EFFECT OF THE pH ON THE ABSORPTION SPECTRUM OF THE ISOLATED D1-D2-CYTOCHROME *b*559 COMPLEX OF PHOTOSYSTEM II

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ABBREVIATIONS: Chl, chlorophyll; Cyt, cytochrome; D1 and D2, core polypeptides of the photosystem II reaction center; MES, 2-(N-morpholino)-ethane-sulphonic acid; P680, primary chlorophyll donor of the photosystem II reaction center; Pheo, pheophytin; RC, reaction center; Tris, tris(hydroxymethyl)aminomethane.

ABSTRACT

The effect of pH on the Q_y absorption band was studied in the isolated D1-D2-cytochrome *b559* complex. The pH-treatments were done on an ion-exchange chromatographic column. The absorption spectra at 77K of the complex treated with acidic pH showed irreversible loss of absorbance at both the blue and the red sides of the Q_y absorption band with minima at 664.5 and 683.5 nm, respectively. These absorption changes were not accompanied by modifications on the Q_x absorption region characteristic of pheophytin pigments. Furthermore, the pigment composition of the D1-D2-cytochrome *b559* complex remained unchanged after this treatment. The effects of basic pH effects were similar to that of the acidic pH, but somewhat more pronounced. These results suggest that chlorophyll pigments absorbing at 664.5 and 683.5 nm are located on or close to the surface of the complex. Freezing/thawing cycle treatment firstly affected the band absorbing at 683.6 nm indicating that it corresponds to the chlorophyll most exposed to the medium in the D1-D2-cytochrome *b559* complex. At lower pH than 5 a small reversible change at 672.5 nm was measured that correlated with a reversible change at 542 nm, indicating that inactive pheophytin *a* would absorb at this wavelength.

INTRODUCTION

Photosystem II (PSII) is a multisubunit complex inserted into the thylakoid membranes of photosynthetic organisms (higher plants, algae and cyanobacteria). It uses light-energy to catalyze a series of electron transfer reactions which result in the splitting of water into molecular oxygen, protons and electrons (for reviews see 1-3). The primary photochemical processes that drive the water-splitting reaction occur in the reaction center (RC) of PSII which in its isolated form consists of the D1 and D2 polypeptides, the α and β subunits of the cytochrome (Cyt) *b*559 and the *psbI* gene product (4,5). This complex, known as the D1-D2-Cyt *b*559 complex, binds in addition to P680 and pheophytin (Pheo) a number of accessory chlorophylls (Chl). At present, the current accepted chlorine content is 6 Chls and 2 Pheos per RC complex, though preparations containing 4 and 5 Chl per 2 Pheo have also been reported (3,6,7). The spectroscopic and functional studies on the isolated D1-D2-Cyt *b*559 complex are complicated by the fact that all the chlorine pigments contribute to the Q_y absorption and fluorescence bands of the complex in the range 670-690 nm [for reviews see Renger (1992) and Barber (1994)]. Although considerable research based on spectroscopic measurements has been done to investigate the assignment of the single pigments, there is still no consensus on the exact positions in the absorption spectrum, particularly with respect to the accessory Chls and Pheo.

An interesting feature of the native biological photosynthetic systems is the redshifting of their pigment absorption bands compared to the pigments in solution. This phenomenon is remarkable in bacterial photosynthetic complexes

and has been the aim of numerous studies (10-12). Two main theories have been proposed to explain it. One of them attributes a good part of the spectroscopic properties of bacteriochlorophyll (BChl) *in vivo* to strong interactions of the BChl with the holder apoprotein (13-15). The other theory relates the redshifting to strong excitonic interactions among closely spaced BChl molecules (16-19). However, the most likely explanation seems to be a combination of both (15,20).

To further investigate the organisation of pigments in their surrounding proteins different methods such as freezing (21), proteolysis (22), treatment with detergents and sodium chloride (12) and chemical modifications with sodium borohydride (23) or ferricyanide (24) were used in studies performed on the bacterial reaction center and antenna complexes. In the PSII RC the excitonic interactions between pigments are much less stronger than that in the case of bacterial RCs, however they have also been aim of interest (25-27). Recently, some of the treatments mentioned above have been applied to investigate the electronic absorption properties of the PSII RC (28,29). These studies have reported on the position of the inactive Pheo (29) in the absorption spectrum of the D1-D2-Cyt *b*559 complex. However, there is still no consensus on the assignment of most of the accessory and “antenna” Chls in the PSII RC. The former pigments correspond to Chls homologous to the accessory BChls in RC from purple bacteria and the second ones to the “extra” Chls present in the PSII RC from higher plants compared to that of bacteria (30). In the present work we report the absorption spectral changes induced at different pH and after freezing/thawing cycles on the D1-D2-Cyt *b*559 complex isolated from higher plants.

MATERIALS AND METHODS

Preparation of the D1-D2-Cyt *b559* complex.- The D1-D2-Cyt *b559* complex was isolated from highly purified PSII membranes (31) according to the method of Nanba and Satoh (4) with some modifications mainly concerning to the detergent concentration. PSII membranes at 1 mg/mL Chl were solubilized with 4% (w/v) Triton X-100 for 2 h, centrifuged at 100,000 x g for 1 h at 4 °C and the resultant supernatant purified by ion-exchange chromatography (DEAE-Toyopearl TSK 650S column) using 1% (w/v) Triton X-100 in the column washing buffer steps (32). The detergent Triton X-100 was subsequently replaced by 0.1% (w/v) *n*-dodecyl- β -*D*-maltoside before a 60-350 mM NaCl linear gradient was applied. The Q_y absorption band maximum of the complex was at 675.5-676 nm at 4 °C indicative of the good quality of the preparation. The stoichiometry of the complex was six Chl *a* and one β -carotene per two Pheo *a* determined according to Eijkelhoff and Dekker (6).

Treatment with solutions at different pH (for details see Figure Legends) were done following two different procedures: a) by adding aliquots of 1 N NaOH and 1 N HCl to the samples and b) by washing the D1-D2-Cyt *b559* with 50 mM MES-NaOH (pH 5.5, 5.0 and 4.0) or 50 mM Tris-HCl (pH 7.5, 8.1, 10.0), 30 mM NaCl and 0.1% (w/v) *n*-dodecyl- β -*D*-maltoside through the ion-exchange column. The isolated preparation was eluted with 200 mM NaCl after the corresponding pH value was reached. In some cases, the column was again washed with 50 mM MES-NaOH (pH 6.5), 30 mM NaCl and 0.1% (w/v) *n*-dodecyl- β -*D*-maltoside to return to the

original conditions before elution. All the steps during the preparation procedure were done in dim green light in a cooled chamber at 4 °C.

Spectroscopy.- Absorption spectra at 4 °C were recorded with a Beckman DU 640 spectrophotometer with the temperature controlled with a Pharmacia circulating water bath. The spectra at 77K were measured in the same spectrophotometer using a variable liquid nitrogen cryostat DN1704 (Oxford Instruments). For measurements at 77K, samples were diluted three times in glycerol.

RESULTS

The influence of the pH on the Q_y absorption band of the D1-D2-Cyt *b559* complex was investigated at room temperature and 77K. Treatments were done by adding aliquots of 0.1 N HCl or 0.1 N NaOH directly to the sample suspensions and by washing steps with buffer solutions at the corresponding pH through an ion-exchange chromatographic column (for details see Materials and Methods and Figure Legends). Both treatments provided similar changes in the Q_y absorption maxima at 4 °C (data not shown). However, the corresponding spectra at 77K showed stronger modifications in the Q_y band in the samples treated in solution compared to those treated through the ion-exchange chromatographic column at both acidic and basic pH ranges. Because the pH conditions in solution were more difficult to control, we show in the present work the results of the experiments performed using the chromatographic column to exchange the pH.

Figure 1 shows the influence of pH 5.5 and 4.0 on the Q_y absorption band of the D1-D2-Cyt *b559* complex at 77K. Both the blue and the red sides of the band narrowed after treatment at acidic pH compared to those of the control spectrum at pH 6.5. The maximum at 678.5 nm shifted to the blue, the shift being 0.5 nm at pH 4.0. In contrast, the maximum at 671.1 nm remained unchanged in all the experimental conditions used. The reported changes on the Q_y absorption band of the D1-D2-Cyt *b559* were not reversible. On the other hand, the pigment composition of the D1-D2-Cyt *b559* complex did not change after the pH-treatment, indicating that the modifications in the absorption spectra are not due to a selective loss of pigments but to changes in its electronic absorption properties. Some partially reversible changes were observed at the Q_y band red-side. The blue-shift at 678.5 nm which was accompanied by an increment of the absorbance

at around this wavelength, was partially reverted when the D1-D2-Cyt *b559* complex preparation at pH 4.0 was subsequently washed with solution at pH 6.5 to get the initial experimental conditions (Fig. 1b).

The Q_x absorption band at around 542 nm assigned to Pheo was also examined. At pH 5.5 no significant modifications were observed in this spectral region, however stronger acidic conditions such as pH 4.0 reduced the intensity of the Pheo band around 40% (Fig. 1b, *inset*). This phenomenon was reversible and the shape of Q_x absorption band at 542 nm in the D1-D2-Cyt *b559* complex treated with pH 4.0 and subsequently with pH 6.5 was comparable to that of the control. The loss of absorbance at 542 nm was also accompanied by the appearance of a new band near to 554 nm and an increment of the absorbance at around 525 nm (Fig. 1b, *inset*). Changes in the pH conditions can modify the redox potential of the medium and consequently contribution of reduced Cyt *b559* could be detected. Considering that at 77K the maximum of Cyt *b559* shifts to 556 nm (33) the new band in the 550-560 nm spectral region could be assigned to a partial reduction of Cyt *b559*. Figure 1c shows in more detail the changes induced by the treatments at acidic pH. The difference absorption spectra between those of the acid-pH-treated and control showed minima at 683.5 and 664.5 nm, being the loss of absorbance of 14.4-15.3% and 3.5-4.5%, respectively. In addition a minor minimum at around 672-674 nm was observed in the difference absorption spectrum between those of the treated at pH 4.0 and non-treated D1-D2-Cyt *b559* complex samples (Fig. 1c). This minimum disappeared when the pH of the sample reverted to the control conditions (Fig. 1c). The reversible change at around 672-674 nm can be correlated to those observed in the Q_x absorption band region at pH 4.0. Similar changes were observed by Shuvalov *et al.* (33) during reduction of

an extra low potential Cyt *b*559. These authors observed a bleaching at 672 nm at 293 K assignable to reduced Pheo. The *inset* of Fig. 1c shows the difference absorption spectrum between the pH 4.0-treated sample after washed with buffer solution at pH 6.5 and that before treatment. This difference spectra indicated that the reversible change measured in the Q_y band corresponded to a maximum at 672.5 nm. In addition, a small reversible change was also observed at 681.7 nm.

The effect of the basic pH on the absorption spectrum of the D1-D2-Cyt *b*559 complex is shown in Fig. 2. In general the modifications induced by the basic pH were stronger than those of acidic pH. At pH 8.5 (Fig. 2a) the Q_y band slightly narrowed compared to that of the control and the decrease of the intensity of the maximum at 678.5 nm was accompanied by an increment of that at 671.5 nm. The loss of absorbance at both the blue and the red sides of the Q_y absorption band were no reversible. However, a reversible change in the center of this band was observed (Fig 2a). The Pheo band at 542 nm was not affected in these conditions. At higher pH values the changes were stronger as shown in the Figure 2b. At pH 10.0 the Q_y absorption band shifted significantly to shorter wavelengths, the maximum at 678.5 nm disappeared being the new maximum of the Q_y band at 670.6 nm. The presence of a shoulder at 679-680 nm is indicative that not all the species that absorb at around these wavelengths are affected by the treatment. The absorption changes were partially reversible as shown in the spectrum measured after washing the sample with the control buffer at pH 6.5 (Fig. 2b). Part of the absorbance at the red side of the Q_y absorption band at around 680 nm was recovered, being the new maxima at 678.0 nm. Two species have been proposed to contribute to the band at 680 nm, P680 and active Pheo (2,3,8). Since no changes were observed at 542 nm, the reversible change at 679-680 nm could be

assigned to P680. It has previously been reported that P680 is susceptible to suffer reversible induced changes by biochemical treatments such as detergent exchange (28). Reversible modifications in the Q_x band region of Pheo were also induced as occurred at pH 4.0 (Fig. 1b, *inset*). A new band at 553 nm was observed (Fig. 2b, *inset*) which could be assigned to the reduced Cyt *b*559 (33,34). This band disappeared again when the original experimental conditions were restored. The difference absorption spectra between those of the basic pH treated and control samples are shown in Fig. 2c. The changes caused by the basic pH treatment were more pronounced than those at acidic pH. The treatment at pH 8.5 (Fig. 2c) caused a loss of absorbance with minima at 683.7 and 663.8 nm of 11% and 7.5%, respectively. These values are comparable to that calculated for the treatment at pH 4.0, although the effect on the blue side of the spectrum seem to be slightly higher. At pH 10.0 the minima were at 681.6 and 665.1 nm and the associated loss of 16.9 and 4.2%, respectively. Part of the loss of absorbance at Q_y absorption band at basic pH was reversible (Fig. 2c, *inset*). The difference spectra between the pH 10.0 treated samples after washing with buffer solution at pH 6.5 and that at pH 10.0 showed maxima at 680.5 nm. The calculated maxima corresponded to reversible changes induced by basic pH. As occurred at acidic pH such modifications could be assigned to P680.

The second derivatives of the absorption spectra measured after the different pH-treatments is shown in Fig. 3. The acidic pH (Fig. 3a) modified more selectively the 680 nm spectral region and particularly the red side of the band absorbing at this wavelength. The shoulder at 683 nm was the first to disappear after the subsequent pH-treatments. After that, the minimum at 680.7 nm shifted to 679.8 nm at pH 5.5 and 679.3 nm at pH 4.0. Although in general the modifications

at basic pH were more pronounced, the second derivative spectrum showed that the results at pH 8.1 (Fig. 3b) are comparable to those at pH 5.5 (Fig. 3a). It seems again that the band absorbing at 683 nm is firstly affected.

Freezing methods have also been used to investigate the properties of photosynthetic pigment-protein complexes (21). It is known that the stability and integrity of the D1-D2-Cyt *b*559 complex is affected by freezing and storage procedures. In order to study in more detail which is the effect of freezing in this new preparation we measured the absorption spectra of the D1-D2-Cyt *b*559 complex at 277 and 77K complex after different freezing/thawing cycles at 193K and 77K. Figure 4 shows that freezing in liquid nitrogen (77K) conserved more intact the D1-D2-Cyt *b*559 complex preparations than at 193K. The Q_y absorption band maximum measured at 277K remained quite stable after eight freezing/thawing cycles at 77K in contrast to what occurred at 193K. The comparison of the absorption spectra measured at 77K after the different treatments at 193K showed that the Chl pigment(s) absorbing at 682-684 nm was(were) the most affected and no significant changes were caused on the Pheo molecules (Fig. 5). The loss of absorbance after four and eight freezing/thawing cycles at 683.6 and 682.7 nm was 10% and 21%, respectively. The values are in the same order of magnitude than those calculated after acidic pH treatment.

DISCUSSION

The experiments based on the modifications of the pH conditions in the isolated D1-D2-Cyt *b559* complex samples reported in this work revealed that the absorption bands with maxima at 683.5 and 664.5 nm that contribute to the Q_y absorption band are selectively first modified by both acidic and basic pH treatments. These changes were observed even with slight pH changes from 6.5 to 5.5 or 8.5. The maxima affected by the milder treatments were assigned to Chls since no changes at the Pheo Q_x band region were observed in the same conditions. The fact that the pigment composition of the D1-D2-Cyt *b559* complex was not modified during the pH treatment suggest that the observed spectral changes are not due to the loss of specific pigments. Thus, the spectral changes must be explained by some modifications of the protein matrix.

The stability of proteins is highly dependent on pH since hydrogen bonds, which are essential for the secondary structure of most proteins, are very sensitive to pH changes of the environment and associated protonations and deprotonations. Changes in the PSII RC due to pH have also been investigated in the past (35,36). It is also known that hydrogen bonds are formed between some amino acid residues and Chls in the D1-D2-Cyt *b559* complex which have a structural role in binding and orienting the pigment molecules (37). Particularly, Chl absorbing at 683.5 nm was the most affected by slight changes in the pH conditions. Furthermore, the percentage of ~15% loss of absorbance at this wavelength was comparable to the calculated contribution (~14%) of a gaussian with maximum at 683.2 nm to the absorption spectra of the isolated D1-D2-Cyt *b559* complex reported by Konermann and Holzwarth (30). This finding indicated that the chromophore absorbing at this wavelength was quantitatively modified.

The nature of this chromophore absorbing at the red-edge of the absorption spectrum has been discussed in recent years (25-27,30,38,39). The presence of a shoulder at 684 nm is a typical spectral feature of most PSII RC preparations obtained in different laboratories. However, those preparations clearly differed from each others in the size of the contribution of this band to the total spectra (30). Based on these findings, some authors postulated that the shoulder at 684 nm could be caused by additional Chls not actually belonging to the PSII RC (30). However, samples with 6Chl/2 Pheo and clearly free of contamination (30,32) also presented a pronounced shoulder at 684 nm, indicating that this band is not caused by a red-absorbing Chl contaminant. On the other hand, it has recently shown that the absorption spectrum of PSII RC particles containing 5Chl/RC reveals that the shoulder near 684 nm is more pronounced than in any other PSII RC preparation.

Excitonic coupling due to pigment-pigment interactions have also been described in the D1-D2-Cyt *b*559 complex. The dimeric nature of P680 has been extensively discussed. Based on hole-burning measurements at low temperatures, the Q_y band of P680 absorption spectrum consists of two excitonic bands one peaking at around 667 nm and the other at 681 nm (26). These results are in good agreement with circular dichroism (35,40) and triplet-*minus*-singlet absorption difference spectra (27). The fact that the Chls absorbing at 683.5 and 664.5 nm are selectively affected by pH treatments could suggest that both pigments are excitonically coupled. Kwa *et al.* (27) reported that the triplet-*minus*-singlet absorption spectrum ascribed to P680 can be interpreted in terms of a dimer with two distinguishable distributions around 681 and 684 nm which contribute to the low-energy exciton band and the upper dimer component peaking at 667 nm.

More recently, spectroscopic data on 5Chl PSII RC preparation indicated that the low energy shoulder at 684 nm originates, at least in part, from the primary electron donor P680 (41). This model is not consistent with our freezing/thawing cycle treatments, except if we consider that only a minor part of the absorbance at 684 nm corresponds to P680 and other species absorb at this wavelength. Our treatments caused selective loss of absorbance at 683 nm that was not accompanied by significant effect on the blue side of the Qy absorption band (Fig. 5b). The 684 nm absorption band has been also found to be easily disrupted by the ionic detergent Triton X-100 (26).

Spectral gaussian deconvolution analyses and site-selection fluorescence spectroscopic measurements have identified the lowest energy pigment in the PSII RC as an accessory Chl which is not related to P680 and is analogous to one of the accessory BChl in the bacterial RC (30,38). The fact that the Chl absorbing at 683 nm was the pigment first affected by pH-treatments (Fig. 1c, 2c, 3) seems to indicate that it should be in a protein region highly exposed to the medium. Furthermore, these considerations are also supported by our freezing/thawing cycle experiments. Indeed, the Chl absorbing at 683 nm was the most susceptible to damage by the freezing/thawing cycle procedure at 193K, suggesting that it should be more exposed to the surface of the complex. Distances of 9.9 and 10.4 Å between D1-accessory Chl and D1-Chl(P680) and active D1-Pheo, respectively have been calculated (37). Although there is no data concerning to the analogous distances from D2-accessory Chl to D2-Chl(P680) and inactive D2-Pheo the values should be similar. Thus, these accessory Chls would correspond to the inner chromophores which together with the P680 and the Pheo constitute the “core pigment” of the PSII RC.

In the present work we have showed that Chl absorbing in the blue side of the absorption spectra is also affected by the pH but the freezing/thawing cycle treatments did not cause significant changes in this spectral region. These findings suggest a location of the pigment absorbing at 667-670 nm spectral region exposed to the medium but more protected by the matrix than that absorbing at 684 nm. The highest energy pigment in the PSII RC has been assigned by others to one of the "extra" Chls called "antenna" Chls in the D1-D2-Cyt *b*559 complex (30). These results are in agreement with spectroscopic data on the 5Chl-RC preparation (7,41). They indicated that the Chl removed belongs to a pool of Chl absorbing at 670 nm likely to be peripherally bound.

The treatment with stronger pH conditions as pH 4.0 caused additional reversible modifications in the Q_x Pheo spectral region which were accompanied by reversible changes in Q_y absorption band at around 672-674 nm. Since, there is a general consensus that the active Pheo absorbs close to 680 nm, this maximum should correspond to the inactive Pheo. At present, there is no consensus on the exact position of the inactive Pheo and it has been proposed that it absorbs at 671 (42) and 680 nm (29). Our results support the conclusion that the inactive Pheo most probably has a maximum in the 672-674 nm spectral region.

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FIGURE LEGENDS

Figure 1.- Effect of the acid pH on the Q_y band region of the absorption spectrum of the D1-D2-Cyt *b*559 complex. Control at pH 6.5 (a and b, —), treated at pH 5.5 (a, ---) and pH 4.0 (b, ---), and after washing the pH-treated samples with buffer at pH 6.5 (a and b,). *Inset*: Q_x absorption band region of the spectra. c) Difference absorption spectra by subtracting the absorption spectra of D1-D2-Cyt *b*559 complex samples at pH 5.5 (1), pH 4.0 (3), and after washing the pH 5.5 (2) and 4.0 (4) treated samples with solution buffer at pH 6.5, respectively, from that non-treated D1-D2-Cyt *b*-559 complex. *Inset*: Difference absorption spectrum between the pH 4.0 treated sample after washing with buffer solution at pH 6.5 and that at pH 4.0. Measurements were done at 77K and to do that samples were diluted three times in glycerol. For details see Materials and Methods.

Figure 2.- Effect of the basic pH on the Q_y band region of the absorption spectrum of the D1-D2-Cyt *b*559 complex. Control at pH 6.5 (a and b, —), treated at pH 8.1 (a, ---) and pH 10.0 (b, ---), and after washing the pH-treated samples with buffer at pH 6.5 (a and b,). *Inset*: Q_x absorption band region of the spectra. c) Difference absorption spectra by subtracting the absorption spectra of the D1-D2-Cyt *b*559 complex samples at pH 8.1 (1), pH 10.0 (3), and after washing the pH-8.1 (2) and 10.0 (4) treated samples with solution buffer at pH 6.5, respectively, from that non-treated D1-D2-Cyt *b*-559 complex. *Inset*: Difference absorption spectrum between the pH 10.0 treated sample after washing with buffer solution at pH 6.5 and that at pH 10.0. Measurements were done at 77K. Samples were diluted three times in glycerol. For details see Materials and Methods.

Figure 3.- Second derivative absorption spectra of the Q_y band region of the D1-D2-Cyt *b559* complex in control conditions at pH 6.5 (a and b, 1), and treated with buffer at pH 5.5 (a, 2), 4.0 (a, 4), 8.1 (b, 2), 10.0 (b, 4) and after washing the pH 5.5 (a, 3), 4.0 (a, 5), 8.1 (b, 3) and 10.0 (b, 5) treated samples with buffer solution at pH 6.5.

Figure 4.- Dependence of the Q_y absorption maximum of the D1-D2-Cyt *b559* complex on the subsequent freezing/thawing cycle treatment at 193K (●) and 77K (○). Samples were resuspended in 50 mM Mes-NaOH (pH 6.5) and 0.1% *n*-dodecyl- β -*D*-maltoside. Absorption spectra were recorded at 4 °C after treatment.

Figure 5.- a) Absorption spectra in the Q_y band region of the D1-D2-Cyt *b559* complex at 77K after subsequent freezing/thawing cycle treatments at 193K. *Inset*: Q_x absorption band region of the spectra. b) Difference absorption spectra between those of the treated and control samples. Number of freezing/thawing cycles 0 (—), 4 (----), 8 (.....).

Fig.1

Fig. 2

Fig. 3

Fig. 4

Fig. 5