Identification of the Amino Acids Involved in the Functional Interaction between Photosystem I and Ferredoxin from *Synechocystis* sp. PCC 6803 by Chemical Cross-linking*

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Cécile Leong, Pierre Sétif, Bernard Lagoutte, and Hervé Bottin

From the Service de Bioénergétique/URA 1390 (CNRS), Département de Biologie Cellulaire et Moléculaire, C. E. A.

Saclay, 91191 Gif sur Yvette Cedex, France

Ferredoxin isolated from the cyanobacterium *Synechocystis* sp. PCC 6803 has been chemically cross-linked to purified photosystem I from the same organism. The reaction was catalyzed by N-ethyl-3-(3-dimethylaminopropyl)carbodiimide in the presence of N-hydroxyoxysuccinimide. A short reaction time and neutral pH values can be used in the presence of the two reagents, ensuring the integrity of both of the proteins and the iron-sulfur cluster of the ferredoxin. The only covalent complex detected comprised ferredoxin and the photosystem I (PSI)-D subunit, as identified by antibodies probing after electrophoresis. Electron paramagnetic resonance measurements of this covalent complex have shown that the cross-linked ferredoxin was entirely photoreducible by photosystem I and that the molar ratio of ferredoxin to PSI was close to 1. Extensive sequencing of the peptides obtained after proteolysis of the purified cross-linked product led to the identification of a covalent bond between glutamic acid 93 of ferredoxin and lysine 106 of the PSI-D subunit.

Soluble ferredoxin is a small (10 kDa) and strongly acidic protein containing a [2Fe-2S] cluster. A high degree of conservation is found among ferredoxins that have a similar function (Rogers, 1987; Matsubara and Wada, 1988; Knaff and Hirasa, 1991). The ferredoxin sequence of *Synechocystis* sp. PCC 6803 has been recently determined (Bottin and Lagoutte, 1992). The PSI reaction center is a large complex (approximately 300 kDa) containing at least 12 different subunits and about 100 chlorophyll molecules (Goebel and Bryant, 1991). The three-dimensional structure of both partners has been determined either at high resolution, for four different cyanobacterial ferredoxins (Tsukihara et al., 1981, 1996; Rypniewski et al., 1991; Jacobson et al., 1993), or at low resolution, in the case of PSI from the cyanobacterium *Synechococcus* (Krauss et al., 1993). PSI is embedded in the photosynthetic membrane but also contains several protruding peripheral subunits. The redox components involved in the first steps of photoinduced electron transfer are located in the reaction center core, which is largely hydrophobic and comprises the large subunits PSI-A and PSI-B (Goebel and Bryant, 1991). Following light excitation, an electron moves from the primary donor (a dimer of chlorophyll a known as P700) to a series of electron acceptors. One of these (F,) is a [4Fe-4S] cluster with a very low redox potential, located at the interface between PSI-A and PSI-B. The terminal acceptors of PSI, two [4Fe-4S] clusters named F_A and F_B, are carried by a small subunit named PSI-C. Electron transfer then proceeds presumably directly from F_A or (and) F_B to soluble ferredoxin. Ferredoxin also has many different soluble partners, such as FNR, ferredoxin-thioredoxin reductase, nitrite reductase, and glutamate synthase (Knaff and Hirasawa, 1991).

On the cytoplasmic side of the membrane, two peripheral subunits of PSI were found to play some role in the interaction with soluble ferredoxin. Cross-linking experiments using EDC suggested that the PSI-D subunit is involved in the docking of ferredoxin (Zanetti and Merati, 1987; Zilber and Malkin, 1988; Andersen et al., 1992a), and a role for PSI-E in the interaction of PSI with ferredoxin (Andersen et al., 1992b; Sonoike et al., 1993; Rousseau et al., 1993) and FNR (Andersen et al., 1992b) has also been suggested. The functional properties of the electron transfer between PSI and ferredoxin have been investigated (Hervas et al., 1992; Rousseau et al., 1993) and indicate that the interaction between both partners is stabilized by electrostatic interactions, in agreement with cross-linking experiments. Furthermore, the psa-D gene from *S. sp.* PCC 6803 has been cloned and sequenced (Reilly et al., 1988), and its inactivation has shown the importance of PSI-D subunit in both the activity and the stability of the PSI reaction center (Chitnis et al., 1989).

We modified the cross-linking conditions by using both N-hydroxyoxysuccinimide ester (sulfo-NHS) and N-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The use of these two reagents enhances the yield of the cross-linking reaction while preserving the integrity of both PSI and ferredoxin. This together with the high stability of ferredoxin from *S. sp.* PCC 6803 allowed the isolation of a functional, one to one, covalent complex between PSI and ferredoxin.

**EXPERIMENTAL PROCEDURES**

*Chemicals—n-dodecyl β-D-maltoside, N-hydroxyoxysuccinimide, and N-ethyl-3-(3-dimethylaminopropyl)carbodiimide were purchased from Biomol (Germany), Fluka (Swiss), and Pierce Chemical Co., respectively. Other chemicals were purchased from Sigma.*

*Protein Purification—Ferredoxin was isolated from *S. sp.* PCC 6803 and purified according to Bottin and Lagoutte (1992). Thylakoid membranes (1 mg/ml) were solubilized with 1% (w/v) n-dodecyl maltoside in 20 mM MES, pH 6.5, 10 mM CaCl_2, 10 mM MgCl_2 for 30 min at 4 °C as described by Rögner et al. (1990). The solubilized material was loaded onto a sucrose density gradient consisting of four layers (8, 24, 32, and 40% sucrose (w/v)) in the same buffer as above containing 0.5 M mannitol.*
nitol and 0.04% (w/v) n-dodecyl β-maltoside then centrifuged for 16 h in a fixed angle rotor (150,000 x g at 4°C, Beckman Ti 45). The upper green band, consisting in highly enriched PSI particles, was dialyzed against 20 mM Tricine/NaOH, pH 7.5, 0.04% n-dodecyl β-maltoside and concentrated by ultrafiltration (Centriprep 100, Amicon).

Chemical Cross-linking Reaction—PSI particles (0.2 mg chlorophyll/ml) were incubated in the presence or absence (control PSI particles) of 10 μM ferredoxin with 2 mM EDC, 2 mM sulfo-NHS in 20 mM HEPES/NaOH, pH 7.5, 5 mM MgCl2, and 0.03% n-dodecyl β-maltoside for 30 min at room temperature. The reaction was stopped by the addition of 0.5 M ammonium acetate to a final concentration of 0.1 M. The PSI-ferredoxin covalent complex was then washed with 20 mM Tricine/NaOH, pH 7.8, containing 0.03% n-dodecyl β-maltoside by ultrafiltration with a Centriprep 100 filter (Amicon) to eliminate chemical reagents and free ferredoxin and to concentrate the PSI particles. The washing procedure was repeated several times.

PSI-D-Ferredoxin Complex Extraction and Purification—The cross-linked sample was diluted to 0.25 mg of chlorophyll/ml with 5 mM Tris/Cl, pH 8, 1 mM EDTA, and the peripheral subunits PSI-D and PSI-E dissociated by the addition of NaSCN at a final concentration of 2 M. After 1 h of gentle stirring, PSI-E and PSI-D-ferredoxin complex were separated from the remaining core complex by ultrafiltration over a Centriprep 100 filter and then concentrated to a 100 M final volume. The concentrated extract was size-fractionated by high pressure liquid chromatography on a TSK 2000 column (LKB) equilibrated with the sample buffer 0.1 M Tris/Cl, pH 8, 2 mM NaSCN, 1 mM EDTA. The purity of the cross-linked PSI-D-ferredoxin complex was tested at each step of the purification by electrophoresis and immunoblots using a variety of antibodies.

Peptide Mapping—Cysteine residues of ferredoxin, in the PSI-D-ferredoxin complex, were derivatized by 4-vinylpyridine as described (Friedman et al., 1970). The sample was then cleaved after glutamic acid residues by staphylococcal V8 proteinase (Houmard and Drapeau, 1972). Peptides were purified by reverse-phase high pressure liquid chromatography on a Delta-Pak C-18 column (Waters), eluted by a linear gradient of acetonitrile (0–55% (v/v)) in water containing 0.1% (v/v) trifluoroacetic acid. A pulsed liquid sequencer 477A from Applied Biosystems was used for all sequence determinations.

Electrophoresis—Minislab gel electrophoresis (Bio-Rad apparatus) was used for protein characterization. Sample buffer and acrylamide to bisacrylamide ratio were as described (Laemmli, 1970), with a final concentration of acrylamide of 66 in the stacking gel and of 15% in the separating gel. The gels were run at 20 mA each tube at a chlorophyll concentration of about 700 pg/ml. Comparisons were made between the covalent PSI-ferredoxin complex and a control PSI preparation, which has been treated as for cross-linking (same incubation and washing steps) but in the absence of ferredoxin. Samples were prepared as described above and concentrated by ultrafiltration (Centriprep 100, Amicon) to a chlorophyll concentration of about 800 μg/ml. EPR spectra were also recorded for purified ferredoxin (about 100 μM) after reduction by 10 mM sodium dithionite in 50 mM Tricine/NaOH, pH 8.0. Double integration of the signals was performed for quantitative analysis using the software provided by Bruker, under conditions (30 K, 2 mW) that ensured that none of the iron-sulfur EPR signals were saturated by the microwave irradiation. The saturation properties may depend on the conditions of ambient pH or redox potential, this was therefore checked for all conditions used for quantitative analysis. A radical signal around g = 2.002–2.005 is always present and was eliminated before integration.

FIG. 1. SDS-polyacrylamide gel electrophoresis and immunodecoration of PSI reaction center treated with EDC and NHS in the presence or in the absence of ferredoxin. Lanes 1, 3, 5, and 7, PSI reaction center treated with EDC and NHS; and 8, PSI reaction center treated with NHS in the presence of ferredoxin. Proteins were stained with Coomassie Blue (lanes 1 and 2), immunoreacted with anti-ferredoxin antibodies (lanes 3 and 4), with anti-PSI-D antibodies (lanes 5 and 6), or with anti-PSI-E antibodies (lanes 7 and 8).

Different reox posing and illumination conditions were used. White light illumination was provided by an 800-watt tungsten-iodine lamp whose beam was filtered to remove infrared light (water cuvette/Calflex filter). To reach a maximum reduction of the iron-sulfur centers, samples were prepared in the presence of an excess of sodium dithionite (20 mM) in Tricine/NaOH, pH 9.5. They were then illuminated at room temperature for 30 s (~200 mW/cm2 of white light) before freezing under illumination using a solid CO2-ethanol bath (198 K). Similar illumination conditions (~100 mW/cm2) were also used with samples prepared at pH 8 (50 mM Tricine/NaOH) in the presence of 5 mM sodium ascorbate and 300 μM DPIP. For some of the quantitative spin determinations, sodium dithionite at pH 9.5 was also used, and the illumination at room temperature was performed with a lower light intensity (~50 mW/cm2) and was followed by 2 min of dark adaptation at room temperature before freezing in the dark. This latter treatment results in the complete reduction of Fx and Fh, while reducing less than 15% of Fh. For all of the samples in which the number of spins was evaluated quantitatively at 30 K, EPR spectra were also recorded at 10 K to obtain a precise estimate of the amount of reduced Fx.

RESULTS

Cross-linking of PSI Particles and Ferredoxin—Before cross-linking studies, the effects of the reagents were tested on ferredoxin at 2 pH values. Native ferredoxin was incubated with 2 mM EDC and 2 mM sulfo-NHS, in either 20 mM MES at pH 6.5 or 20 mM HEPES at pH 7.5. After 30 min at 20°C, the reaction was stopped by the addition of ammonium acetate (final concentration, 0.1 M). At pH 7.5, ferredoxin remained colored and exhibited the same absorption spectrum as native ferredoxin with characteristic peaks at 330, 422, and 462 nm. In contrast, ferredoxin incubated at pH 6.5 became colorless and no longer exhibited the characteristic absorption peaks of native ferredoxin. The bleaching observed at this weakly acidic pH value is indicative of the loss of the [2Fe-2S] cluster. The former pH conditions (20 mM HEPES at pH 7.5) were therefore chosen for the cross-linking reactions detailed below.

Control PSI particles were first treated under these conditions, without ferredoxin, and analyzed by SDS gel electrophoresis. The electrophoretic profile (Fig. 1, lane 1), is similar to that of a native PSI, showing three major bands at 18, 17, and 13 kDa and a faint diffuse one of lower molecular mass previously attributed to PSI-C. The 13- and 17-kDa components are, respectively, PSI-E and PSI-L, and the 18-kDa component was shown to result from the superposition of PSI-D and PSI-F (Rousseau et al., 1993). When the same PSI treatment was conducted in the presence of ferredoxin, two additional bands appeared with molecular masses of 34 and 32 kDa, and the intensity of the 18-kDa band (including PSI-D) showed a considerable decrease (Fig. 1, lane 2). Immunological assays
using antibodies against ferredoxin, PSI-D subunit, or PSI-E subunit allowed the precise identification of the new bands in the 30–40-kDa range. Whereas the 34-kDa band was recognized both by anti-ferredoxin and anti-PSI-D antibodies (Fig. 1, lanes 4 and 6), the band at 32 kDa was only detected by anti-PSI-D antibodies (lane 6). This band could correspond to a multimere of the PSI-D subunit. A faint band is still present at the original position of the PSI-D subunit (18 kDa) (Fig. 1, lane 6), although at a considerably lower level than in the control experiment (lane 5). Two bands were also revealed using specific antibodies against PSI-E subunit (lanes 7 and 8). The major band was located at about 13 kDa in both control (lane 7) and cross-linked samples (lane 8); this corresponds to the PSI-E subunit. Another band at 28 kDa (lane 8) of weaker intensity was not labeled by anti-PSI-D or anti-ferredoxin antibodies. We thus conclude that a specific cross-linking reaction occurred with a good yield between ferredoxin and the PSI-D subunit.

**EPR Spectroscopy**—The presence of ferredoxin in the cross-linked complex was also analyzed by comparing the EPR spectra with those observed in a control PSI preparation that had been similarly treated but in the absence of ferredoxin (see “Experimental Procedures”). A preliminary experiment showed that the treated control PSI preparation exhibits EPR characteristics, which are indistinguishable from an untreated PSI preparation. When normalized to the chlorophyll concentration, the amplitudes and the g-values of the (FA-, FB-) and Fx- signals were identical in both samples indicating that no degradation of the iron-sulfur centers of PSI occurs during the treatment with EDC and NHS. In the following experiments, EPR spectra were systematically compared between these two different kinds of samples (control and cross-linked) submitted to the same redox and illumination treatments.

Two different methods were used for identifying unambiguously the signal of the reduced [2Fe-2S] cluster of ferredoxin in the covalent complex. In a first series of experiments, the samples were prepared under highly reducing conditions and frozen under illumination. Control experiments performed at 10 K demonstrated that iron-sulfur centers FA, FB, and Fx were reduced by such a pretreatment, as it is expected (data not shown). Fig. 2 shows the EPR spectra of these samples recorded at 50 K. At this temperature the bound iron-sulfur centers of control PSI are barely detectable, as can be seen from the spectrum obtained with the control sample (Fig. 2b). A small signal is observed, but it can be attributed to both baseline distortion and the broad lines of the fast-relaxing [4Fe-4S] clusters of PSI. However, in addition to these last signals, the covalent complex exhibits the characteristic spectrum of reduced ferredoxin, with g-values of 2.05, 1.96, and 1.89 (Fig. 2a). This can be seen more clearly when the difference spectrum (Fig. 2, a and b) is compared with the spectrum of reduced soluble ferredoxin recorded under the same conditions (Fig. 2c).

The detection of the [2Fe-2S] cluster of ferredoxin and the almost complete loss of the [4Fe-4S] EPR signal at 50 K are in agreement with previous observations, showing that the [2Fe-2S] clusters can be observed in their reduced state at a much higher temperature than the [4Fe-4S] clusters (Rupp et al., 1978). In a second series of experiments, control and cross-linked complexes were poised at a redox potential, where ferredoxin is reduced (E_{red} = -420 mV) (Bottin and Lagourette, 1992), and FA and FB remain oxidized (E_{ox} = -540/580 mV) (Ke et al., 1973). This was achieved by preparing the samples in darkness in the presence of an excess of sodium dithionite at pH 7.0. The EPR spectra of these samples were recorded at 30 K, to observe signals from both [2Fe-2S] and [4Fe-4S] clusters (Fig. 2, inset). In this case, only the EPR signal of ferredoxin is clearly visible in the cross-linked sample (Fig. 2d) with no signal observable in the control (Fig. 2f).

The relative stoichiometry of ferredoxin to the (FA, FB) clusters, present in the covalent complex, was evaluated by double integration of the EPR signals recorded at 30 K. At this temperature, both signals can be observed under nonpower-saturating conditions. Measurements were made for samples prepared under redox conditions, which allowed the reduction of ferredoxin (if present), FA and FB while maintaining Fx in the oxidized state (less than 15% reduced as seen by control experiments at 10 K, see “Experimental Procedures”). The number of spins observed in the covalent complex is approximately 1.5 times that observed in the control PSI (Table I). Assuming that two spins are present per reaction center in the control PSI (FA and FB, neglecting the small amount of Fx present), this indicates that one additional spin is found per reaction center in the covalent complex, attributable to reduced ferredoxin. It was also found that the presence of reduced Fx had a negligible influence on the spin quantitation. This is most probably due to the fact that the Fx signal is very broad at 30 K, this iron-sulfur center relaxing much faster than FA and FB (Guigliarelli et al., 1989).

The ability of PSI to photoreduce ferredoxin within the covalent complex was verified by illumination in the presence of exogenous electron donors to P700+ (DPNP reduced by ascorbate). Under these redox conditions, ferredoxin remains oxidized in the dark. Illumination was performed at room temperature and the samples frozen under illumination (see “Experimental Procedures”). The EPR spectra resulting from this treatment are shown in Fig. 3. For both the control PSI (trace b) and the covalent complex (trace a). For the control sample, the spectrum obtained corresponds to the complete reduction of both FA and FB. This was verified by a spectrum recorded at 10 K (data not shown) in which the g-values (1.89, 1.92, 1.94, and 2.05) are characteristic of the coupled signal due to the simultaneous reduction of both FA and FB. There was also a small signal attributable to Fx- (less than 15% reduced). Fig. 3 shows the spectrum recorded at 30 K. At this tempera-
EPR conditions are: microwave power, 2 mW, modulation amplitude, 10 G, average of 2 scans. Highly reducing conditions are: the samples were poised with 20 mM sodium dithionite at pH 8.5 and were submitted, to 30 s of white light illumination at room temperature (100 mW/cm²) before freezing under illumination in the presence of 5 mM sodium ascorbate and 300 µM DPIP at pH 8.0. a and b, the two spectra were identical, and the double integrals were given an arbitrary value of 100. d, average of 3 experiments. The spectrum and the integrated intensities were identical with those for the control incubated in the presence of EDC/NHS (not shown).

|                | Control PSI | Cross-linked complex | Cross-linked complex |
|----------------|-------------|----------------------|----------------------|
|                | PSI-ferredoxin | PSI-ferredoxin | PSI-ferredoxin |
| Highly reducing | 100 (±5)     | 150 (±9)             | 129 (±2)             |
| Illumination at | 100 (±5)     | 147 (±9)             | ND                   |
| room temperature |             |                      |                      |

ND, not determined.

**FIG. 3.** EPR spectra of control and cross-linked PSI under moderately reducing conditions after freezing under illumination. Main panel, the control (trace b) and the cross-linked complex (trace a) were studied at pH 7.0 (200 mM MOPS) in the presence of 5 mM sodium ascorbate and 300 µM DPIP after illumination at room temperature and during freezing. Inset, difference between trace a and trace b. Instrument settings are temperature, 30 K; microwave power, 30 mW; modulation amplitude, 10 G; average of 2 scans.

**FIG. 4.** Elution profile of the isolated PSI-D-ferredoxin covalent complex hydrolyzed by the V8 proteinase. Purified PSI-D-ferredoxin covalent complex was hydrolyzed by V8 proteinase under conditions where it mainly cleaves after glutamic residues. The mixture of polypeptides was resolved by reverse-phase high pressure liquid chromatography on a C18 column and eluted by a linear gradient of acetonitrile (0-55% (v/v)) in water containing 0.1% (v/v) trifluoroacetic acid, at a flow rate of 1 ml/min. The peptide elution was recorded at 214 nm. The peak labeled with an arrow eluting at 35% acetonitrile corresponds to the covalent complex partially or not hydrolyzed by V8 proteinase. The peak labeled with a star includes the cross-linked peptides, the sequences of which are noted under the chromatogram. Amino acids circled (Glul-93 of ferredoxin and Lys-106 of PSI-D subunit) have been identified as the amino acids cross-linked by EDC and NHS during the interaction between ferredoxin and PSI particles.
Functional Cross-linking of Ferredoxin to Photosystem I

starting at Ala-31, with an initial amount of 200 pmol. This amount was more than two times the amount found for the two other sequences, allowing unambiguous determination of these last ones. Only three uncertainties could not be avoided (Fig. 5, dotted lines), but they did not affect the identification of the cross-linking site. We clearly identified the C terminus of ferredoxin, from Thr-89 to Tyr-96, and a PSI-D peptide from Val-93 to Glu-109 as the two cross-linked peptides. Comparable initial amounts, close to 65 pm, were found for these two peptides, as expected for a cross-linked product. The missing residues were Glu-93 for the ferredoxin and Lys-106 for PSI-D; we thus concluded that these two residues were cross-linked in the complex. Indeed, the amide bond between the ε-amino group of Lys-106 and the 6-carboxyl group of Glu-93 is not cleaved during Edman reaction, and the resulting dipeptide cannot be identified using the normal analytical procedure.

For the other peaks, the constant lower amount of most of the sequenced PSI-D peptides as compared with the ferredoxin peptides was an unexpected result. This could be due to the presence of internal cross-links in the PSI-D subunit resulting in a poor access and thus a lower efficiency of the V8 proteinase. The larger peak (labeled with an arrow) eluting at a high concentration of acetonitrile (35%) could be the result of such a poor proteolysis.

DISCUSSION

The involvement of the PSI-D subunit in the interaction between PSI and soluble ferredoxin has already been inferred from previous cross-linking studies, all using EDC as the cross-linking reagent. The first studies were made with spinach proteins. In these studies, only PSI-D was found to be cross-linked to ferredoxin. Though demonstrating a specific interaction of ferredoxin with the PSI-D subunit, these experiments suffered from poor functional characterizations, which were either not described quantitatively (Zilber and Malkin, 1988) or which indicate that only 50% of the PSI particles were cross-linked (Merati and Zanetti, 1987; Zanetti and Merati, 1987). A more recent study was described using spinach or barley ferredoxin together with PSI or thylakoids from barley (Andersen et al., 1992a). It was found that subunits PSI-E and PSI-H, in addition to PSI-D, were cross-linked to ferredoxin. The yield of the reaction was rather low, with less than 50% of PSI-D subunit cross-linked, as determined from protein staining of electrophoresis gels. We tried to overcome the difficulty of obtaining cross-linking products with both specificity and high yield by the combined use of sulfo-NHS and EDC. In the present study with S. 6803, a specific cross-linking with the PSI-D subunit was found. EPR spin quantitation showed that under these conditions, the cross-linking is almost complete. Protein staining also confirms that only a very small amount of PSI-D was not cross-linked. These results can be presumably attributed to the fact that addition of sulfo-NHS to EDC allows the cross-linking reaction to occur at a pH value close to neutrality with a high rate, thus providing good conditions to preserve the integrity of ferredoxin and PSI. EDC activates carboxyl groups to form O-acylurea derivatives, which are able to react readily with primary amino groups resulting in the formation of peptide bonds. Upon the addition of NHS, O-acylurea intermediates of EDC are activated in the form of NHS-ester, which is far more resistant to hydrolysis (Staros et al., 1986). This addition of NHS thus permits the use of a shorter reaction time at neutral pH, limiting the reactions of EDC with cysteine or tyrosine residues (Carraway and Koshland, 1968; Carraway and Tripelett, 1970).

Clear evidence for electron transfer within a covalent complex is described in a few cases in the literature, i.e., involving the following complexes: ferredoxin-FNR (Zanetti et al., 1984; Colvert and Davis, 1988; Pueyo et al., 1992); flavodoxin-FNR (Walker et al., 1990; Pueyo and Gomez-Moreno, 1991); cytochrome c55 ferredoxin I (Dolla et al., 1989); cytochrome c reaction centers of purple bacteria (Rosen et al., 1985). In the present study, EPR experiments have shown that the PSI reaction center contained in the PSI-ferredoxin covalent complex is active and capable of photoreducing all of the cross-linked ferredoxin. The molar ratio of PSI to ferredoxin in the cross-linked complex has been measured by two different methods. EPR measurements revealed the presence of one [2Fe-2S] ferredoxin cluster functionally associated to each PSI reaction center. Protein sequencing of the purified ferredoxin-PSI-D complex also revealed a ratio of one to one for each protein. These results suggest that ferredoxin is cross-linked to PSI particles in a position identical or very similar to that occurring in vivo.

The lack of ferredoxin reduction at 198 K in the covalent complex can be interpreted in several ways, which are not exclusive one from the other. It may be due 1) to a slow rate of electron transfer at any temperature, implying that the two partners are complexed in an unfavorable arrangement or that some molecular movements involved in the electron transfer are hindered in the covalent complex; 2) to restriction of some molecular movements as the temperature is lowered, leading to some modification of the relative redox potentials of the two partners and thus making the electron transfer energetically less favorable (see e.g. Ortega and Mathis (1993) for a recent discussion of such effects); 3) to a large activation energy for the electron transfer step. Further work is needed to distinguish between these different possibilities.

It has been recently found that the PSI-E subunit plays an important role for the fast reduction of ferredoxin by PSI (Rousseau et al., 1993). However, in accordance with the first cross-linking studies of ferredoxin and PSI (Zanetti and Merati, 1987; Zilber and Malkin, 1988) and in contradiction with a
We have identified the amino acids involved in the cross-linking of ferredoxin and to PSI as glutamic acid 93 from ferredoxin and lysine 106 from the PSI-D subunit. Most of the peptides recovered after V8 proteolysis have been sequenced, with residues linked in close electrostatic regions of ferredoxin and PSI-D that are highly conserved in linking of ferredoxin and to PSI as glutamic acid 93 from ferredoxin.

Furthermore, Lys-106 is part of a sequence that appears to be involved in the interaction of ferredoxin with the PSI reaction center and with FNR. This is consistent with observations that show the PSI-ferredoxin complex is unable to photoreduce NADP+ (Zanetti et al., 1987; Andersen et al., 1992a). This would preclude the formation of a tertiary complex between PSI, ferredoxin, and FNR, the existence of which was deduced from measurements of rotational diffusion (Wagner et al., 1982), unless such a complex would allow some limited reorientation of ferredoxin compatible with efficient electron transfer with PSI and FNR. Recently, DePascale et al. (1993) found that some other amino acids, also located around the iron-sulfur cluster of ferredoxin, could be involved in the interaction with FNR.

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Note Added in Proof—It is interesting to note that the C-terminal acidic cluster of ferredoxin has recently been found to be essential for efficient electron transfer to FNR (Hurley et al., 1993).

REFERENCES

Andersen, B., Koch, B., and Scheller, H. V. (1992a) Physiol. Plant. 84, 154-161
Andersen, B., Scheller, H. V., and Meijer, B. L. (1992b) FEBS Lett. 311, 169-173
Bottin, H., and Lagoutte, B. (1992) Biochim. Biophys. Acta 1101, 48-56
Carraway, K. L., and Koshland, D. E., Jr. (1968) Biochim. Biophys. Acta 160, 272-277
Carraway, K. L., and Triplett, R. B. (1970) Biochim. Biophys. Acta 200, 564-566
Chitnis, P. R., Reilly, P. A., and Nelson, N. (1989) J. Biol. Chem. 264, 18381-18385
Colvert, K. K., and Davis, D. J. (1988) Photochem. Photobiol. 47, 231-245
DePascale, A. R., Jelisavcic, I., Ackermann, F., Koppelen, W. H., Hirasawa, M., Knaff, D. B., and Boasgard, H. R. (1993) Protein Science 2, 1126-1135
Dolla, A., Guerlesquin, F., Bruschi, M., Guigliarelli, B., Asso, M., Bertrand, P., and Biochem. Biophys. Acta 975, 385-398
Friedman, M., Krull, L. H., and Cavins, J. F. (1970) J. Biol. Chem. 245, 3866-3871
Goldbeck, J. H., and Bryant, D. A. (1991) in Current Topics in Bioenergetics (Lee, C. P. ed.) Vol. 16, pp. 83-137, Academic Press, New York
Guigliarelli, B., Guillaussier, J., Bertrand, P., Gayda, J. P., and Stief, P. F. (1989) J. Biol. Chem. 264, 602-6025
Hervás, M., Navarro, J. A., and Tolín, G. (1992) Photoschem. Photobiol. 56, 319-324
Hirasawa, M., Chang, K.-T., and Knaff, D. B. (1991) Arch. Biochem. Biophys. 286, 171-177
Housman, J. D., and Drapeau, G. R. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3506-3509
Hurley, J. K., Salmon, Z., Meyer, T. E., Fitch, J. C., Casanovitch, M. A., Markley, J. L., Cheng, H., Xia, B., Chae, Y. K., Medin, M., Gomez-Moreno, C., and Tolín, G. (1993) Biochemistry 32, 9345-9354
Jacobson, B. L., Chae, Y. K., Markley, J. L., Raymont, I., and Holder, H. M. (1993) Biochemistry 32, 6788-6793
Ke, B., Hansen, B. E., and Beinert, H. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 2941-2945
Knaff, D. B., and Hirasawa, M. (1991) Biochim. Biophys. Acta 1056, 93-125
Krause, N., Hinrichs, W., Witt, I., Fromme, P., Pitzkow, L., Dauter, Z., Betzel, C., Wilson, K. S., Witt, H. T., and Sienerger, W. (1993) Nature 361, 326-330
Laemmli, U. K. (1970) Nature 227, 680-685
Lagoutte, B., and Vallon, (1992) J Ferredoxins of Spirulina platensis (Tsukihara et al., 1981), Aphanathece sacrum (Tsukihara et al., 1990), and Anabaena sp. PAC 7120 (Rypniewski et al., 1991; Jacobson et al., 1993) is involved in cross-linking FNR. Thus the same sequence appears to be involved in the interaction of ferredoxin with the PSI reaction center and with FNR. This is consistent with observations that show that the PSI-ferredoxin complex is unable to photoreduce NADP+ (Zanetti et al., 1987; Andersen et al., 1992a). This would preclude the formation of a tertiary complex between PSI, ferredoxin, and FNR, the existence of which was deduced from measurements of rotational diffusion (Wagner et al., 1982), unless such a complex would allow some limited reorientation of ferredoxin compatible with efficient electron transfer with PSI and FNR. Recently, DePascale et al. (1993) found that some other amino acids, also located around the iron-sulfur cluster of ferredoxin, could be involved in the interaction with FNR.

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The Cyanobacteria (Pay, F. and Van Baalen, C., eds) pp. 35-67, Elsevier, Amsterdam
Ragot, M., Nixon, P. J., and Diner, B. A. (1990) J. Biol. Chem. 265, 6189-6196
Rosen, D., Okamura, M. Y., Alpers, E. C., Valkirs, G. E., and Fober, G. (1983) Biochemistry 22, 335-341
Rousseau, F., Stief, P., and Lagoutte, B. (1993) EMBO J. 12, 1755-1765
Rupp, H., Rie, K. K., Hall, D. D., and Campsack, B. (1979) Biochim. Biophys. Acta 537, 255-269
Rynpiewski, W. R., Breiter, M. M., Wiesbahn, G. H., B. H.-M., Markley, J. L., Raymont, I., and Holder, H. M. (1991) Biochemistry 30, 4126-4131
Schägger, H., and Von Jagow, G. (1987) Anal. Biochem. 166, 366-379
Snooke, K., Hatanaka, H., and Kato, S. (1993) Biochim. Biophys. Acta 1141, 67-73
Starrs, J. V., Wright, R. W., and Swingle, D. M. (1986)Anal. Biochem. 156, 220-222
Tsukihara, T., Fukuyama, K., Nakamura, M., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., Hase, T., and Nishimura, H. (1991) Biochem. 190, 1765-1773
Tsukihara, T., Fukuyama, K., Mizushima, M., Horioka, T., Kusunoki, M., Katsube, Y., Hase, T., and Matsubara, H. (1990) J. Mol. Biol. 216, 399-410
Wagner, H., Carrillos, N., Junge, W., and Vallespo, N. H. (1992) Biochim. Biophys. Acta 680, 317-330
Walker, M. C., Pueyo, J. J., Gomez-Moreno, C., and Tolín, G. (1990) Arch. Biochem. Biophys. 281, 76-83
Zanetti, G., and Merati, G. (1987) Eur J. Biochem. 169, 143-146
Zanetti, G., Aliverti, A., and Curti, B. (1984) J. Biol. Chem. 259, 6153-6157
Zanetti, G., Morelli, D., Ronchi, S., Negri, A., Aliverti, A., and Curti, B. (1988) Biochim. Biophys. Acta 971, 3753-3763
Zilber, A. L., and Malkin, R. (1988) Plant Physiol. 88, 810-814

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