The Cysteine-proximal Aspartates in the F X-binding Niche of Photosystem I

EFFECT OF ALANINE AND LYSINE REPLACEMENTS ON PHOTOCYTHEROTROPHIC GROWTH, ELECTRON TRANSFER RATES, SINGLE-TURNOVER FLASH EFFICIENCY, AND EPR SPECTRAL PROPERTIES*

(Received for publication, July 22, 1998, and in revised form, January 3, 1999)

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The FX electron acceptor in Photosystem I (PS I) is a highly electron-negative (E_m = −705 mV) interpolytropic [4Fe-4S] cluster ligated by cysteines 556 and 565 on PsAD566 and cysteines 574 and 583 on PsAA in Synechocystis sp. PCC 6803. An aspartic acid is adjacent to each of these cysteines on PsAD566 and adjacent to the proline-proximal cysteine on PsAA. We investigated the effect of D566PsaB and D557PsaA on electron transfer through FX by changing each aspartate to the neutral alanine or to the positively charged lysine either singly (D566APsaB, D566KpasaB, D557APsaB, and D557KpasaB) or in pairs (D557PsaA/D566APsaB and D557KPsaB/D566APsaB). All mutants except for D557KPsaB/D566APsaB grew phototrope and D557APsaB/D566APsaB was impaired under low light. The doubling time was increased, and the chlorophyll content per cell was lower in D557KPsaB and D557APsaB/D566APsaB relative to the wild type and the other mutants. Nevertheless, the rates of NADP* photoreduction in PS I complexes from all mutants were no less than 75% of that of the wild type. The kinetics of back-reaction of the electron acceptors on a single-turnover flash showed efficient electron transfer to the terminal acceptors FA and FB in PS I complexes from all mutants. The EPR spectrum of FX was identical to that in the wild type in all but the single and double D566APsaB mutants, where the high-field resonance was shifted downfield. We conclude that the impaired growth of some of the mutants is related to a reduced accumulation of PS I rather than to photosynthetic efficiency. The chemical nature and the charge of the amino acids adjacent to the cysteine ligands on PsAD566 do not appear to be significant factors in the efficiency of electron transfer through FX.

Iron-sulfur clusters are widely represented in various proteins and multiprotein complexes and, in the majority of instances, are involved in electron transfer reactions. Cubane ([4Fe-4S]) clusters are 1-electron carriers that function between two of the three oxidation states, 2 or 3 (+ or +2/+ for review, see Ref. 1). The midpoint potentials of the near-geometrically identical [4Fe-4S] clusters are modulated by different protein environments and can range from +350 to −705 mV (versus H2O). One of the lowest potential redox-active iron-sulfur clusters is FX (E_m = −705 mV) (2), which functions as an intermediate electron acceptor in PS I. FX is a relatively rare form of an interpolytropic [4Fe-4S] cluster, where two cysteine ligands are provided by PsA and two cysteine ligands are provided by PsB (3, 4). The EPR spectral properties of FX are unusual in that the average of the g-values 2.096, 1.853, and 1.757 is lower than in typical low potential cubane clusters, and its line widths are broad relative to the other two [4Fe-4S] clusters (FA and FB), which serve as the terminal low potential electron acceptors in PS I. The EPR spectrum of FX shows a low-temperature optimum (~8 K) and a relatively high microwave half-saturation power (P_1/2 > 200 mW at 8 K), which indicates an efficient spin relaxation mechanism (5). It is not known which features of the protein are responsible for the unusual redox and spectroscopic properties of FX.

Unlike the non-heme iron that bridges the two subunits of the quinone-type reaction centers (represented by Photosystem II and the reaction centers of purple bacteria) and that does not participate in the electron transfer, FX serves as an indispensable component of the electron transport chain of PS I (6, 7). According to our current understanding, light-induced electron transport between the PS I-bound cofactors follows a linear path: P700 (Chl a dimer) → A0 (Chl a monomer) → A1 (4Fe-4S) cluster → FA (4Fe-4S cluster) → FB (4Fe-4S cluster) → F0 (4Fe-4S cluster), P700, A0, A1, and FX are located on the PsA/PsbB reaction center heterodimer, and FA and FB are located on an extrinsic, ferredoxin-like protein termed PsAC (for review, see Ref. 8). A comparison of the FX domain among eukaryotes and prokaryotes shows that the cysteine ligands on PsAD566 are located in a nearly invariant region of 100 amino acids that surround and include helices VIII and IX (8) (helices j (or j') and k (or k') in current nomenclature (9)). An additional interesting feature of FX is that an aspartate residue is adjacent to each conserved

* This work was supported by Grant 96-37306-2632 from the United States Department of Agriculture, National Research Initiative Competitive Grants Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: FX, FA, and FB, [4Fe-4S]-type electron acceptors bound to Photosystem I chlorophyll-protein complex; A1, the secondary electron acceptor in Photosystem I, a phylloquinone; PS I, Photosystem I; Chl, chlorophyll; MES, 4-morpholineethanesulfonic acid; DM, dodecyl β-maltoside; DM-PS I, Photosystem I complex isolated using n-dodecyl β-maltoside (containing FX, FA, and FB iron-sulfur clusters); DCPIP, 2,6-dichlorophenolindophenol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; OD, optical density.

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cysteine on PsaB (PCDGPGGGGTCD) and adjacent to the proline-proximal cysteine on PsaA (PCDGPGGGGTCTQ). According to the 4.5-Å electron density map, the FX cluster is located just inside the membrane region of the stromal side of two membrane-spanning α-helices. Even though the low potential FX cluster operates between +2 and +1 oxidation states, a more comprehensive view incorporates the charge of the four cysteine thiolate ligands, [(4Fe-4S)(S-Cys)]3−2−, whereupon the overall charge on the FX cluster can be considered to increase from −2 to −3 on reduction. There is a high electrostatic price paid for stabilizing a charge in the normally low protein dielectric, and such highly charged sites are usually located on the surface of proteins. Since FX is located just within the membrane phase, one explanation of its low reduction potential is that the charge is destabilized due to the low dielectric medium. A second explanation for its low reduction potential is that the cysteine-proximal aspartic acid residues may be deprotinated in the membrane (although there are no experimental data to support this point), and they may be positioned so that they interact electrostatically with the negatively charged iron-sulfur clusters. It follows that the additional work to move an electron into this environment would translate to a lower reduction potential of FX. Substitutions of these aspartates with amino acids differing in charge may alter the reduction potential and hence the efficiency of electron transfer through FX.

The effects of ligand substitution on the properties of a variety of iron-sulfur clusters were recently reviewed (10). The ability to substitute oxygen for sulfur ligands has been demonstrated in PS I reaction centers by substitution of serines for the cysteine ligands to FX with serines in Synechocystis sp. PCC 6803. This change did not prevent the assembly of PS I or increase relative to the wild type. The FX region has also been altered in Chlamydomonas reinhardtii by visual best-fit comparison with the experimental data. Site-directed Mutagenesis—Oligonucleotides were designed to effect the mutation of pLS3531 (11) were then used to transform strain ΔB-RCIPT. Spectinomycin-resistant colonies were selected under light-activated heterotrophic growth conditions as described (17).

Growth of Cells, Oxygen Evolution Rates, and Chlorophyll Content in Whole Cells—Cell growth rates, oxygen evolution rates, and chlorophyll content in the whole cells were determined as described (18). For large-scale culture, wild-type and mutant cells were grown under light-activated heterotrophic growth conditions in carboys with 15 liters of BG-11 medium supplemented with 5 mM glucose. In the case of mutants, the medium was supplemented with spectinomycin (20 mg ml−1, Sigma). After harvesting with a Sorvall continuous flow rotor (DuPont), the cells were stored in BG-11 medium with 15% (v/v) glycerol at −95 °C. For oxygen evolution measurements, the light from a slide projector was passed through a copper sulfide solution and filtered with a red cutoff filter (λ > 640 nm), providing saturating photon flux density of 480 μmol m−2 s−1. PS I-mediated oxygen uptake rates were determined using the isolated thylakoid membranes as described (11).

Isolation of Thylakoid Membranes and PS I Complexes—Thylakoid membranes were isolated from Synechocystis sp. PCC 6803 according to the procedure described (19). The cells were broken in buffer containing 20 mM MES, pH 7.2, 800 mM sucrose, and a protease inhibitor (phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, pepstatin, and leupeptin). Membranes were solubilized in 1% n-dodecyl b-t-o-maltoside (DM) (Calbiochem) at 4 °C for 1 h at a Chl concentration of 1 mg ml−1. Trimeric PS I complexes were isolated from the lower green band after sucrose density (0.1–1.0 M sucrose) ultracentrifugation of the solubilized membrane suspension for 24 h at 4 °C. The isolated PS I complexes were dialyzed overnight in 50 mM Tris-HCl, pH 8.3, resuspended in the same buffer containing 15% (v/v) glycerol and 0.05% DM; frozen as small aliquots in liquid nitrogen; and stored at −95 °C.

Electron Paramagnetic Resonance Spectroscopy—EPR studies were performed with a Bruker ECS-106 X-band spectrometer with a standard-mode resonator (ST 8615) that had either a grid or a quarter-wave-stub for light entry. Cryogenic temperatures were maintained with a liquid helium cryostat and an ITC-4 temperature controller (Oxford Instruments, Oxford, United Kingdom). The microwave frequency was measured with a Hewlett-Packard 5340A frequency counter, and the magnetic field was calibrated using DPPH (a,a′-diphenyl-β-tert-butyl hydrazyl) as the standard. Sample temperatures were monitored by a calibrated thermometer located 3 mm beneath the bottom of the quartz sample tube and referenced to liquid nitrogen. Samples were illuminated with a 150-watt xenon arc source (Model 66057, Oriel Corp., Stratford, CT) passed through 5 cm of water and a heat-absorbing color filter to remove the near-IR light. Samples used for EPR measurements contained 1 mg ml−1 Chl, 1 mM sodium ascorbate, and 30 mM DCPIP in 50 mM Tris, pH 8.3. Samples requiring reduced F5 and F6 were suspended to 1 mg ml−1 Chl in 250 mM glycine, pH 10.5, prior to the addition of 50 mM sodium hydrosulfite.

Differential EPR spectra of FX were obtained by subtracting the spectrum of a sample treated in darkness with sodium hydrosulfite at pH 10.5 (to reduce F5 and F6) from a sample that was treated identically, but frozen during illumination to additionally reduce FX. The resonances observed between 340 and 360 mT are derived from FX, as determined by band simulations of EPR spectra that were performed using WINEPR SimFonia Version 1.25 (Bruker EPR), where the g-values were obtained by visual best-fit comparison with the experimental data.

Room Temperature Transient Absorbance Spectroscopy—Transient absorbance changes of F5 of 0.826 nm (ΔA365) were measured in the microsecond to tens-of-seconds time domain with a laboratory-bds time domain with a double-beam spectrophotometer as described previously (21). Samples for optical experiments were suspended under anaerobic conditions at 50 μg ml−1 Chl in 25 mM Tris buffer, pH 8.3, with 4 μM DCPIP, 10 mM sodium ascorbate, and 0.03% DM. The photocurrents generated by UDCT PIN-10D photodiodes were converted to photovoltages using 1-kOhm resistors and amplified using an EG&G Model 113a differential amplifi-
TABLE I

Characteristics of whole cells, thylakoid membranes, and PS I complexes of wild-type and mutant strains.

| Strain | Characteristics of whole cells and thylakoid membranes | Rates in isolated PS I complexes |
|--------|--------------------------------------------------------|---------------------------------|
|        | Rate of whole chain O2 evolution by cells*             | Rate of PS I-mediated O2 uptake by thylakoids* | Flavodoxin reduction | Flavodoxin-mediated NADP+ reduction | Ferredoxin-mediated NADP+ reduction |
| Wild-type | 532 | 3.25 | 2410 | 580 | 299 | 397 |
| D566A | 18.6 | 636 | 2.85 | 4020 | 600 | 264 | 336 |
| D566K | 20.1 | ND | 3.21 | 2600 | 560 | 240 | 294 |
| D557A | 18.6 | 411 | 2.11 | 2870 | 590 | 240 | 408 |
| D557K | 27.8 | 336 | 1.92 | 2840 | 500 | 240 | 360 |
| D557A/D566A | 32.8 | 673 | 2.03 | ND | 520 | 280 | 400 |
| D557K/D566A | 633 | 0.96 | ND | 580 | 280 | 400 |

* Per Chl content (in μmol mg\(^{-1}\) h\(^{-1}\)) measured upon illumination at the rate of 60 μE m\(^{-2}\) s\(^{-1}\).

RESULTS

Growth Rates and Physiological Characteristics of Wild-type and Mutant Strains—Mutant cells of Synechocystis sp. PCC 6803 were tested for their ability to grow on BG-11 plates with and without supplemental glucose. Doubling times and chlorophyll/cell ratios were determined using mid-log phase liquid cultures grown photoautotrophically upon bubbling with air (Table I). All single aspartate mutant strains had the ability to grow mixotrophically with supplemental glucose at rates close to the wild-type strain. Strains D566A\(_{\text{psaB}}\), D566K\(_{\text{psaB}}\), and D557A\(_{\text{psaB}}\) grew photoautotrophically at wild-type rates at light intensities ranging from 2.5 to 60 μE m\(^{-2}\) s\(^{-1}\). Strain D557K\(_{\text{psaB}}\) did not grow or grew extremely slowly at 2.5 μE m\(^{-2}\) s\(^{-1}\) (and the colony was much smaller), but it grew at wild-type rates at 5.5 and 11 μE m\(^{-2}\) s\(^{-1}\). At the higher light intensities of 20 and 60 μE m\(^{-2}\) s\(^{-1}\), however, D557K\(_{\text{psaB}}\) again grew more slowly than the wild type. The chlorophyll content of the whole cells of the single aspartate mutants was similar to that of the wild type, except for D557K\(_{\text{psaB}}\), which had about two-thirds of the wild-type chlorophyll content per cell (Table I). Whole chain oxygen evolution rates of light-activated heterotrophic grown cells of the single aspartate mutants were within 20% of those of the wild type, except for D557K\(_{\text{psaB}}\), for which the rates were somewhat lower. The rates of methyl viologen-mediated O\(_2\) uptake by thylakoid membranes of the single mutants were likewise similar to or slightly reduced.
compared with the wild-type rates. The amounts of PsaA, PsaB, PsaC, PsaD, and PsaE polypeptides assayed by Western blotting were about the same in the wild type and the single aspartate mutants on a chlorophyll basis (25). The ability to grow photautotrophically, to accumulate chlorophyll, and to evolve O2 at rates comparable to the wild type indicates accumulation of high levels of PSI and PSII in the single aspartate mutants.

Double aspartate mutant strains grew mixotrophically with supplemental glucose at rates close to the wild-type strain except for D557K/PsaB/D566APsaB, which grew at all but the highest tested light intensity of 60 μE m−2 s−1. We noted that both double mutant strains bleached early when grown mixotrophically between light intensities of 7.5 and 22 μE m−2 s−1. Strain D557A/PsaB/D566A/PsaB grew slowly photautotrophically, but D557K/PsaB/D566A/PsaB failed to grow photautotrophically at all light intensities ranging from 2.5 to 60 μE m−2 s−1. The chlorophyll content of the photautotrophically growing cells of D557A/PsaB/D566A/PsaB was about two-thirds of that of the wild type (Table I). Whole chain oxygen evolution rates of light-activated heterotrophic grown cells of the double aspartate mutants were 20–25% greater than those of wild type. This finding implies that PS I is functional in the nonphotautotrophic D557K/PsaB/D566A/PsaB double mutant. As shown in Table I, the chlorophyll levels in mid-log phase photautotrophic cells were significantly lower in D557K/PsaB and D557A/PsaB/D566A/PsaB, the two mutant strains that had significantly longer doubling times compared with the other strains. This suggests that reduced chlorophyll levels in these two strains may be responsible for the slow growth.

Steady-state Rates of Electron Transfer in PSI Complexes—Steady-state rates of electron transfer were measured in the wild-type and mutant DM-PS I complexes by reduction of flavodoxin, by reduction of NADP+ mediated by flavodoxin, and by reduction of NADP+ mediated by ferredoxin. All assays employed cytochrome c6, the physiological electron donor to F570+, and the ionic strength of the media was optimized to support the highest rates of electron transfer. The rates of flavodoxin photoreduction in wild-type DM-PS I complexes were 580 μmol mg−1 Chl h−1, and the rates of ferredoxin- or flavodoxin-mediated NADP+ photoreduction were ~300 and 400 μmol mg−1 Chl h−1, respectively (Table I). The rates of flavodoxin photoreduction in DM-PS I complexes from the four single aspartate mutants and the two double aspartate mutants were in the range of 500–600 μmol mg−1 Chl h−1, similar to the wild-type values. When flavodoxin-mediated NADP+ photoreduction was measured, all aspartate mutants showed rates better than 240 μmol mg−1 Chl h−1, and when ferredoxin-in-mediated NADP+ photoreduction was measured, the rates ranged from 340 to 410 μmol mg−1 Chl h−1. Thus, impairment of photautotrophic growth in certain mutants is not accompanied by a decline in whole chain electron transfer rates in either the single or double aspartate mutant strains.

Low Temperature EPR Spectra of F A and F B—F A and F B were irreversibly photoreduced when dark-frozen membranes or DM-PS I complexes from the single mutants D566A/PsaB, D566K/PsaB, D557A/PsaB, and D557K/PsaB and the double mutants D557A/PsaB/D566A/PsaB and D557K/PsaB/D566A/PsaB were illuminated at 15 K. The principal g-values of F A at 2.048, 1.944, and 1.851 and F B at 2.069, 1.930, and 1.881 and the ratio of photoreduced F A to F B of ~2.5:1 are identical to those in wild-type membranes and DM-PS I complexes (data not shown). F A and F B were completely photoreduced and showed the characteristic interaction spectrum of F A/B with g-values of 2.048, 1.940, 1.921, and 1.885 when membranes or DM-PS I complexes of the single and double mutants were frozen during illumination. The EPR spectral properties of F A− and F B− are therefore unaffected by substitutions of the cysteine-proximal aspartic acid residues on PsaB even though extramembrane loops in the F R region of PsaA and PsaB appear to be in close contact with PsaC (26).

Low Temperature EPR Spectra of F X—The EPR spectrum of F X in the wild-type DM-PS I complex is shown in Fig. 1A. The numerical simulation yields an acceptable match to the F X spectrum in the non-occuled region of the spectrum upfield from F A and F B, with g-values of 2.096, 1.853, and 1.757 and line widths of 9.2, 8.8, and 84 mT, correspondingly. The resonance with g = 2.002 corresponds to the F α cation radical (not fully shown on the vertical scale used in the figures). The g-values and line widths of the EPR spectra of the membranes isolated from the single lysine mutants D557K/PsaB (Fig. 1B, inset) and D566K/PsaB (Fig. 1C, inset) and the single alanine mutant D557A/PsaB (Fig. 1D, inset) are similar to those of F X in wild-type membranes. The high-field resonance occurs in the g = 1.744–1.764 region in these mutants; however, the mid-field and low-field regions are not depicted in membranes due to spectral overlap with F B/F D. However, the EPR spectra of DM-PS I complexes isolated from D557K/PsaB and D557A/PsaB (Fig. 1, B–D) are broadened, and they cannot be numerically simulated with any degree of accuracy. These spectra may result from a superposition of two or more conformers, which may result from increased conformational flexibility in the detergent-isolated PSI complexes.

The g-values of the EPR spectra of membranes isolated from the single alanine mutant D566A/PsaB (Fig. 1E, inset) and the double alanine mutants D557A/PsaB/D566A/PsaB (Fig. 1F, inset) and D557K/PsaB/D566A/PsaB (Fig. 1G, inset) are shifted down-field relative to F X in wild-type membranes. In particular, the high-field resonance occurs near g = 1.795 compared with g = 1.755 in the wild type. The g-values and line widths of the spectra of F X in the isolated DM-PS I complexes from the three D566A/PsaB mutants (Fig. 1, E–G) appear similar to those of F X in the membranes. The relaxation behavior is not greatly affected as judged by the low temperature optima (9 K) at 100 mW (~3 dB) of microwave power and by the inability to microwave power-saturate the signal at 9 K (data not shown). The similarity of the EPR spectra in all three mutant strains indicates that the amino acid in the position 566PsaB has a major influence on the electronic properties of F X.

Room Temperature Absorbance Spectroscopy—Fig. 2 (A–G) shows ΔA280, kinetics of the DM-PS I complexes from the wild type and the aspartate mutants measured in the presence of the artificial electron donor, reduced DCPIP. As detailed previously (21, 27), the slowest component in the wild-type DM-PS I complex (Fig. 2A) has a lifetime of 2.2 s, and it arises from F700− reduction by the external electron donor, ascorbate-reduced DCPIP. The major pathway of F700− reduction following a single-turnover flash is charge recombination with [F A/F B]−, and the kinetics of this back-reaction in wild-type PS I complexes are approximated by two components with lifetimes of ~25 and 112 ms (Fig. 2A). In most cases, another component with a lifetime of ~250–300 ms (221 ms in the preparation used in this work) can usually be resolved. At present, we cannot unambiguously ascertain whether this component represents F700− reduction from an external donor or a back-reaction with endogenous acceptor. It should be noted, however, that kinetic components with lifetimes longer than 100 ms were not observed in absorbance changes in the near-IR (ΔA) and photovoltage (ΔV) decay measurements performed in the absence of exogenous mediators (21). Faster components (Fig. 1A) are typically present in all PS I preparations, they usually make up 10–20% of the overall ΔA amplitude and may
FIG. 1. EPR spectra of wild-type (A) and mutant strains D557K_{PsaB} (B), D566K_{PsaB} (C), D557A_{PsaB} (D), D566A_{PsaB} (E), D557A_{PsaB}/D566A_{PsaB} (F) and D557K_{PsaB}/D566A_{PsaB} (G). DM-PS I complexes are depicted in the main spectra, and thylakoid membranes are depicted in the insets. The spectra were obtained with F_{X} and F_{Y} pre-reduced with hydrosulfite at pH 10.5 and illuminated during freezing to 9 K. Background spectra were recorded in chemically reduced dark-adapted samples frozen to 9 K and subtracted from the light-induced spectra. Spectrometer settings were as follows: temperature, 9 K; microwave power, 40 mW; microwave frequency, 9.4521 (A), 9.4476 (B), 9.4499 (C), 9.4496 (D), 9.4487 (E), 9.3591 (F), and 9.3593 (G) GHz; receiver gain, $2 \times 10^{4}$; modulation amplitude, 32 G at 100 kHz; magnetic field, 3600 G with scan width of 1000 G; and four scans averaged. The magnetic field positions of the resonances in the double mutants (F and G) are shifted in resonant frequency compared with the single mutants (B–E) as a consequence of adding a quarter-wave optical stub to the cavity window. The wild-type spectrum of F_{X} was simulated (dashed lines) using g-values of 2.086, 1.853, and 1.755.
be derived from the back-reactions of $F_X^-$ and $A_1^-$ in a population of damaged PS I complexes. The lifetimes of 2.6 ms and 543 $\mu$s are characteristic of the back-reactions from $F_X^-$ when $F_A$ and $F_B$ are absent, and the 89-$\mu$s component is characteristic of $A_1^-$ back-reaction when $F_X$, $F_A$, and $F_B$ are absent (21, 28).

The $\Delta A_{826}$ kinetics of D566KpsaB (Fig. 2C), D557ApsaB (Fig. 2D), and D566ApsaB (Fig. 2E) are similar to those of the wild-type (A), D557KpsaB (B), D566KpsaB (C), D557ApsaB (D), D566ApsaB (E), D557ApsaB/D566ApsaB (F), and D557KpsaB/D566ApsaB (G) PS I complexes in the presence of 1 mM sodium ascorbate and 4 $\mu$M DCPIP in 50 mM Tris-HCl, pH 8.3, and illuminated by saturating laser flashes (16 mJ) at 50-s intervals. Horizontal arrows point to the absorbance change values calculated as the sum of the initial amplitudes (Ampl.) of the components with lifetimes longer than 7 ms.
type PS I complex. The overall ΔA826 absorbance change in D557KPSaB (Fig. 2B) shows a much higher initial amplitude than in the wild type (5.9 versus 3.75 mOD) or the other single aspartate mutants. However, the contribution of the ~20-μs component to the overall P700− decay kinetics in D557KPSaB is much greater than the contribution of kinetically similar components in D566A PSaB, D566KPSaB, or D557A PSaB. Unlike the millisecond-to-seconds components, the 20-μs component did not saturate when the excitation flash intensity was increased to 150 mJ (data not shown), indicating that the decay of an antenna chlorophyll triplet (3Chl) most likely contributes to the ΔA826 in this time range. If only those components with lifetimes longer than 7 ms are taken into account when calculating the overall absorbance change, then the resulting amplitude of 2.4 mOD (Fig. 2B) lies in the same range as the corresponding value calculated in the wild type and other mutants at equal chlorophyll concentrations (this value is indicated by horizontal arrows pointing to the absorbance change axis in Fig. 2).

Therefore, the efficiency of electron transfer to Fb appears to be either unaffected by the mutation in D557KPSaB or affected to the same extent as in other mutants. The 2.4-mOD absorbance change in D557KPSaB corresponds to a reasonable Chl a/P700 value of ~121 calculated based on a differential extinction coefficient of P700 at 826 nm (29). This ratio is close to the Chl a/P700 value of 91 that is obtained in the wild type (Fig. 2A) or in the other mutants where Chl a/P700 falls within the range of 97–122 (Fig. 2, C–G). These values are compatible with the high whole chain electron transfer rates in D557KPSaB calculated on an equal chlorophyll basis (Table I). The implication of this result is that the absorbance change that decays with a lifetime of ~20 μs in D557KPSaB is not related to P700 photochemistry, but is rather due to the decay of 3Chl generated at flash intensities that saturate charge separation in the reaction center. In this respect, the kinetic properties of D557KPSaB are qualitatively different from those of the C565SPSaB mutant, which was shown to have a decreased quantum efficiency of electron transfer via Fx (13). The decrease in quantum efficiency was manifest in C565SPsaB by a considerably lower amplitude of the sum of components with lifetimes slower than 7 ms relative to the wild type at equivalent chlorophyll concentrations (30).

In D557KPSaB, the component with a lifetime of ~15–20 μs still had a relatively high contribution of 26.5% to the total absorbance change at flash intensities as low as 0.2 mJ, energies that produce a much lower (<10%) contribution of tens-of-microseconds components in the wild type (21). As shown in Fig. 3, at a flash intensity of 0.2 mJ, the amplitude of the absorbance change in D557KPSaB calculated on the basis of components with lifetimes slower than 7 ms was similar to that of the other single mutants. This finding further elaborates an equally efficient electron transfer from P700 to Fx/Fb in this mutant. At the same time, the decrease in the overall absorbance change upon reduction of the excitation flash energy occurs mainly in the fast 15–20-μs components (Fig. 3). This finding implicates that 3Chl decay is a significant component in this time range. Although some contribution of ΔA1− back-reaction cannot be rigorously excluded in D557KPSaB, it is most likely that the fastest components represent uncoupled antenna chlorophyll molecules, which are responsible for generation of 3Chl states even at low flash energies.

The ΔA826 absorbance change in D557A PSaB/D566A PSaB (Fig. 2F) and D557KPSaB/D666A PSaB (Fig. 2G) also shows a higher initial amplitude than the wild type (4.9 and 4.1 versus 3.75 mOD). Similar to D557KPSaB, both double aspartate mutants have a higher contribution of 3Chl to the decay kinetics than the wild type. Applying a lower flash energy to the double
aspartate mutants led to a preferential decrease of the fastest (10–15 μs) component, without completely saturating the amplitudes of the F₂/F₁-related components (data not shown). When the microsecond kinetic phases are neglected, the remaining amplitudes of 3.0 and 3.2 mOD, along with a high contribution of the components related to the [F₆/F₅] back-reaction, indicate relatively efficient electron transfer from P₇00 to F₇/F₈ in the double aspartate mutants.

**DISCUSSION**

**Relationship between Growth and Electron Transfer**—Our studies of D557PsaB and D566PsaB show that the ability of *Synechocystis* sp. PCC 6803 to grow photoautotrophically depends on the position and identity of the substituting residue. There was no phenotypic difference from the wild type when aspartate 566PsaB or 557PsaB was substituted with alanine. However, when aspartates 566PsaB and 557PsaB were both changed to alanine, the organism grew slower than the wild type. This shows that a single change to a neutral amino acid has no detectable consequence, but the cumulative effect of a double change to two neutral amino acids leads to a noticeable change in photoautotrophic growth characteristics. There was also no phenotypic difference from the wild type when aspartate 566PsaB was changed to lysine, but when aspartate 557PsaB was changed to lysine, the organism grew slowly and failed to grow at low light intensity. When aspartate 566PsaB was changed to alanine and aspartate 557PsaB was changed to lysine, the organism failed to grow at any light intensity. Hence, position 557PsaB is more sensitive to the identity of the substitututed amino acid than is position 566PsaB. These results in *Synechocystis* sp. PCC 6803 are different from those obtained with D562N mutant of *C. reinhardtii* (comparable to D557 in *Synechocystis* sp. PCC 6803), in which no PS I complexes accumulated in the mutant cells (15).

Yet, despite the effect of the studied mutations on growth characteristics, there is little or no effect on the rate or efficiency of electron transfer through F₅X. The findings on the functional properties of the aspartate mutants can be summarized as follows. First, the reduction of F₅ and F₆ in the single and double aspartate mutants occurs quantitatively at cryogenic temperatures. The ratio of reduced F₅ to F₆ after dark-freezing and illumination at 15 K is the same as in the wild type, and the interaction spectrum of F₅ and F₆ after photoaccumulation is identical to that in the wild type. Second, light-driven rates of NADP⁺ photo-reduction in the single and double aspartate mutants are comparable to those in the wild type, provided that a physiologically relevant donor (cytochrome c₉ and acceptor (ferredoxin or flavodoxin) are used. The most adversely affected mutant, D566APsaB/D566APsaB, shows rates of flavodoxin-mediated and ferredoxin-mediated NADP⁺ reduction that are within 75 and 80%, respectively, of the wild type, and the interaction spectrum of F₅ and F₆ after photoaccumulation is identical to that in the wild type. However, the most adversely affected mutant, D566APsaB/D566APsaB, shows rates of flavodoxin-mediated and ferredoxin-mediated NADP⁺ reduction that are within 75 and 80%, respectively, of the wild type, and the interaction spectrum of F₅ and F₆ after photoaccumulation is identical to that in the wild type. When the microsecond kinetic phases are neglected, the remaining amplitudes of 3.0 and 3.2 mOD, along with a high contribution of the components related to the [F₆/F₅] back-reaction, indicate relatively efficient electron transfer from P₇00 to F₇/F₈ in the double aspartate mutants.

**Differences in Chlorophyll Content in Aspartate Mutants**—Given the efficient electron transfer through F₅X, the proximal cause behind the early bleaching of the double mutants and the incapability of the double mutant D557PsaB/D566PsaB to grow photoautotrophically is not due to inefficiency in electron transfer through PS I. However, it was found that D557KPsaB and D557APsaB/D566APsaB, the two slow growing strains, had about two-thirds chlorophyll content compared with the other strains in actively dividing photoautotrophic cells (Table I). This suggests that reduced chlorophyll levels in these two strains correlate with the slow growth. The lower chlorophyll levels may reflect lower PS I levels in these cells since most of the chlorophyll in cyanobacteria is bound to PS I. The implication is that these two mutations, although having no detectable effects on electron transfer efficiency, make PS I complexes less stable. It may be further speculated that the stability of PS I is even lower in the double mutant D557KPsaB/D566APsaB, which could not grow photoautotrophically. Lower levels of PS I in these three mutants could be largely responsible for the observed growth phenotypes.

**Differences in EPR Spectra between Membranes and Detergent-isolated PS I Complexes**—When the isolated membranes are examined, the g-values and line widths of F₅X in the D557APsaB/D566APsaB and D557KPsaB/D566APsaB mutants appear identical to those in the wild type. When PS I complexes are solubilized with n-dodecyl β-maltoside, the high-field g-value (the only reliable resonance, since the middle-field resonance is often obscured by F₆/F₅) remains centered around g = 1.75–1.76, but the line width broadens considerably. We suggest that conformational flexibility accounts for the heterogeneity of F₅X in the detergent-isolated PS I complexes. The spectrum likely represents an envelope in which the g-tensor is distributed due to the presence of a relatively large number of conformers. We speculate that the presence of a salt bridge involving D557PsaB and D566PsaB with an unidentified basic residue on a nearby helix may bring conformational stability to the extramembrane region that binds F₅X. The breaking of these bonds, by substituting either a positively charged amino acid such as lysine or a neutral amino acid such as alanine, would lead to a greater flexibility of the structure. A larger number of conformations, which would reflect a high degree of flexibility of the detergent-solubilized reaction centers, would become available and would be frozen-in at low temperature, leading to an inhomogeneously broadened set of lines. Notable differences from the wild type are found in the single and double D566APsaB aspartate mutants. Although the line widths are not affected, the g-values have moved downfield, with gₓ shifting from 1.758 to 1.795. This change derives from an alteration in the electronic properties of the F₅X cluster. The alanine/alanine (D557PsaB/D566APsaB) and lysine/alanine (D557KPsaB/D566APsaB) double mutants are nearly identical to the D566APsaB single mutant, indicating that D566APsaB is a major EPR spectral determinant of the g-tensor for F₅X in the wild type. The change from aspartate to positively charged lysine appears to have a minimal effect on the spectral properties of F₅X, except for the linewidth broadening that occurs when PS I complexes are removed from the membrane using a detergent. It is interesting that substitution of aspartate with alanine, a neutral amino acid with a hydrophobic side group, rather than lysine, a positively charged amino acid, results in the shifts in the g-values of F₅X.

**Functional Role of Cysteine-adjacent Aspartates in F₅X-binding Site**—The question behind this study was whether elimination of the electrostatic contribution by nearby charged aspartate residues may induce a sufficiently large change in the thermodynamic properties of F₅X to influence the efficiency of electron transfer from A₃⁻ to F₅/F₆. The results of this study indicate that the chemical nature and the charge of the amino acids adjacent to the cysteine ligands on PsaB are not significant factors in the ability of F₅X to accept an electron from A₃⁻ or to donate an electron to F₅/F₆. It would be reasonable to conclude that either the aspartate residues are not charged or,
if they are charged, their influence on those factors that control the electron transfer efficiency through $F_X$ is negligible. Although we have not undertaken the direct measurement of the midpoint potential of $F_X$ in these mutants, relevant data indicate that, in certain instances, the midpoint potential of an intermediate electron carrier is not the major factor determining the efficiency of forward electron transfer in a chain of electron carriers. The presence of a thermodynamically uphill electron transfer step from $F_A$ to $F_B$ in PS I (27, 31) gives an example that an unfavorable Gibbs free energy change between cofactors does not preclude efficient forward electron transfer from the PS I complex to ferredoxin or flavodoxin. This step, of course, occurs in the context of an overall favorable change in Gibbs free energy from $A_0^-$ to the terminal acceptor, ferredoxin or flavodoxin. The same consideration likely applies to electron transfer among the tetraheme cytochromes in the Rhodopsseudomonas viridis reaction center (32) and among the three iron-sulfur clusters in the nickel-iron hydrogenase of Desulfovibrio gigas (33). We suggest that apart from direct redox titration, single-turnover flash efficiency monitored by $P_{700}^+$ re-reduction kinetics is the single best parameter for detecting changes in the thermodynamic properties of the electron acceptors.

Since the single-turnover flash efficiency for photooxidation of $F_X/F_B$ in certain aspartate mutants is, at best, only slightly reduced compared with that in the wild type, the equilibrium constant between $F_X$ and $F_A$ will remain no less than 10. This translates to a midpoint potential of $F_X$ in the aspartate mutants that is more electronegative than the midpoint potential of $F_B$ by at least 60 mV. Hence, there is a certain dynamic range available for a change in the reduction potential of $F_X$ from the wild-type value of $-705 \text{ mV}$ (2) without major consequence on electron distribution between the terminal iron-sulfur clusters. Direct measurement of the $F_X$ reduction potential in the $F_X$ niche mutants may be needed to resolve the basis for major differences in the electron transfer efficiencies between individual electron acceptors that were found especially in the cysteine-to-serine mutants (13), but also for the minor differences that were seen in some of the aspartate mutants (this work). Nevertheless, this work shows that the efficiency of electron transfer through $F_X$ remains relatively unaffected by the presence or absence of cysteine-proximal aspartate residues on PsaB.

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