim. Biophys. Acta 1060, 106–114.
[22] Strain, H.H. and Svec, W.A. (1966) in: The Chlorophylls (Vernon, L.P. and Seely, G.R., Eds.), pp. 21–66, Academic Press, New York.
[23] Holt, A.S. (1959) Plant Physiol. 34, 310–314.
[24] Wolf, H. and Scheer, H. (1971) Liebigs Ann. Chem. 745, 87–98.
[25] Klimov, V.V., Klevanik, A.V., Shuvalov, V.A. and Krasnovskii, A.A. (1977) FEBS Lett. 82, 183–186.
[26] Barber, J. and Melis, A. (1990) Biochim. Biophys. Acta 1020, 285–289.
[27] Doring, G., Renger, G., Vater, J. and Witt, H.T. (1969) Z. Naturforsch. 24b, 1139–1143.
[28] Van Gorkom, H.J., Tamminga, J.J., Haveman, J. and Van der Linden, I.K. (1974) Biochim. Biophys. Acta 347, 417–438.
[29] Braun, P., Greenberg, B.M. and Scherz, A. (1990) Biochemistry 29, 10376–10387.
[30] Mimuro, M., Tomo, T., Nishimura, Y., Yamazaki, I. and Satoh, K. (1995) Biochim. Biophys. Acta 1232, 81–88.
[31] Breton, J. (1990) in: Perspectives in Photosynthesis (Jortner, J. and Pullman, B., Eds.) pp. 23–38, Kluwer, Dordrecht.