Essential Role of a Single Arginine of Photosystem I in Stabilizing the Electron Transfer Complex with Ferredoxin*

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PsaE is one of the photosystem I subunits involved in ferredoxin binding. The central role of arginine 39 of this 8-kDa peripheral polypeptide has been established by a series of mutations. The neutral substitution R39Q leads to a 250-fold increase of the dissociation constant \( K_d \) of the photosystem I-ferredoxin complex, as large as the increase induced by PsaE deletion. At pH 8.0, this \( K_d \) value strongly depends on the charge of the residue substituting Arg-39: 0.22 \( \mu \)M for wild type, 1.5 \( \mu \)M for R39K, 56 \( \mu \)M for R39Q, and more than 100 \( \mu \)M for R39D. The consequences of arginine 39 substitution for the titratable histidine were analyzed as a function of pH. The \( K_d \) value of R39H is increased 140 times at pH 8.0 but only 5 times at pH 5.8, which is assigned to the protonation of histidine at low pH. In the mutant R39Q, the association rate of ferredoxin was decreased 3-fold compared with wild type, whereas an 80-fold increase is calculated for the dissociation rate. We propose that a major contribution of PsaE is to provide a prominent positive charge at position 39 for controlling the electrostatic interaction and lifetime of the complex with ferredoxin.

Photosystem I (PSI) is a multisubunit complex catalyzing the light-driven electron transfer from reduced plastocyanin to oxidized ferredoxin (Fd). It is found in oxygen-evolving photosynthetic organisms, including cyanobacteria, eukaryotic algae, and higher plants. In cyanobacteria, the PSI complex can be isolated as monomers or trimers (1). It is partly embedded in the membrane bilayer and constituted of 11 subunits, whose nomenclature (PsaA to PsaF and PsaI to PsaM) is derived from the gene names. The large subunits PsaA and PsaB are organized as a central heterodimeric core that houses most of the chlorophylls involved in light trapping and the redox cofactors required in electron transfer. Numerous structural details on the organization of this core have been gained from the successive improvements in the X-ray structure of PSI trimers from Synechococcus elongatus (16).

Cyanobacterial PsaE is a slightly basic, water-soluble protein that contains 69–75 residues. Although the cyanobacterial proteins are significantly smaller than their mature homologues from higher plants (91–101 amino acids), the common sequence of PsaE proteins is highly conserved (17, 18). No significant homology to other proteins has been found in sequence data bases. NMR studies in solution of the overexpressed PsaE from Synechococcus sp. PCC 7002 have shown that the isolated polypeptide is composed of a rigid, five-stranded \( \beta \)-barrel, with an extended flexible loop between the third and fourth \( \beta \)-strands (19). Recently, the representative NMR model of PsaE was fit into the electron density map of PSI. The superimposition of the NMR and X-ray model structures of PsaE reveals both to be almost identical in the core region (16).

PsaE-deleted strains of synechocystis and Synechococcus were originally reported to have no obvious phenotypic alterations (20). It was later shown that the PsaE subunit was necessary for an efficient reduction of Fd (21). Subsequently, more careful physiological investigations revealed that the growth rate of the deletion mutant from Synechococcus sp. PCC 7002 was considerably reduced at low light intensity or low carbon dioxide level (22). This phenotype suggested that the PsaE subunit might play a role in cyclic electron transport.

Apart from these functional implications, different biochemical studies pointed to a stabilizing role of this subunit on the stromal side of PSI (23, 24). Some cross-linking experiments on higher plants PSI indicated a proximity of PsaE and ferredoxin (11, 12). PsaE, -D, and -E have now been clearly localized by electron microscopy in a ridge of 30 Å height protruding from the membrane bilayer on the stromal side of PSI (13, 14). This ridge is in contact with the electron acceptor proteins, ferredoxin or flavodoxin (10, 15). PsaC occupies a central position in this ridge. This subunit carries the terminal electron acceptors of PSI, F_A and F_B ([4Fe-4S] clusters), the latter being the direct electron donor to the [2Fe-2S] center of soluble Fd. In electron density maps, PsaC is flanked by two compact regions ascribed to PsaD and PsaE, PsaD facing toward the trimer axis C3 and PsaE being the more distant and located outside (Refs. 2 and 16; Fig. 5).

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The abbreviations used are: PSI, photosystem I; \( \beta \)-DM, dodecyl \( \beta \)-d-maltoside; Fd, ferredoxin; WT, wild type; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
product between PsAE and Fd was never reported.

It becomes clear now that all the three subunits PsAC, PsAD, and PsAE participate to some extent in the formation of the ferredoxin site. A specific role of PsAD and PsAE on the association and dissociation rates of Fd has been proposed from studies on deleted mutants, with a 100-fold decreased affinity for Fd when PsAE is absent (26). More detailed information on some specific residues of PsAD and PsAC (12, 27) involved in this process are also available, but no precise amino acid of PsAE has yet been described as essential for this interaction. In the present work, we clearly show by a series of single site-directed mutations that a unique arginine at position 39 plays a central role in the PsAE-mediated Fd interaction with PSI.

**EXPERIMENTAL PROCEDURES**

**Biological Samples**—Recombinant ferredoxin was overexpressed in *Escherichia coli*. The fed1 gene of *Synechocystis* sp. PCC 6803 was cloned into a pRSET expression vector. Transformed *E. coli* BL21 (DE3) cells were grown at 30 °C in LB medium supplemented with 100 μg/ml ampicillin. A 48-h growing time is required to get a sufficient yield of Fd. The inducer isopropyl-1-thio-β-D-galactopyranoside has been found dispensable when using the LB medium (28). Cells were harvested, resuspended in a Tris-HCl buffer (50 mM Tris-HCl, 0.1 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 0.1% β-mercaptoethanol, pH 8.5), and broken in a French press. Cellular debris were sedimented at 9000 g for 10 min at 4 °C, and the whole cell extract was stepwise precipitated with ammonium sulfate (40%, 55%, and 70% saturation). Ferredoxin is purified from the last supernatant as already described (29, 30). Ferredoxin from the last purification step was concentrated by ultrafiltration and usually frozen at a concentration of 200 μM in a low ionic strength buffer (5 mM Hepes, pH 7.0). Approximately 2–5 mg of purified ferredoxin was obtained from 1 liter of induced cells.

Variant PsAE-PSI were isolated from recombinant *Synechocystis*. Thylakoid membranes were obtained from French press broken cells after extensive washing with ice-cold 20 mM Tricine, 1 mM EDTA, pH 7.8. PSI was obtained after solubilization with 1% (w/v) β-DM and purified on a sucrose density gradient (31). The upper green band consisting of highly enriched monomeric PSI particles was dialyzed against 20 mM Tricine/NaOH, pH 7.8, and 0.03% β-DM and concentrated by ultrafiltration. The chlorophyll concentration was determined in 80% acetone. The last step of the purification procedure was anion-exchange chromatography on a Mono Q column essentially as described (33). The only modification was the substitution of ammonium sulfate instead of magnesium sulfate as eluting salt to avoid any contamination by Mg²⁺, whose concentration is a critical parameter for the reduction of Fd.

**Site-directed Mutagenesis**—The *psae* gene was previously cloned into the Bluescript SK+ plasmid to produce pFB-CE (21). Codon exchanges, leading to amino acid substitutions, were carried out by site-directed mutagenesis by the method of Kunkel (32). For this technique, 20-base oligonucleotide primers were constructed such that a codon change resulted in an amino acid substitution. The desired nucleotide substitution in the genes encoding the mutant proteins was confirmed by dideoxynucleotide sequencing of the complete gene.

The strain of *Synechocystis* sp. PCC 6803 deleted for the *psae* gene (6803 Δ*psae*) was used to raise site-directed mutants of PsAE. Mutants were generated by direct transformation of the 6803 Δ*psae* with the pFB-CE constructs and grown for a 24-h period in liquid BG11. Selection of transformants was carried out by serial dilution in the presence of increasing chloramphenicol concentrations (from 1 to 30 μg/ml). Once resistance to 30 μg/ml chloramphenicol was established, the selection procedure was continued through further 8–10 rounds of liquid subcultures.

**Direct Sequencing of Polymerase Chain Reaction-amplified Genomic DNA**—Genomic DNA from liquid cultures of *Synechocystis* variants was isolated as described previously (33) and used for control sequencing. A 360-base pair fragment encompassing the full *psae* sequence was amplified using the polymerase chain reaction procedure (12), and sequencing reactions were carried out by the dideoxy termination method using the cycle sequencing kit (Roche Molecular Biochemicals).

**Protein Electrophoresis and Western Blotting**—Electrophoreses were made on mini-slab gels (Bio-Rad apparatus) using the Tris/Tricine buffer system (34). The concentration of P700 was measured in all mutated PSI samples, by flash- absorption spectroscopy at 820 nm, and a constant amount of 125 pmol of P700 was loaded for each sample after 1 h dissociation in the loading buffer at 45 °C. Electroblotting of the proteins was made overnight at 4 °C using two layers of Immobilon P membranes (Amersham Pharmacia Biotech) and a constant current of 10 mA. PsAE was probed with a polyclonal antibody (21) revealed by an alkaline phosphatase anti-mouse IgG conjugate (Sigma) and a luminescent substrate (Immuno-star, Bio-Rad). Under these conditions, variable Fd reduction by the site-directed PsaE mutants were recovered on the two successive membrane layers. This could reflect different behaviors as a result of the different mutations and a possible heterogeneity of the local electric field. It underlines the necessity of a careful survey of the blotting conditions before getting confident quantitations. Quantitation was made by adding the integrated optical densities on the two x-ray films. The yields of recovery for all six mutated Polypeptides were found between 89 and 104% of the WT, which is in the range of cumulated errors of the technique (Table I).

**Flash-absorption Spectroscopy**—Measurements were made as described previously, in cuvettes of either 1-cm or 2-mm optical path length (26, 35). 2-mm cuvettes were used for mutants of low affinity, using high Fd concentrations. Kinetic curves were corrected according to Sefi and Bottin (35), so that they correspond solely to electron transfer from the terminal acceptor of PSI (F₅₀₋₇₀, F₇₀₋₉₀) to Fd. For PSIIs having a relatively high affinity for Fd (Kₐ ≤ 1 μM), it is not possible to obtain second-order rate constants from experiments made under pseudo-first order conditions (with [Fd] >> [PSI]). In that case, determination of second-order rate constants was performed with equal concentrations of PSI and Fd (~0.24 μM), allowing use of an analytical function to fit the data (36). All kinetic measurements were performed at a constant ionic strength of 56 mM, taking into account all contributions from the buffer, sodium ascorbate (1–3 mM) and added salts. Constant buffer and MgCl₂ concentrations were used throughout the experiments (20 and 5 mM, respectively). The NaCl concentration (30 mM) was adjusted in order to provide an ionic strength of 56 mM.

Dissociation constants were determined from the dependence of the sum of first-order amplitudes upon Fd concentration. For mutants R39D and R39E of PsAE, this was not attempted for two different reasons as follows: first, the first-order amplitude was rather small and became only observable at Fd concentrations above 30 μM. Under these conditions, the second-order process is sufficiently fast and predominant to impede a precise determination of the first-order decay. Second, the first-order amplitude increases only slightly between 32 and 64 μM Fd, which is the maximal Fd concentration that was tested (R39D, from 30 to 32% of total amplitude of Fd reduction; R39E, from 19 to 23%). This suggests that a PSI-Fd complex cannot be formed in 100% of PSI, even at very high Fd concentrations. Although a precise analysis is beyond the precision of our data, it seems that the maximum (asymptotic) proportion of PSI-Fd complexes is less than 50% in both R39D and R39E.

**RESULTS**

Fd reduction by WT PSI has been studied in *Synechocystis* 6803 by flash-absorption spectroscopy at room temperature in the 460–600-nm region (see under “Experimental Procedures”) (35, 36). The wavelength of 580 nm has been selected in this work to minimize signals not related to Fd reduction on the microsecond time scale. All measurements were made by subtracting a reference signal without Fd, so that the kinetics directly represent Fd reduction (35, 36). Three different first-order phases of Fd reduction have been reported with half-times (t½) of ~500 ns and 15 and 110 μs (36). These phases correspond to electron transfer within PSI-Fd complexes prepared before the flash excitation; it may reflect the presence of three, closely related, subclasses of complexes. A second-order phase of Fd reduction has been also previously measured, which is thought to correspond to a diffusion-limited process (kₐ = 2–5 × 10⁶ M⁻¹ s⁻¹) (35). The dissociation constants Kₐ for the PSI-Fd complex were calculated from the dependence of first-order amplitudes upon Fd concentration (35, 36). A recent modification in the final purification step of PSI led to a somewhat decreased Kₐ of the PSI-Fd complex (0.22 μM instead of ~0.5 μM at pH 8.0). An equivalent decrease was not observed for the PsAE-deleted mutant (52 μM for both types of preparations).

**Fd Reduction by the Site-directed PsaE Mutants R39Q and R42Q**—A number of site-directed mutations on positively charged residues were previously made on PsAE. They were
more or less drastic, sometimes resulting in the non-integration of PsaE, but none of them led to interesting phenotypes in terms of ferredoxin reduction (21). Two highly conserved arginines, Arg-39 and Arg-42, escaped this first series of mutations and were first replaced in the present work by the neutral glutamine. Fd reduction by PSI prepared from the R39Q and R42Q mutants is illustrated in Fig. 1. At a Fd concentration of about 1 μM, Fd reduction is very similar in R42Q and WT PSI with a dominating first-order process (traces b and c, respectively). The three first-order phases previously found for WT PSI are recovered in the R42Q mutant (τ1/2 < 1, 20, and 125 μs). The Kf values for the PSI-Fd complex were in the same range for WT and R42Q (0.22 and 0.41 μM, respectively). In contrast with R42Q, Fd reduction is dramatically affected in R39Q. In the presence of ~1 μM Fd, no first-order phase is present, and only a second-order process of Fd reduction is observed on a slower time scale (not shown). However, at much larger Fd concentrations, a first-order phase becomes visible (trace e of Fig. 1, [Fd] = 16 μM). The first-order components are nevertheless small at this Fd concentration (left part of trace e), and a second-order process is still predominant (right part of trace e). This process is very similar to that previously observed with PsaE-minus PSI (trace f; Ref. 26). This close behavior is further confirmed at higher Fd concentrations up to 64 μM. The Kf for R39Q was calculated from the fit of the total first-order amplitude dependence versus Fd concentration (Fig. 2, upper part). A value of 56 μM was found, which is very close to the Kd value of 52 μM for PsaE-minus PSI. Second-order rate constants kobs were also calculated from linear fits of the observed rate of the slowest phase (Fig. 2, lower part): a 3-fold decrease in kobs is observed for R39Q (0.84 × 108 M⁻¹ s⁻¹) compared with WT (2.7 × 108 M⁻¹ s⁻¹) (Table I). A decrease of about 2-fold is also found compared with PsaE-minus (1.5 × 108 M⁻¹ s⁻¹). The different characteristics of Fd reduction at pH 8.0 are given for WT, PsaE-minus, R39Q, and R42Q in Table I, together with characteristics of the other mutants studied in the present

### Table I

Characteristics of Fd reduction by WT and PsaE site-directed mutants of PSI

| Amount of PsaE subunit detected by immunoblot | Second-order rate constant kobs | Dissociation constant Kd | τ1/2 of first-order Fd reduction |
|-----------------------------------------------|-------------------------------|-------------------------|--------------------------------|
| WT                                           | 100%                          | 10² M⁻¹ s⁻¹             | 0.22                           | <1/20/125 |
| R42Q                                         | 92%                           | 10² M⁻¹ s⁻¹             | 0.41                           | idem wt   |
| R39K                                         | 101%                          | 3.0 M⁻¹ s⁻¹             | 1.5                            | idem wt   |
| R39H                                         | 97%                           | 8.7 M⁻¹ s⁻¹             | 31                             | <1/20     |
| R39Q                                         | 91%                           | 8.4 M⁻¹ s⁻¹             | 56                             | <1/25     |
| R39D                                         | 89%                           | 0.27 M⁻¹ s⁻¹            | >100                           | idem wt   |
| R39E                                         | 104%                          | 0.18 M⁻¹ s⁻¹            | >100                           | idem wt   |
| PsaE-minus                                   | 0%                            | 1.5 M⁻¹ s⁻¹             | 52                             | <1/10     |

* a<10; this relatively large uncertainty is due to the measuring conditions (1 experiment with [PSI] = [Fd] = 0.24 μM).

|               | b | c     | d     | e     | f     |

Not measured.

* Measured from a linear fit of kobs of slow phase versus [Fd] (Fig. 2, bottom).

* Not measured, amplitude too small.
work. The amounts of the PsaE polypeptides, as found by immunoblotting (see under “Experimental Procedures”), are also reported for all mutants in Table I, showing a WT level of integration of the PsaE subunit in all site-directed mutants.

**Fd Reduction by the Site-directed PsaE Mutants R39X—**

Arginine 39 was then changed for residues of the same or reverse charge, and also for histidine, a titratable residue at physiological pH. A kinetic trace of the fast reduction process is shown for mutant R39K in Fig. 1 (trace a; [Fd] = 2 μM). In this trace, fast first-order components are observed on a 400-μs time scale, but the signal amplitude is smaller than for WT or R42Q (traces c and b, respectively) and represents only about 50% of ferredoxin reduction (~50% being due to a slower second-order process, which contributes only marginally on this time scale). A quantitative analysis of R39K kinetics shows a 7-fold decrease in affinity compared with WT (Kd = 1.5 μM; Table I) but no modification of the second-order rate constant k on (3.0 × 10^{8} \text{ M}^{-1} \text{s}^{-1}). R39H mutated PSI is much more affected and behaves like R39Q PSI (traces b and a, respectively, upper part of Fig. 3) with a 140-fold decrease in Fd affinity (Table I) and a similarly 3-fold decreased second-order rate constant (0.87 × 10^{8} \text{ M}^{-1} \text{s}^{-1}). This suggests that His-39 is in a neutral deprotonated form at pH 8.0. A more detailed study of the R39H mutant is described below.

When Arg-39 is replaced by an acidic residue (Asp or Glu), Fd reduction becomes even less efficient than in R39Q or R39H mutants (R39D, trace d of Fig. 1, Table I). At all Fd concentrations that were studied (up to 64 μM), the second-order rate constant is largely dominant, suggesting that Kd value is larger than 100 μM (more than 500-fold decrease in affinity). With both R39D and R39E mutants, a quantitative determination of Kd was not attempted (see under “Experimental Procedures”).

A semi-quantitative analysis of our data indicates that the maximum (asymptotic) proportion of PSI-Fd complexes is less than 50% in both R39D and R39E. Compared with R39Q and R39H, the second-order rate constants k on of R39D and R39E are also significantly smaller, with values of 0.27 and 0.18 × 10^{8} \text{ M}^{-1} \text{s}^{-1}, respectively (lower part of Fig. 2).

**pH Dependence of Fd Reduction by R39H PSI—**
The kinetics of Fd reduction by R39Q, R39H, and WT PSI at pH values of 5.8 and 8.0 are shown in Fig. 3. At both pH values, Fd reduction was studied in the presence of 5 mM MgCl2, and the amount of NaCl was adjusted in order to get the same ionic strength (I = 56 mM; see under “Experimental Procedures”). At pH 5.8 and in the presence of 8 μM Fd (upper part of Fig. 3), Fd reduction by WT PSI occurs almost only through fast first-order processes (trace c), whereas a second-order process is largely dominant with both R39Q and R39H PSI (traces a and b, respectively).

The lower part of Fig. 3 compares the same kinetics at pH 5.8 in the presence of 1 μM Fd. Whereas the kinetics of Fd reduction by WT and R39Q PSI (traces c’ and a’) are similar to those observed at pH 8.0, Fd reduction by R39H PSI is much faster, with a deca signal observed 300 μs after the flash (trace b’). A quantitative analysis shows that about half of the Fd reduction process is now first-order at this Fd concentration. A comparison of the first-order amplitude of Fd reduction by WT and R39H PSI was performed at different pH values between 5.5 and 9.2 and at constant ionic strength (I = 56 mM) and MgCl2 concentration (5 mM). The pH dependence of the Kd value is shown for both types of PSI in the upper part of Fig. 4. For WT PSI, the Kd value is almost constant between pH 5.8 and pH 8.0, with values ranging from 0.20 to 0.22 μM, and deviates from this value only at the extreme pH values (0.13 and 0.29, respectively, at pH values of 5.56 and 8.45). The Kd value then starts to increase faster as the pH is raised further (0.64 μM at pH 9.23). For R39H PSI, the pH dependence of Kd is much steeper than for WT and varies continuously between pH 5.8 (Kd ~1.05 μM) and pH 9.23 (Kd ~120 μM). A 30-fold increase in Kd is found when going from pH 5.8 to pH 8.0, in contrast with the constant Kd value observed for WT. This can be explained by protonation of the histidine residue occurring in this pH range. His-39 is probably highly protonated at pH
5.8, as the $K_r$ values are almost equivalent at pH 5.5 and 5.8 (1.10 and 1.05 μM, respectively).

As an additional control to ascertain the role of a protonable residue at this position, the effect of the neutral mutation R39Q was also studied at pH 5.8. Just a weak pH effect on the $K_r$ was observed for R39Q PSI (decreased from 56 μM at pH 8.0 to 24 μM), supporting a main role of the engineered histidine at position 39 in the pH dependence of the $K_r$ value.

In a simplified approach (see “Appendix” and “Discussion”), we considered that His-39 protonation does not significantly affect any other protonation events in its vicinity (either on PSI or on Fd). The effect of this histidine protonation is then interpreted relatively to the WT as follows: the ratio $\rho = K_r$(R39H)/$K_r$(WT) exhibits 2 different values when residue His-39 is either in its protonated ($\rho_2$) or unprotonated ($\rho_1$) forms. As shown under “Appendix,” this change in $\rho$ is directly related to a change in the acidity constants of His-39 when PSI is in a free form ($K_{a1}$) or associated to Fd ($K_{a2}$) (see Equation 1).

$$\rho_2/\rho_1 = K_{a2}/K_{a1} \quad \text{(Eq. 1)}$$

The ratio $K_r$(R39H)/$K_r$(WT) at any pH value will have the value shown in Equation 2.

$$\rho = \rho_1 \times (K_{a1} + [\text{H}^+])/(K_{a2} + [\text{H}^+]) \quad \text{(Eq. 2)}$$

From the fit shown in Fig. 4 (lower part), the two values of $\rho$ are 3.0 and 228 for the protonated and unprotonated forms of H39, respectively. This corresponds to a 76-fold decrease in $K_r$ which can be ascribed to protonation of His-39. The $pK$ values of His-39 found from the same fit are 5.84 and 7.69 for free and Fd-bound PSI, respectively. Although the present model is a crude approximation, it allows a satisfactory fit to our data. A significant interaction between His-39 and other protonable residues of PSI would result in a poorer fit as the $pK$ values of these residues should be involved in the pH dependence. The second-order rate constant $k_{on}$ of Fd reduction was also measured at pH 5.8 for R39H and WT PSI, for which values of 4.3 ± 1.0 and 5.0 ± 1.0 × 10^9 M⁻¹ s⁻¹ were obtained, respectively. The large increase in $k_{on}$ of R39H when going from pH 8.0 to 5.8 (5-fold compared with less than 2-fold for WT) is in line with partial protonation of the histidine side chain which is expected from the calculated $pK$ for histidine in free PSI.

**DISCUSSION**

The peripheral PsaE subunit of PSI was previously shown to participate in the formation of the electron transfer complex with Fd (21). In a recent detailed study on a PSI lacking PsaE, the role of this polypeptide on the lifetime of the complex has been clearly indicated (26). Together with PsaC and PsaD, PsaE contributes to the formation of the Fd site on PSI, the kinetic complexity of the reduction process possibly reflecting closely related subclasses in the Fd/PSI population (35). Among the numerous basic residues of PsaE, some were already mutated without significant effects on the function (21). In the present work, using a series of site-directed mutations, we establish the central role of the positive charge carried out by arginine 39. Such a central role was not found for the proximate arginine 42, as illustrated by neutral mutations of both residues to glutamine. Whereas the R42Q mutation results in almost no change of the $K_r$ (0.41 μM as compared with 0.22 μM for the WT), the same substitution of the arginine 39 has important consequences as follows: the $K_r$ for Fd binding increases up to 56 μM, close to the 52 μM value obtained when the PsaE polypeptide is deleted (26). In terms of affinity, the function of PsaE thus appears to rely exclusively on the presence of Arg-39. Nevertheless, in both PsaE-minus and Arg-39 mutants, a complex reduction kinetic is still observed at high Fd concentrations, with submicrosecond and microsecond first-order reduction phases. This suggests that enough specific interactions with the other subunits participating in the Fd site, like PsaC and PsaD, are still ensuring the formation of a functional and efficient electron transfer complex.

The primordial role of a positive charge at position 39 of PsaE was further investigated using conservative, neutral, or charge reversion mutants. The basic lysine is just partly able to substitute for arginine, as the affinity is decreased by a factor of 7, arguing for a specific role of the arginine side chain in the WT system. The effect of this conservative mutation remains moderate as compared with the 250-fold decreased affinity observed for the neutral glutamine substitution. Acidic replacements (Asp or Glu) are even more perturbing than the neutral one, inducing affinity decreases close to 3 orders of magnitude. The histidine mutation was designed to follow the effect of a positive charge formation as a function of the pH decrease, based on the likely protonation of the imidazole in the commonly used pH range of 8.0 to 5.8. With histidine in the unprotonated state (pH 8.0), the affinity for Fd ($K_r = 31 \mu M$) is close to that of the neutral R39Q mutation. Once in the protonated state (pH 5.8), histidine behaves like lysine in terms of affinity ($K_r = 1 \mu M$). As for the lysine, the protonated form of histidine imperfectly replaces arginine.

The progressive effect of His-39 protonation as a function of the pH decrease was tentatively separated from other protonation events in the PSI-Fd complex by calculating the $K_r$ ratio between R39H and WT PSI (lower part of Fig. 4). This assumes that protonation of His-39 does not affect surrounding charges contributing to the PSI-Fd complex, which is supported by the control experiments. Moreover, this assumption is in line with the available structural data on free PSI as follows: (i) in the average NMR structure of isolated PsaE (19), arginine 39 is exposed at the surface, and the closest charged residue is aspartate 20, with its carboxyl group 13 Å away from the guanidinium; (ii) most recent x-ray data (16) on PSI crystals do not indicate the proximity of other polypeptide chains of PSI. The above assumption also seems true for ferredoxin in the complex, whose residues close to position 39 do not seem strongly affected by the presence of a positive charge. This is shown by the parallel behavior of the WT and R39Q when the pH decreases from 8.0 to 5.8.

The consequences of His-39 protonation on Fd binding were thus interpreted in comparison to the WT system ($K_r$ ratios). This allowed us to calculate a 76-fold increase in affinity due to the single protonation of histidine 39. This affinity change is accompanied by a $pK$ modification of His-39, from 5.84 in free PSI to 7.69 in Fd-bound PSI. This indicates that the protonated form of His-39 is stabilized within the PSI-Fd complex compared with free PSI. Together with the effects induced by the other mutations, these results strongly suggest that the positive charge at position 39 is actually involved in interactions with Fd residues within the PSI-Fd complex. From the R39H titration, the decrease in binding energy associated to loss of the positive charge at position 39 is around 11 kJ/mol (>4 kT), which is fairly large but quite reasonable if one assumes that Arg-39 is involved in electrostatic interaction with an (or group of) acidic residue(s) at the Fd surface (see Ref. 37 for review).

Arginine is known to be quite complex in terms of length, hydrophobicity of its long aliphatic side chain, and specific electrostatic character due to the charge delocalization. Therefore, when replacing arginine by either lysine or histidine, secondary but quite important effects due to differences in solvation, hydrophobic character, number of salt bridges with residues on Fd, or steric constraints may be important at position 39 of PsaE. Further investigations are necessary to probe...
such effects.

Neutral mutation of Arg-39 can be compared with a similar mutation of the lysine 106 of PsaD, a residue previously found cross-linked to Fd (9). In the latter case, the neutral mutation K106A just induced a 2-fold decrease of the $K_d$ (12) corresponding to 1.7 kJ/mol. A multiple change of charged residues of PsaD seems required to get an effect as important as observed in the present study, second-order process of Fd reduction was also observed, and the corresponding association rate constants were determined (Table I). The mutant R39K showed almost no change in its association rate with Fd. The mutant R39Q exhibited a 3-fold decrease in its association rate with Fd which is quite low compared with the 250-fold decrease in $K_d$. From $K_d = k_{off}/k_{on}$ (26), the R39Q substitution appears to induce an approximately 80-fold increase in $k_{off}$. Concerning the substitutions by an acidic residue, an additional 4-fold decrease in the association rate with Fd is observed as compared with that found for the loss of the positive charge. Since the $K_d$ values could not be determined quantitatively, no estimation of the effects on $k_{off}$ was attempted. The role of a positive charge on position 39 therefore seems less important in guiding of Fd toward its binding site through long range electrostatic steering than in the control of the lifetime of the Fd-Psi complex, in line with the model predicted from the study of the PsaE-deleted mutant (26).

APPENDIX

Model 1 below describes the binding equilibrium of Fd to PSI in two different forms corresponding to the protonation of a single residue (histidine at position 39 of PsaE in the present case), with dissociation constants $K_{d1}$ and $K_{d2}$ when PSI is protonated (PSIH$_{H^+}$) or not (PSI). The acidity constants of the protonable residue are $K_{a1}$ and $K_{a2}$ for free and Fd-bound PSI, respectively. (Fd$\cap$PSIH$_{H^+}$) and (Fd$\cap$PSI) are the complexes that are formed when the residue is protonated or not, respectively (see Equations 3–6).

\[
K_{d1} = \frac{[\text{PSIH}_{H^+}] [\text{Fd}]}{[\text{PSIH}_{H^+} \cap \text{PSI}]} \\
K_{d2} = \frac{[\text{PSI}] [\text{Fd}]}{[\text{PSI} \cap \text{PSI}]} \\
K_{a1} = \frac{[\text{PSI}] [H^+]}{[\text{PSIH}_{H^+}]} \\
K_{a2} = \frac{[\text{PSI}]}{[\text{PSIH}_{H^+}]} \\
\]

MODEL 1

\[K_{d1} = [\text{PSIH}_{H^+}] [\text{Fd}] / [\text{PSIH}_{H^+} \cap \text{PSI}] \quad \text{(Eq. 3)}\]
\[K_{d2} = [\text{PSI}] [\text{Fd}] / [\text{PSI} \cap \text{PSI}] \quad \text{(Eq. 4)}\]
\[K_{a1} = [\text{PSI}] [H^+] / [\text{PSIH}_{H^+}] \quad \text{(Eq. 5)}\]

\[K_{a2} = [\text{PSI}] / [\text{PSIH}_{H^+}] \quad \text{(Eq. 6)}\]
Arginine 39 of PsaE

\[
K_d = [\text{Fd} \cap \text{PSI}] / [H^+] / [\text{Fd} \cap \text{PSI}] \tag{6}
\]

with \([H^+]\) being the proton concentration.

The above dissociation and acidity constants are linked by Equation 7.

\[
K_d / K_{a_1} = K_d / K_{a_1} \tag{7}
\]

At any given pH, the following identity can be easily checked (Equation 8).

\[
([\text{PSI}] + [\text{PSI}]_1) \times ([\text{Fd}]_1 + [\text{Fd}]_2) = K_d \times (K_{a_1} + [H^+]) + [H^+] / (K_d + [H^+]) \tag{8}
\]

The left expression of Equation 8 corresponds to the dissociation constant \(K_d\) at any pH value, which can be therefore calculated as a function of the \(K_{a_1}\), \(K_{a_2}\) and pH values. An equivalent formula has been detailed for the ionization equilibria of an enzyme with two different conformations (detailed in Ref. 39).

During a pH titration of \(K_d\), several PSI and Fd residues are expected to change their protonation states, leading to changes in \(K_d\) for the PSI-Fd complex. In order to titrate separately the influence of the protonation state of histidine 39 in the R39H mutant of PsaE, the change in \(K_d\) ascribed to other changes should be subtracted. For doing that, we consider the \(pH\) dependence of the ratio \(p = K_d(\text{R39H}) / K_d(\text{WT})\) instead of \(K_d(\text{R39H})\). This assumes that one can separate, during the pH titration, the effect of the charge on His-39 from all other changes in protonation states that could affect Fd binding. In other words, the \(p \delta\) associated with His-39 and other residues (on PSI and ferredoxin) are separated into additive components. This is certainly a highly oversimplified model that is not of general validity. However, this model seems operative to get a quantitative idea of the importance of the positive charge on residue 39 for Fd binding. The experimental observations and the control experiments that allow us to validate this model are further detailed under “Results” and “Discussion.”

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