Biochimica et Biophysica Acta, 1056 (1991) 93–125

ADONIS 000527289100056E

BBABIO 43316

Review

Ferredoxin-dependent chloroplast enzymes

David B. Knaff and Masakazu Hirasawa

Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX (U.S.A.)

(Received 27 July 1990)

Key words: Ferredoxin; Chloroplast enzyme; Photosynthesis

Contents

Correspondence: D.B. Knaff, Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409-1061, U.S.A.

I. Introduction

The [2Fe-2S]-containing protein, ferredoxin, functions as the central molecule for distributing electrons originating from the oxidation of water during oxygenic photosynthesis to a number of ferredoxin-dependent enzymes in the chloroplasts of higher plants and algae and also in cyanobacteria. This review will begin with a brief summary of the current state of our knowledge of the essential properties of higher plant, algal and cyanobacterial ferredoxins. As a full understanding of the role of ferredoxin in oxygenic photosynthesis not only requires an outline of how reduced ferredoxin is utilized, but also requires a description of how reduced ferredoxin is made available, the second section of the review will summarize what is known of the mechanism by which the Photosystem I reaction center reduces ferredoxin. With this information in hand, a description of four of the best-characterized chloroplast ferredoxin-dependent enzymes (ferredoxin : $NADP^+$ oxidoreductase; ferredoxin : nitrite oxidoreductase; glutamate synthase and ferredoxin : thioredoxin reductase) will be presented. Emphasis will be placed on the electrostatic interaction of these enzymes with ferredoxin.

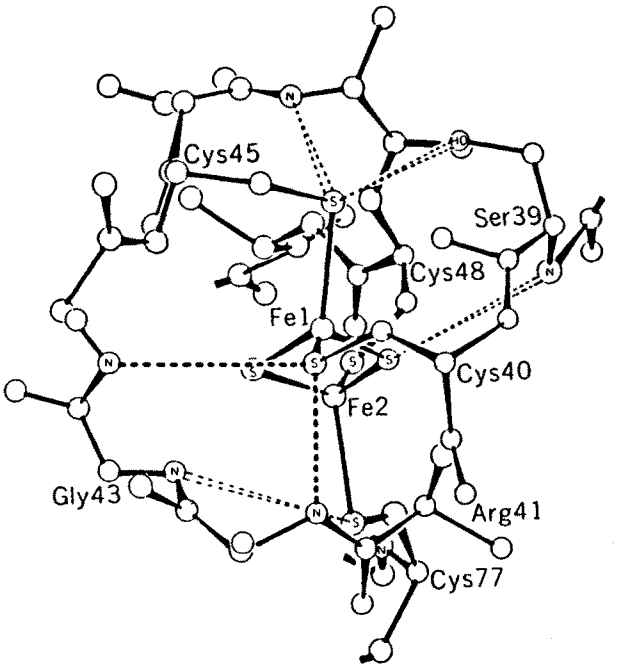

Fig. 1. The [2Fe-2S] cluster of *S. platensis* ferredoxin. The two irons of the cluster are designated Fe1 and Fe2. Two sulfurs are inorganic sulfide and the other sulfurs coordinated to iron arise from cysteines 40, 45, 48 and 77. From Ref. 20a.

II. Ferredoxin

Ferredoxins are widely distributed in biological systems, being found in microorganisms, plants and animals [1]. Although the name ferredoxin was first applied to a bacterial iron-sulfur protein [2], by 1962 it had become clear that proteins isolated from higher plants and known by a variety of names [3–8] were related to the bacterial protein through the presence of similar iron-sulfur prosthetic groups. Thereafter, the name ferredoxin began to be applied to both bacterial and plant proteins. The functional relationship of the plant and bacterial ferredoxins became clear when it was demonstrated that a bacterial ferredoxin could replace the plant protein as an electron carrier in $NADP^+$ photoreduction catalyzed by chloroplast membranes [8], even though it was established later that the bacterial ferredoxin contained a [4Fe-4S] prosthetic group rather than the [2Fe-2S] cluster found in chloroplast ferredoxins [1,9]. The discussion in this section will be limited to a treatment of the chemistry of 'plant-type' ferredoxins, but readers interested in the chemistry of 'bacterial-type' ferredoxins should consult Refs. 10–13.

Ferredoxins found in the chloroplasts of higher plants and algae and in cyanobacteria are monomeric proteins with molecular masses of approx. 12 kDa [14–18]. The proteins contain, per molecule of protein, two non-heme irons and two inorganic (acid labile) sulfides arranged in a single cluster. Each of the irons is tetracoordinate, with the two inorganic sulfides bridging the irons and four cysteinyl sulfurs, two per iron, also serving as ligands [1,9]. Although there are no X-ray crystal structures available for any higher plant or algal ferredoxins, the three-dimensional structure of the homologous ferredoxin from the cyanobacterium *Spirulina platensis* is known [19,20]. The structure of the [2Fe-2S] cluster of *S. platensis* ferredoxin, shown in Fig. 1, is likely to be very similar to those of plant and algal ferredoxins, as all these ferredoxins have very similar optical, circular dichroic and electron paramagnetic resonance (EPR) spectra [1,9].

Ferredoxins serve as low-potential, one-electron carriers with E_m values ranging from -310 to -455 mV [9,21,22]. Oxidized forms of [2Fe-2S]-containing ferredoxins exhibit no EPR spectrum because the presence of two anti-ferromagnetically coupled, high-spin ($S = 5/2$) ferric irons gives the cluster a net spin state of $S = 0$ [14,22]. The reduced proteins exhibit characteristic EPR spectra (values of 2.03, 1.94 and 1.86 are typical for g_z, g_y and g_x) due to the net $S = 1/2$ spin state of the cluster that arises from the addition of one electron to the $S = 0$ oxidized cluster [22]. EPR spectra of reduced parsley ferredoxin in which the naturally occurring isotopes of iron and inorganic sulfide were replaced by isotopes that have a nuclear magnetic moment indicate that the single electron taken up on reduction is substantially delocalized over the entire cluster [23].

Amino acid sequences are available for more than 40 [2Fe-2S]-containing, higher plant ferredoxins [24]. These proteins contain from 93 to 99 amino acids in a single

polypeptide chain, with alanine being present at the amino terminus in almost all cases. Sequences are also available for more than 20 [2Fe-2S]-containing, cyanobacterial ferredoxins [25] and for several [2Fe-2S]-containing ferredoxins from red algae [26]. There is a conservation of the spacing between the four cysteines that serve as ligands to the cluster irons. (The four cysteines that serve as cluster ligands are located at residues 39,44,47 and 77 in the spinach ferredoxin sequence – Ref. 24.) The region in the vicinity of the [2Fe-2S] cluster is the most highly conserved portion of ferredoxin, with regions of the protein remote from the cluster showing considerably more variability (Table I, Refs. 24, 27, 28). Ferredoxins isolated from plants, algae and cyanobacteria are, in general, highly acidic proteins. As can be seen from Table I, several of the acidic amino acids found in ferredoxins occur at highly conserved positions. The possible role of these conserved acidic residues in the interaction of ferredoxin with ferredoxin-dependent enzymes will be discussed below.

The tertiary structure of ferredoxin from the cyanobacterium *S. platensis* is known to 0.25 nm resolution [19,20]. Because of the amino acid sequence homology of this cyanobacterial ferredoxin with plant and algal ferredoxins [24], it is likely that the tertiary structures of other ferredoxins will show considerable similarities to that of *S. platensis*. Fig. 2 illustrates the overall tertiary structure of the cyanobacterial ferredoxin. A detailed view of the network of hydrogen bonds to amino acids that serve as ligands to the iron-sulfur cluster is presented in Fig. 1. Fig. 3 shows the location of the acidic amino acid groups of spinach ferredoxin on a model in which the spinach ferredoxin amino acid sequence has been juxtaposed on the *S. platensis* ferredoxin structure [29].

In higher plants and algae the gene coding for ferredoxin is a nuclear gene [30] and ferredoxin is synthesized on cytoplasmic ribosomes in the form of a precursor protein with an amino-terminal sequence that is required for targeting the precursor for import across

TABLE I

Amino acid sequences of ferredoxins

The amino acid position numbers are based on the spinach sequences and the alignment was chosen to maximize homologies. The positions of the four cysteine residues that serve as ligands to the [2Fe-2S] cluster are indicated by *. The spinach sequence is from Ref. 15 and the sequences for the proteins from wheat, parsley, alfalfa, elder, taro and *S. platensis* are from Refs. 24a–f, respectively.

	5	10	15	20	25	30
Wheat (Triticum asetium)	ATYKV	KLVTP	EGEVE	LEVPD	DVYIL	DAAEE
Spinach (Spinacia oleracea)	AAYKV	TLVTP	TGNVE	FQCPD	DVYIL	DAAEE
Parsley (Petroselinum sativum)	ATYNV	KLITP	DGEVE	FKCDD	DVYVL	DQAEE
Alfalfa (Medicago sativa)	ASYKV	KLVTP	EGTQE	FECPD	DVYIL	DHAEE
Elder (Sambucus nigra)	ASYKV	KLITP	DGPQE	FECPD	DVYIL	EHAEE
Taro (Colocasia esulenta)	ATYKV	KLVTP	SGQQE	FQCPD	DVYIL	DQAEE
Cyanobacteria (Spirulina platensis)	ATYKV	TLINE	AEGINE	TIDCD	DDTYI	LDAAE

35	40	45	50	55	60	65	70
EGIDL	PYSCR	AGSCS	SCAGK	LVSGE	IDQSD	QSFLD	DDQME
EGIDL	PYSCR	AGSCS	SCAGK	LKTGS	LNQDD	QSFLD	DDQID
EGIDI	PYSCR	AGSCS	SCAGK	VVSGS	IDQSD	QSFLD	DEQMD
EGIVL	PYSCR	AGSCS	SCAGK	VAAGE	VNQSD	GSFLD	DDQIE
LGIDI	PYSCR	AGSCS	SCAGK	LVAGS	VDQSD	QSFLD	DEQIE
VGIDL	PYSCR	AGSCS	SCAGK	VLVGD	VDQSD	GSFLD	DEQIG
EAGLDL	PYSCR	AGACS	TCAGT	ITSGT	IDQSD	QSFLD	DDQIE
	*	*	*				

75	80	85	90	95	
AGWVL	TCHAY	PKSDI	VIETH	KEEEL	TA
EGWVL	TCAAY	PVSDV	TIETH	KEEEL	TA
AGYVL	TCHAY	PTSDV	VIETH	KEEEI	V
EGWVL	TCVAY	AKSDV	TIETH	KEEEL	TA
EGWVL	TCVAY	PKSDW	TIETH	KEEEL	TA
EGWVL	TCVAY	PVSDG	TIETH	KEEEL	TA
AGYVL	TCVAY	PTSAC	TIKTH	QEEGL	Y
	*				

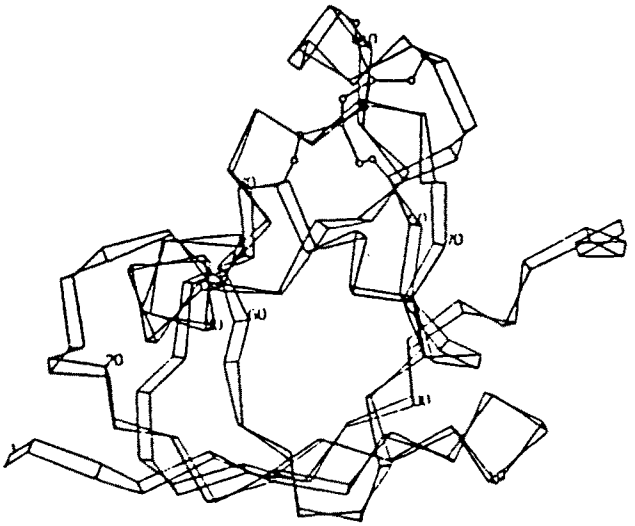

Fig. 2. The tertiary structure of *S. platensis* ferredoxin. Adjacent α-carbons are connected by ribbons. The [2Fe-2S] cluster atoms, the cysteine sulfur ligands to the cluster and the β-carbons of these cysteines are shown as open circles. From Ref. 20.

the chloroplast membrane into the stromal space [30]. Transport of the ferredoxin precursor across the chloroplast envelope requires ATP as an energy source [30]. The signal sequence is subsequently cleaved by a specific protease to generate the mature peptide form [31]. The insertion of the [2Fe-2S] cluster into the apoprotein in vivo probably occurs via an enzyme-catalyzed reaction that takes place in the chloroplast stroma [32]. However, in vitro studies indicate that the cluster can spontaneously assemble if the apoprotein, ferrous iron and inorganic sulfide are present under reducing conditions [9].

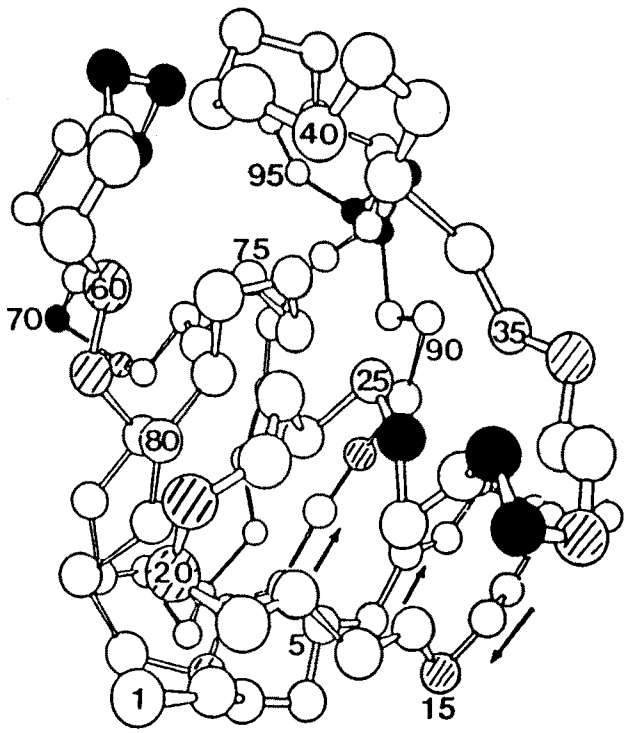

Fig. 3. Location of amino acid residues with carboxylate side-chains in spinach ferredoxin. The amino acid sequence of spinach ferredoxin was superimposed on the structure of *S. platensis* ferredoxin. Only the α-carbons are shown and those of aspartate and glutamate residues are either in balck or are cross-hatched. The residues in black represent possible sites of interaction with FNR and other ferredoxin-dependent plant enzymes. From Ref. 29.

III. Ferredoxin reduction by Photosystem I

III-A. Photosystem I function and primary reactions

The reduction of ferredoxin in chloroplasts is accomplished by the Photosystem I reaction center, which catalyzes the transfer of one electron from the cuprous form of the copper-containing protein plastocyanin to ferredoxin [33]. Although one does not usually think of the Photosystem I reaction center as a ferredoxin-dependent enzyme, this multi-subunit, integral membrane complex does function to catalyze electron flow to ferredoxin, albeit in a fashion that requires photons as an additional substrate. (The quantum yield for the Photosystem I-mediated reduction of ferredoxin is one electron transferred per absorbed photon. Ref. 34.) In *Euglena,* some cyanobacteria, and some algae under conditions of copper depletion, a soluble *c*-type cytochrome substitutes for plastocyanin as the electron donor for ferredoxin photoreduction [35,36].

The initial charge separation reaction of the Photosystem I reaction center involves the photooxidation of P700 (probably a dimer of chlorophyll *a*) and the reduction of the primary acceptor, A_0 (probably a chlorophyll *a* monomer), with a characteristic time of approx. 10 ps [37–43]. The electron is subsequently transfered from reduced A_0 to the secondary electron acceptor, A_1, in a time ranging from 40 to 200 ps depending on the preparation used [37,38,40,41]. The chemical identity of A_1 is still a matter of some controversy [37,44]. Considerable evidence, including results obtained from resolution and reconstitution experiments, has been gathered supporting the identification of A_1 as one of the two phylloquinone (vitamin K-1) molecules found in the Photosystem I reaction centers of plants and cyanobacteria [37,43,45–57]. Reduced-*minus*-oxidized optical difference spectra of A_1 are consistent with A_1 being a phylloquinone [45,46], as are EPR spectra of photoreduced A_1 [43,47–49,57]. However, some experiments in which phylloquinone is destroyed by ultraviolet irradiation showed little effect on the primary photochemistry of Photosystem I, suggesting that phylloquinone is neither A_1 nor an obligate electron carrier in Photosystem I [58,59]. One possible explanation for this disagreement concerning the role of phylloquinone may be that electron transfer through Photosystem I occurs via different pathways at physio-

logical temperatures and at the cryogenic temperatures often used to study the early events of Photosystem I [59a]. Clearly, additional research will be necessary if the chemical identity of A_1 and the role of phylloquinone in the early electron transfer events of Photosystem I are to be established in an unambiguous manner.

III-B. Iron-sulfur center X

The time for electron transfer from reduced A_1 to the next electron acceptor of the Photosystem I reaction center has been estimated to be in the range from 15 to 200 ns [46,60]. There is now agreement that the component that functions as the electron acceptor after A_1 in Photosystem I is a [4Fe-4S] iron-sulfur cluster, referred to as Center X or F_x [33,37,61–70]. F_x was originally identified by the EPR spectrum of the reduced cluster (*g* values of 2.08, 1.88 and 1.78 are usually observed – Ref. 61, 62) and more recently obtained reduced-*minus*-oxidized optical difference spectra are also consistent with the identification of F_x as an iron-sulfur cluster [63]. The availability of Photosystem I reaction center 'core protein' preparations that contain F_x as the only iron-sulfur cluster [63] has allowed the unambiguous identification of this center as a [4Fe-4S] cluster by X-ray edge spectroscopy (EXAFS) and Mössbauer spectroscopy [64,65], confirming the conclusion of earlier Mössbauer studies conducted with less purified Photosystem I preparations [66]. The conclusion that F_x is a [4Fe-4S] cluster is consistent with the observation that resolved Photosystem I core protein preparations that contain this center as the only iron-sulfur prosthetic group contain approx. 4 inorganic sulfides and 4 non-heme irons per P700 [67,68]. Oxidation-reduction titrations of F_x indicate that it is among the strongest known reductants present in biological systems. (Presumably the earlier acceptors in the Photosystem I reaction center, A_0 and A_1, have more negative E_m values than that of F_x, but these E_m values have not yet been measured.) Oxidation-reduction titrations of F_x, using the EPR signal of the reduced cluster to monitor the redox state of the cluster, indicate an E_m value of -705 mV for F_x [69]. A less negative E_m value of -670 mV was calculated for F_x from the E_h dependence of the magnitude of absorbance changes associated with the back reaction between $P700^+$ and F_x^- [63]. The reason for the discrepancy between these two values for the E_m of F_x is not known. It is possible that local coulombic effects can affect the oxidation-reduction properties of F_x, as its E_m value is apparently shifted to -610 mV when other reduced iron-sulfur clusters (the clusters referred to as Centers A and B, see below) normally present during determinations of the E_m value of F_x are removed from the Photosystem I reaction center [63].

Analyses of EPR signals [70] support the conclusion based on non-heme iron and acid-labile sulfide content [67,68] that F_x is present in the Photosystem I reaction center in amounts equimolar to P700. F_x is present, along with acceptors A_0 and A_1, in Photosystem I core protein preparations that contain only two peptides [64,67], one copy each [71–75] of the products of the *psaA* and *psaB* genes [76–83]. In photosynthetic eukaryotes, both the *psaA* and *psaB* genes are located on chloroplast DNA [76–81]. The two largest, homologous subunits of the Photosystem I reaction center, the *psaA* and *psaB* proteins, thus constitute a heterodimer that contains P700 and the three earliest electron acceptors associated with Photosystem I. The demonstration that F_x is a [4Fe-4S] cluster requires that the heterodimer peptides provide 4 cysteine residues to act as ligands to the cluster irons [9]. An examination of amino acid sequences for these two large subunits of the Photosystem I reaction center, deduced from the gene sequences [76–83], indicates that higher plant *psaB* proteins contain only two cysteines and higher plant *psaA* proteins contain four cysteines, only two of which are highly conserved. These two highly conserved *psaA* cysteines are found in a completely conserved, 12 amino acid long portion of the protein that is not only identical in all eight known *psaA* sequences, but is also identical to the sequence found in *psaB* that contains the two cysteines found in this protein [68]. It thus has been

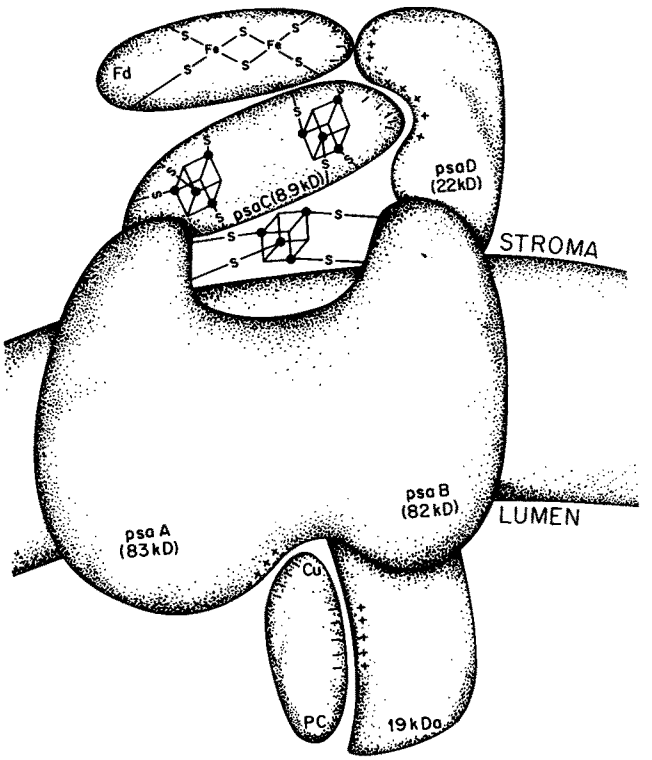

Fig. 4. Proposed topography for the Photosystem I reaction center. The model, courtesy of Prof. Richard Malkin, is reprinted with permission from Ref. 83a.

proposed [63,71,72,72a] that the [4Fe-4S] cluster of F_x bridges the two subunits of the heterodimeric Photosystem I reaction center core, with the *psaA* and *psaB* proteins each providing two cysteine ligands to the cluster. This likely bridging arrangement of F_x between the two large subunits of the Photosystem I reaction center is shown in Fig. 4 [83a]. The molecular masses of the two largest subunits given in Fig. 4, 82 kDa and 83 kDa, are those calculated from the amino acid sequences, rather than the lower apparent molecular masses calculated from migration distances during polyacrylamide gel electrophoresis under denaturing conditions [63,67,68,71–74,84].

III-C. Iron-sulfur centers A and B

Fig. 4 also shows the probable arrangement of three smaller subunits of the Photosystem I reaction center with molecular masses of 9, 19 and 22 kDa, respectively. The three subunits are coded for by the *psaC* [68,77,78,85–87], *psaF* [88,89] and *psaD* [90–93] genes, respectively. In photosynthetic eukaryotes, the *psaC* gene is located on the chloroplast DNA, while the *psaD* and *psaF* genes are located on nuclear DNA [85–92]. There is now good agreement that the 9 kDa *psaC* gene product contains the two [4Fe-4S] clusters, known either as Centers A and B, or F_A and F_B, that serve as the final electron acceptors within the Photosystem I reaction center [68,77,78,85–87,94,95]. Amino acid sequences for the *psaC* gene product indicate the presence of eight conserved cysteine residues with the appropriate spacing for two sets of four cysteines that function as ligands to [4Fe-4S] clusters in iron-sulfur proteins for which X-ray crystal structures are available (Fig. 5). The EPR spectrum, non-heme iron and acid-labile sulfide content of the isolated *psaC* protein also are consistent with the presence of two [4Fe-4S] clusters being present in the protein [68,77,78,85–87,94,95]. Earlier EPR and Mössbauer spectroscopic investigations of F_A and F_B had also suggested that both were [4Fe-4S] rather than [2Fe-2S] clusters [96,97]. It has recently been demonstrated that the purified spinach *psaC* protein can restore F_A and F_B function to a cyanobacterial Photosystem I core protein from which the *psaC* protein had been removed by treatment with chaotropic agents [94,95], conclusively establishing the subunit location of these two iron-sulfur clusters.

The original identification of F_A and F_B as electron acceptors associated with the Photosystem I reaction center was based on EPR spectra of Photosystem I

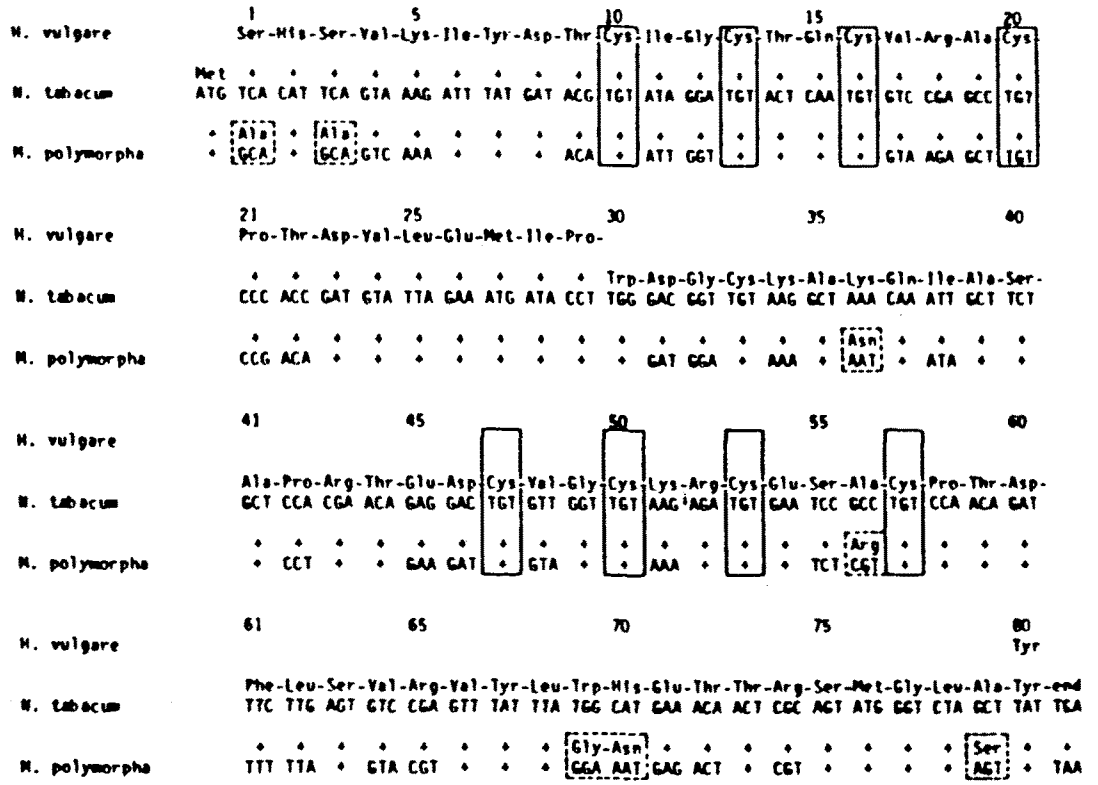

Fig. 5. Comparison of amino acid sequences for the *psaC* gene products. The sequence for the barley (*H. vulgare*) protein, shown using three-letter abbreviations for the amino acids, comes from amino acid sequencing of the isolated peptide. The sequences for tobacco (*N. tabacum*) and liverwort (*M. polymorpha*) are shown as the gene sequences, with the DNA bases grouped as codons. Positions where the amino acid deduced from the *N. tabacum* and *M. polymorpha* gene sequences and the amino acid found in the barley protein are identical and are indicated by a +. Differences in amino acid sequences are indicated by dashed boxes. Cysteine residues with appropriate positions for serving as ligands to the two [4Fe-4S] clusters are shown in solid boxes. From Ref. 68.

components that could be photoreduced at cryogenic temperatures [98–105]. This early work identified two iron-sulfur clusters, each present in an amount equal to that of P700 in the Photosystem I reaction center [70,100,102]. Oxidation-reduction titrations revealed the presence of two different clusters: F_A with an E_m value of approx. −540 mV [101,103] and g values 2.05, 1.94 and 1.86 [98,101,103–105] and F_B with an E_m value of approx. −590 mV [101,103] and g values of 2.07, 1.93 and 1.88 [101,103–105]. Magnetic interactions between the two clusters, presumably made possible by their close proximity, result in shifts in the g values observed for one reduced cluster when the other cluster becomes reduced [101,103–106]. Although the initial observations made with spinach Photosystem I indicated that only F_A accumulated in the reduced form during illumination at cryogenic temperatures [98–100], subsequent experiments, some conducted with Photosystem I preparations isolated from other organisms, revealed that reduced F_B could also accumulate during low-temperature illumination [102,104,105,107]. These observations, and others [108,109], have resulted in some uncertainty as to whether the electron transfer pathway from F_x to soluble ferrodoxin proceeds linearly through F_A and F_B or whether there is a branch in the pathway at this point [37]. For the sake of simplicity, a linear scheme for the light-dependent transfer of electrons from plastocyanin to ferredoxin, carried out by Photosystem I is given in Fig. 6, with the order of the components assigned on the basis of both kinetic studies and E_m values. It should be kept in mind that this linear scheme may overly simplify the actual pathway of electron flow. The detailed kinetics of the electron transfer steps from F_x^- to F_A and/or F_B are not known. Optical absorbance changes presumed to be associated with the reduction of these centers (originally attributed to a component referred to as P430 [110–112] have been used to argue that electrons leaving P700 arrive at one of the *psaC* protein prosthetic groups in less than 100 ns. However, it is possible that the spectral changes originally attributed to the reduction of F_A and/or F_B may actually arise from the photoreduction of F_x [113].

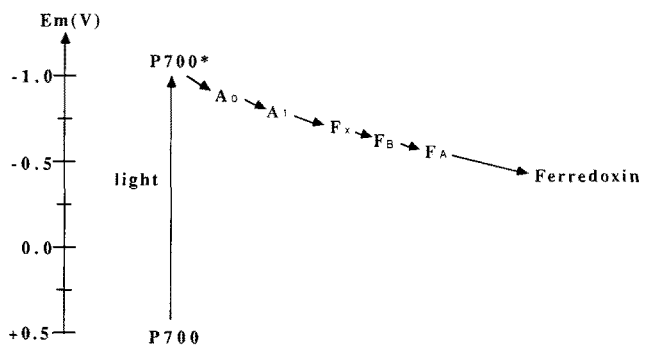

Fig. 6. Electron transfer in the Photosystem I reaction center. The vertical positions of the electron carriers indicate their estimated E_m values.

III-D. Docking proteins

The reduction of ferredoxin by the Photosystem I reaction center probably requires that this soluble, stromally-located, iron-sulfur protein dock, even if transiently, with at least one reaction center subunit. Recent cross-linking experiments have implicated the 22 kDa, *psaD* gene product in binding ferredoxin to the Photosystem I reaction center [114,115]. Cross-linking of spinach ferredoxin to spinach thylakoid membranes or to Photosystem I reaction center preparations with the water soluble carbodiimide *N*-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) resulted in the appearance of new peptide, detectable after electrophoresis, with an apparent molecular mass of approx. 40 kDa that was recognized by an antibody raised against ferredoxin [114,115] and by an antibody raised against the *psaD* gene product [115]. Similar results were obtained with Photosystem I in the cyanobacterium *Synechococcus* sp. PCC 6302, where the *psaD* gene product (M_r = 17.5 kDa in cyanobacteria compared to 22 kDa in spinach) could be specifically cross-linked to spinach ferredoxin [116].

The cross-linked adduct between ferredoxin and the spinach Photosystem I reaction center was able to catalyze the photoreduction of equine cytochrome *c* (a reaction known to be ferredoxin-dependent) in the absence of exogenous ferredoxin [114,115], suggesting that ferredoxin had been cross-linked to its physiological binding site and could be photoreduced. The observation that the cross-linked adduct cannot catalyze $NADP^+$ photoreduction [114,115] is consistent with the hypothesis that the photoreduced, cross-linked ferredoxin is immobilized and unable to diffuse to its binding site on ferredoxin : $NADP^+$ oxidoreductase (FNR). However, experiments to be discussed below suggest the possibility that ferredoxin may be able to form a ternary complex between the Photosystem I reaction center and FNR. If this were to be the case, an alternative explanation for the inability of the cross-linked ferredoxin/reaction center adduct to photoreduce $NADP^+$ must be provided.

A comparison of the amino acid sequences of four *psaD* proteins [116] shows striking conservation of positively charged amino acids, particularly of lysine residues (Fig. 7). As ferredoxin is a highly acidic protein (see above), it is likely that several of these conserved *psaD* lysines are involved in the electrostatically-stabilized binding of ferredoxin to the Photosystem I reaction center under physiological conditions and serve as amino group donors in the EDC-dependent cross-linking reaction with ferredoxin. The amino acid sequence of the amino-terminal portion of the tomato *psaD* pro-

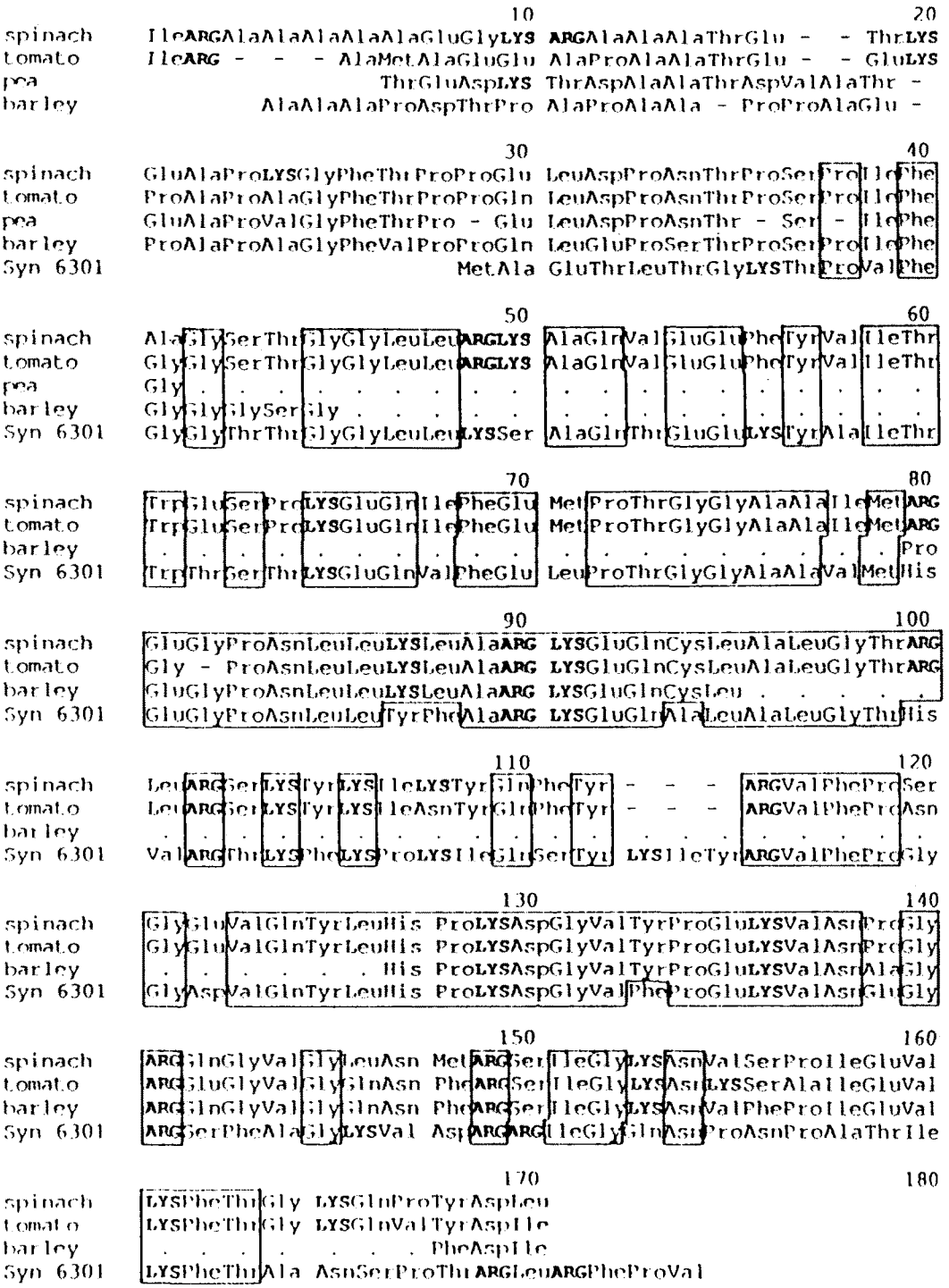

Fig. 7. Amino acid sequences of *psaD* gene products. Dashes represent alignment spaces inserted to maximize homologies and dots represent undetermined sequence portions. Conserved regions are surrounded by boxes. From Ref. 116.

tein precursor does not contain any regions characteristic of proteins that are imported across the thylakoid membrane [90]. The protein is rather polar and contains no obvious regions that could form a membrane-spanning helix [90]. These observations, and the results of labeling studies with a membrane-impermeant probe [117], are consistent with the proposed location of the protein on the stromal-facing side of the Photosystem I reaction center (Fig. 4).

Experiments similar to those described above for the *psaD* gene product have been used to clarify the role of the *psaF* gene product in binding plastocyanin to the Photosystem I reaction center [118–120]. Cross-linking of spinach plastocyanin to the Photosystem I reaction

center of spinach [118,119] or of maize mesophyll [120] chloroplasts using EDC produces only a single cross-linked adduct, identified with immunoblots, in which plastocyanin is bound to the 19 kDa product of the *psaF* gene. It should be mentioned that some uncertainty exists concerning the true function of this subunit, as there is one report that it may function as part of the light-harvesting antenna system associated with Photosystem I [120a]. It is possible that some of the ambiguity in assigning a function to this peptide may arise from the fact that there are at least two peptides with similar molecular masses present in the Photosystem I reaction center.

As described above, in some organisms a soluble *c*-type cytochrome replaces plastocyanin as the direct electron donor to $P700^+$. It has been demonstrated that in the cyanobacterium *Synechococcus* sp. PCC 6301, this soluble electron donor, cytochrome *c*-553, is specifically cross-linked to a 17 kDa subunit of the Photosystem I reaction center [116]. Although the N-terminal amino acid sequence of the *Synechococcus* sp. PCC 6301 17 kDa protein does not show any significant homology to that of the plastocyanin-binding, spinach *psaF* gene product, the observation that an antibody raised against the spinach protein recognizes the cyanobacterial protein [116] indicates that the two proteins are structurally related. The complete amino acid sequence of the cyanobacterial *psaF* gene product would be of interest from a comparative point of view.

The cross-linking of plastocyanin to the Photosystem I reaction center in spinach appears to conserve the orientation of plastocyanin found under physiological conditions, as the cross-linked plastocyanin can reduce photooxidized $P700^+$ with a halftime identical to that seen in situ [119]. The observation that plastocyanin, pretreated to block side-chain carboxyl residues, cannot be cross-linked to the spinach *psaF* protein [118] suggests that negatively charged groups on plastocyanin are involved in the interaction of this soluble electron donor with the *psaF* protein. Studies of the effect of ionic strength on the rate of $P700^+$ reduction by plastocyanin suggest that plastocyanin and its Photosystem I docking target are oppositely charged [121]. As plastocyanin will be negatively charged at all pH values likely to be found in the thylakoid lumen [121,122], its docking protein should provide a region of net positive charge. Predictions made from the amino acid sequence of the spinach *psaF* protein indiciate that it contains a net excess of seven positively charged amino acid residues [88,119]. The N-terminal leader sequence of the *psaF* protein precursor is consistent with transport of the protein across the thylakoid membrane [89], suggesting a location for this Photosystem I reaction center component facing towards the thylakoid lumenal space (Fig. 4) where plastocyanin is known to be oxidized [121].

In summary, we now have a reasonably complete picture of the number and identity of the electron-carrying prosthetic groups of the Photosystem I reaction center, of the peptides to which they are bound, and of the sequence of electron transfer steps involved in the photoreduction of soluble ferredoxin. A picture of the topography of the Photosystem I reaction center is beginning to emerge and recent advances in the crystallization of this reaction center [123,124] promise more detailed structural information in the near future. Readers interested in more details than are presented above should consult Refs. 84, 124a,b for excellent reviews. With this knowledge of how ferredoxin is reduced, we will turn to a discussion of how reduced ferredoxin is utilized in the chloroplast stroma for the reduction of $NADP^+$, of nitrite, of thioredoxin and for the reductive conversion of glutamine plus 2-oxoglutarate to glutamate.

IV. $NADP^+$: ferredoxin oxidoreductase

IV-A. Structure

Ferredoxin: $NADP^+$ oxidoreductase (EC 1.18.1.2, hereafter abbreviated FNR) has been isolated and extensively characterized from a number of plant, algal and cyanobacterial sources [125]. The primary function of FNR in vivo is to catalyze electron flow from ferredoxin, reduced by the light-driven reactions of Photosystem I that were described above, to $NADP^+$ [125]. The proteins are monomeric, with molecular masses near 35 kDa, and contain a single, non-covalently bound FAD prosthetic group. Procedures are available for removing the FAD group and then reconstituting the enzyme by readdition of FAD [126,127]. There have been reports that the purified enzyme exists in multiple forms [128,129]. These forms are probably not true isozymes, as some are interconvertable and appear to differ only in the oxidation state of sulfur-containing amino acids [129]. Heterogeneity may also arise from proteolysis of FNR at the amino terminus during the isolation and purification of the enzyme [130,130a]. Amino acid sequences are available for FNR from spinach [130,131], pea [132], ice plant (*Mesembryanthemum crystallinum* – Ref. 133) and the cyanobacterium *Spirulina* sp. [134]. In the case of *Spirulina* sp. FNR, the sequence was obtained from amino acid sequencing of the protein itself. In the case of spinach FNR, identical sequences were obtained from the protein and from an amino acid sequence deduced from the nucleotide sequence of the corresponding gene. The gene coding for the mature form of spinach FNR has recently been cloned and expressed in *Escherichia coli* [134a]. For pea and ice plant, the amino acid sequences were deduced from the gene sequences. Table II summarizes the currently available sequences. In photosynthetic eukaryotes, FNR is coded for by a nuclear

TABLE II

Amino acid sequences of FNR

The amino acid position numbers are based on the spinach sequence and the alignment was chosen to maximize homologies. The spinach, pea, *M. crystalium*, *Spirulina* sp. and *A. variabilis* sequences are from Refs. 130, 133, 134, 131 and 165, respectively.

	10	20	30	40	50
1.Spinach	Q IASDVEAPP	PAPAKVEKHS	KKMEEGITVN	KFKPKTPYVG	RCLLNTKITG
2.Pea	IR-QVTT--EAP-	---AKVVKHS	KKQDENIVVN	KFKPKEPYVG	KCLLNTKITG
3.*M. crystalium*	IRA-VASDVEAPV	---AKVEKHS	KKMEEGVIVN	KYKPKNPYTG	RCLLNTKITG
4.*Spirulina*			--AKTDIPYN	I YKPKNPY IG	KCLSNEELVR
5.*A. variabilis* (partial)				- FRPNAP FIG	K?ISNEPLVK

	60	70	80	90	100	110
1.	DDAPGETWHM	VFS-HEGEI PY	REGQSVGVIP	DGEDKNGKPH	KLRLYSIASS	AL GDFGDAKS
2.	DDAPGETWHM	VFS-HEGEVPY	REGQS IGIVP	DGI DKNGKPH	KLRLYSIASS	A I GDFGDSKT
3.	DDAPGETWHM	VFS-HEGEI PY	REGQSVGPIV	EGI DKNGKPH	KLRLYSIASR	PL GDFGDSKT
4.	EG GTGTVR HL	I FDI SGGDLRY	LEGQS IGI IP	PGTDNNGKPH	KLRLYSIAST	RHDGHVDDKT
5.	?G GI ??VQ HL	KVD				

	120	130	140	150	160	170
1.	VSLCVKRLI Y	TN-DAGET IKG	VCSNFLCDLK	PGAEVKLTGP	VGKEMLMPKD	PNATIIMLGT
2.	VSLCVKRLVY	TN-DAGEVVKG	VCSNFLCDLK	PGSEVKITGP	VGKEMLMPKD	PNATVIMLGT
3.	VSLCVKRLI Y	TN-DNGE IVKG	VCSNFLCDLK	PGSEVVLTGP	VGKEMLMPKD	PNATIIMLAT
4.	VSLCVRQLE Y	KHPETGETV IG	VCSTYLCNLE	AGADVAITGP	VGKEMLL PED	EDATIIMMAT

	180	190	200	210	220	230
1.	GTGIAPFRSF	LWKMFFEKHD	DYKFNGLAWL	FLGVPTSSSL	LYKEEFEKMK	EKPADMFRLD
2.	GTGIAPFRSF	LWKMFFEKHE	DYQFNGLAWL	FLGVPTSSSL	LYKEEFEKMK	EKAPENFRLD
3.	GTGIAPFRSF	LWKMFFEKHD	DYKFNGLAWL	FLGVPTSSSL	LYKEEFEKMK	EKAPENFRLD
4.	GTGIAPFRAF	LWR IFKEQHE	DYKFKGLAWL	FFGI PYSPNI	LYQ QELEE LQ	EEFP ENFRLT

	240	250	260	270	280	290
1.	FAVSREQTNE	KGEKMYIQTR	MAQYAVELWE	MLKKDNTYVY	MCGLKGMEKG	IDDIMVSLAA
2.	FAVSREQVND	KGEKMYIQTR	MAQYAEELWE	LLKKDNTFVY	MCGLKGMEKG	IDDIMVSLLA
3.	FAVSREQTNE	KGEKMYIQTR	MAQYDRELWE	LLKKDNTYVY	MCGLKGMEKG	IDDIMVSLAA
4.	LAI SREQQNP	EGGKMYIQDR	IKENADQLWE	LI QKPNTHTY	I CGLKGMEGG	IDEGMSAAAG

	300	310	
1.	AEGI DW IEYK	RQLKKAEQWN	VEVY
2.	KDGI DWIEYK	RTLKKAEQWN	VEVY
3.	EDGI DWFDYK	KQLKKAEQWN	VEVY
4.	KFDVDWSDYQ	KELKKKHRWH	VETY

gene and synthesized on cytoplasmic ribosomes as a precursor protein containing an N-terminal leader sequence that targets it for transport across the chloroplast envelope [31,131–133]. The leader sequence is cleaved by a specific proteinase [31] and the mature protein becomes attached to the stromal-facing side of the thylakoid membrane [125–135].

FNR appears to be attached to the thylakoid membrane by a specific, intrinsic binding protein that is accessible from the stromal side of the thylakoid membrane [125,136–140a]. It has been suggested that the 17.5 kDa FNR-binding protein may be identical to a 16.5 kDa protein, previously characterized as a component of the chloroplast oxygen-evolving system [141].

However, recent immunological and amino acid sequence data indicate that the two proteins are not identical [141a]. The N-terminal amino acid sequence of this 17.5 kDa, putative FNR-binding protein also indicates that it is not identical to either the *psaE* or *psaF* gene product components of the Photosystem I reaction center [141a]. An additional 10 kDa protein, named 'connectein', also may be involved in the binding of FNR to the thylakoid membrane [141b,c]. FNR behaves as an allotopic protein [137], with the isolated, purified enzyme differing from membrane-bound FNR in several properties [142–145]. Furthermore, the affinity of the enzyme for its substrates appears to be regulated through energization of the thylakoids [142,143]. These extremely interesting aspects of FNR fall outside the scope of this article, but the interested reader can consult Ref. 125 for an excellent review of this topic.

The X-ray crystal structure of spinach FNR has been solved at a resolution of 0.37 nm [146]. Although no atomic details were available at this resolution, certain important features of the protein could be discerned. The molecule contains two different structural domains and a model of the $NADP^+$-binding region, located near the carboxy terminus of the enzyme, could be constructed [146,146a]. The proposed $NADP^+$-binding site is exposed to the solvent, as predicted from other observations [147], and is shaped into a 'Rossmann fold', a structure often seen at the nucleotide-binding sites of dehydrogenases and kinases [146]. The dinucleotide pocket provided by this 'fold' consists of five parallel pleated strands connected by α-helical regions [146a]. These earlier interpretations, based on the relatively low resolution structure then available, have been confirmed in a refined 0.22 nm resolution structure that has recently become available (Karplus, P.A., personal communication, and Karplus, P.A., Daniels, M. and Herriott, J.R., unpublished data). Neither the exact orientation of $NADP^+$ within the pocket nor any details of the FAD-binding geometry could be discerned from the 0.37 nm resolution structure. However, information concerning these aspects of FNR will be available from the forthcoming 0.22 nm resolution structure.

IV-B. $NADP^+$ binding

Convincing evidence that FNR contains separate binding sites for $NADP^+$ and ferredoxin was obtained by first using spectral perturbations to demonstrate complex formation between the enzyme and each of its substrates separately and then demonstrating the formation of a ternary complex between FNR, $NADP^+$ and ferredoxin [148–150]. Negative cooperativity between the two binding sites has been reported, with the binding of $NADP^+$ causing a decrease in the affinity of FNR for oxidized ferredoxin and the binding of oxidized ferredoxin causing a decrease in binding affinity for $NADP^+$ [149,149a]. Studies of $NADP^+$ binding using spectral perturbations [148–155] have demonstrated, among other things, that there is one binding site for $NADP^+$ on the enzyme, that NADPH is bound more tightly ($K_d \approx 1$ μM) than is $NADP^+$ ($K_d = 14$ μM [155]) and that the 2′-adenosyl phosphate and one or both of the phosphate moieties of the pyrophosphate bridge of $NADP^+$ contribute significantly to the strength and specificity of pyridine nucleotide binding [155]. The measured difference in the binding affinities of $NADP^+$ and NADPH for the enzyme leads to the prediction that the E_m value of the pyridine nucleotide should shift to a more positive value on binding to the enzyme. Oxidation-reduction titrations of the $NADP^+$/FNR complex are somewhat difficult to interpret because of ambiguities in the assignment of electron distribution between the pyridine nucleotide and the FAD group of the enzyme [155]. However, these titrations appear to show that the E_m value of the enzyme-bound pyridine nucleotide is approx. 40 mV more positive than that of the free $NADP^+$/NADPH couple [155]. Thus binding of the substrate makes electron transfer from the $FADH_2$ group of the enzyme to $NADP^+$ (i.e., in the physiologically significant direction) thermodynamically more favorable. At pH 8.0, there is a ΔE_m of approx. 80 mV for the two electron transfer from the $FADH_2$ of the enzyme ($E_m = -380$ mV [155–157]) to the bound $NADP^+$. Binding to $NADP^+$ to FNR had no detectable effect on the E_m value of the enzyme's FAD group, indicating that the oxidized and reduced forms of the enzyme do not differ significantly in binding affinity for the pyridine nucleotide substrate [155].

One region of FNR that is likely to be involved in binding $NADP^+$ has been identified [158] by covalent labeling of the enzyme with a pyridine nucleotide analog, 2′,3′-dialdehyde $NADP^+$ (periodate-oxidized $NADP^+$). The analog selectively labels a single lysine residue at position 244 in the spinach enzyme [158]. This lysine, which is present in all four known FNR sequences, is located in a highly conserved section of the enzyme (see Table II). Treatment of FNR with dansyl chloride, a reagent known to modify the ϵ-amino group of lysine, results in an inhibition of pyridine nucleotide-binding by both the spinach enzyme [159] and FNR isolated from the alga *Bumilleriopsis filiformis* [160]. The lysine that is dansylated in the spinach enzyme has been identified as lysine 116 [161]. Modification of a single lysine residue in spinach FNR by the triplet probe eosin isothiocyanate completely inhibited pyridine nucleotide binding to the enzyme [162]. Eosin itself binds to the enzyme competitively with $NADP^+$/NADPH ($K_i \approx 5$ μM [162]). The lysine modified by eosin isothiocyanate has been shown to be one of the seven lysines in the segment containing amino acids 179–228 [162]. Structural calculations predicted that

this portion of FNR contains two helicies that were likely to comprise part of the α/β pyridine nucleotide-binding fold [162]. Thus three lysine residues, all located in different regions of the primary sequence of FNR (lysine 116, lysine 244, and one lysine located between residue 178 and 228), have been implicated in $NADP^+$ binding. The recent 0.22 nm resolution structure of the enzyme locates lysine 244 and the lysines between residues 178 and 228 within the putative $NADP^+$-binding domain (Karplus, P.A., personal communication). Lysine 244 probably interacts with $NADP^+$, but the lysines between residues 178 and 228 do not appear to be close enough to the $NADP^+$-binding site to interact directly with the pyridine nucleotide (Karplus, P.A., personal communication). Lysine 116, although located in the FAD-binding domain of the enzyme, does interact with $NADP^+$ (Karplus, P.A., personal communication).

Evidence obtained from treatment of FNR with reagents known to modify arginine residues has also suggested the involvement of a single arginine residue at the $NADP^+$-binding sites of FNR's from spinach [163], *Bumilleriopsin filiformis* [153,160] and the cyanoabacterium *Anabaena variabilis* [164]. A partial amino acid sequence of the *A. variabilis* FNR suggests that it will prove to be quite homologous to the *Spirulina* sp. enzyme (Ref. 165 and see Table II). The recent 0.22 nm resolution FNR structure confirms the presence of an arginine residue at the $NADP^+$-binding site of the spinach enzyme (Karplus, P.A., personal communication). Spinach FNR has also been shown to be inactivated by treatment with the histidine-modifying reagent, diethyl pyrocarbonate [166]. The observations that carbethoxylation of FNR inhibited $NADP^+$ binding by the enzyme and that $NADP^+$ protected the enzyme against inactivation by diethyl pyrocarbonate were interpreted in terms of a single histidine residue at the $NADP^+$-binding site [166]. This histidine is the only histidine in the enzyme that is rapidly modified by diethyl pyrocarbonate, suggesting that it is relatively exposed to the solvent and that the other four histidines are considerably less accessible [166]. ^{1}H-NMR measurements with spinach FNR indicate that only one of the five histidine residues in the enzyme can be readily titrated. The pK of this histidine decreases when $NADP^+$ binds to the enzyme, identifying the histidine at the $NADP^+$-binding site as the single solvent-exposed histidine [125]. In addition to the presence of positively charged lysine and arginine residues and of a potentially cationic histidine at the $NADP^+$-binding site of the enzyme, it is possible that the pyridine nucleotide-binding site also contains an essential carboxylate group [167,168]. Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium 3′-sulfonate) inactivates the enzyme and eliminates the ability of FNR to bind $NADP^+$ [167]. Both $NADP^+$ and NADPH completely protect spinach FNR against inactivation, with the pyridine nucleotide and the modifying reagent competing for the same site on the enzyme [167,168]. Inhibition could be attributed to the modification of one carboxyl group per molecule of enzyme [167].

IV-C. Ferredoxin binding

FNR is able to form a 1 : 1, electrostatically stabilized complex with ferredoxin [125]. Complex formation has been documented using a wide variety of techniques, including: the measurement of perturbations in absorbance [148,151–153,169,170], circular dichroism [171–173] and fluorescence spectra [172,174]; co-migration during gel filtration chromatography [172,175,176]; analytical ultracentrifugation [152]; isoelectric focusing [159]; membrane ultrafiltration [176] and cross-linking [29,150,177,179]. The complex dissociates at high ionic strengths, as expected for an electrostatically stabilized association. K_d values at physiological ionic strengths have been estimated to range from 1 to 10 μM for the complexes from a number of different species [125,152, 153,180]. The stability of the complex is also pH-dependent [180], with the K_d increasing as the pH increases from 6 to 8. Above pH 9, K_d is pH-independent. The pH-dependence of the binding affinity has been interpreted in terms of the uptake of a single proton during complex formation. The group that is protonated on complex formation has its pK_a shifted from 6 in the free protein to 8 in the complex [180].

Although all the early experiments demonstrating complex formation were performed with both proteins in their oxidized states, complex formation also occurs between the reduced proteins [157,180,181]. The strength of the protein : protein binding in the FNR/ferredoxin complex is affected by the oxidation-reduction state of the proteins, with the oxidized form of ferredoxin binding more strongly to FNR than the reduced form and the reduced form of FNR binding ferredoxin more tightly than does oxidized FNR [180,181]. These effects of oxidation state on binding affinities, like those described above for the $NADP^+$/FNR complex, are reflected in effects of complex formation on the E_m values of both ferredoxin and FNR [157,180]. The changes in E_m values that result from complex formation make electron transfer from reduced ferredoxin to the FAD group of FNR thermodynamically more favorable than would be the case for the uncomplexed proteins (see below).

Evidence from ^{13}C-NMR experiments [182], chemical modification studies [29,183] and cross-linking experiments [29,177,179] have established that ferredoxin contributes the negatively charged groups involved in complex formation with FNR. The NMR experiments, conducted with ^{13}C-enriched ferredoxin isolated from the cyanobacterium *A. variabilis,* indicated that there are

likely to be three ferredoxin glutamate residues at or near the contact region with FNR in the complex formed between the cyanobacterial ferredoxin and spinach FNR [182]. Chemical modification of spinach ferredoxin, in which 3 to 4 carboxyl groups were altered to remove their negative charges by treatment with glycine ethyl ester and a water-soluble carbodiimide, resulted in a substantial decrease in the ability of ferredoxin to bind to spinach FNR [29,183]. Peptide mapping of the modified ferredoxin located three regions of the protein in which carboxyl groups had been modified [29]. These regions (Fig. 3), which were thus identified as putative domains on ferredoxin that might be involved in binding FNR, were: the region containing amino acid residues 26–30; the region containing residues 65–70 and the region from residue 92 to residue 94 [29]. The sequence of three consecutive glutamate residues at positions 92–94 were proposed to be the most likely spot for binding to FNR because of its relative proximity to the [2Fe-2S] cluster of ferredoxin (Fig. 8).

Analysis of the sites of cross-linking in a complex in which ferredoxin and FNR were covalently linked by a water-soluble carbodiimide after the electrostatically-stabilized complex was allowed to form, identified some

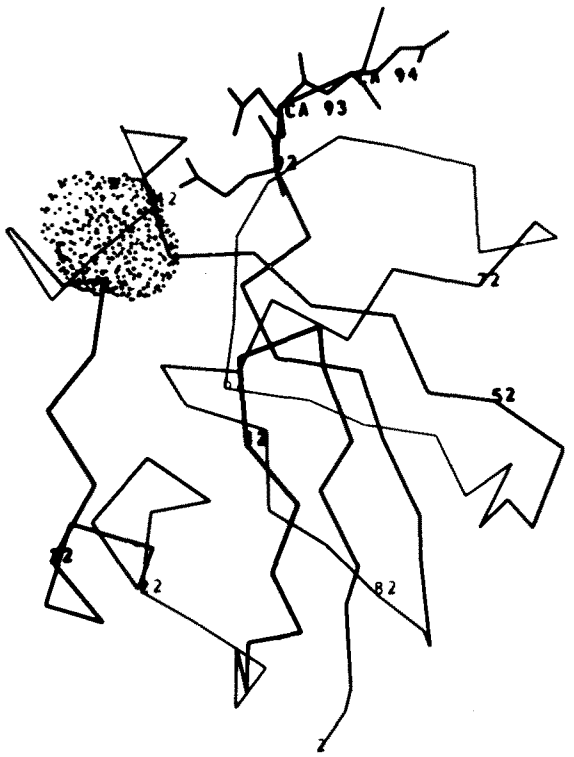

Fig. 8. Computer grpahics model of spinach ferredoxin. The amino acid sequence of spinach ferredoxin isoform I was superimposed on the tertiary structure of *S. platensis* ferredoxin. The side-chains of glutamic acid residues 92, 93 and 94 have been included, but for all other residues only α-carbons are shown. The shaded area delineates the Van der Waal's surface of the [2Fe-2S] cluster. From Ref. 178.

of the specific carboxyl groups on ferredoxin [29,177] and the specific lysine ε-amino groups on FNR involved in linking the two proteins [177]. The major site of cross-linking on ferredoxin was somewhere in the region containing amino acid residues 76 to 97, with the most likely site being one of the three consecutive glutamic acid residues, 92–94 (Fig. 8). As both cross-linking and chemical modification approaches have implicated this acidic region near the carboxy terminus and because of the relative proximity of these amino acids to the [2Fe-2S] cluster, it seems likely that one or more of glutamates 92–94 are in fact involved in the electrostatic binding of ferredoxin to FNR. A second, minor site of cross-linking to FNR was identified as the region from amino acid residue 5 to residue 37 of ferredoxin, with the aspartate residues at positions 20, 21 or 26 being the most likely carboxyl donors in this linkage [177]. Chemical modification studies (see above) also suggest that the region on ferredoxin containing aspartate 26 may be involved in binding to FNR [29].

The cross-linking experiments also allowed the identification of the FNR amino acids that contributed amino groups to the carbodiimide-induced covalent linkages [177]. The FNR residue(s) acting as the amino group donor(s) to the major cross-linking site is lysine 85 and/or lysine 88. Both of these lysines are conserved in all three known sequences for higher plant FNR's [130,132–134]. Lysine 88 is also conserved in the *Spirulina* sp. FNR, whereas lysine 85 of the higher plant FNR's is replaced by an asparagine in the *Spirulina* sp. sequence (Ref. 131 and see Table II). The recent 0.22 nm resolution X-ray structure of spinach FNR indicates that these lysines are in relatively close proximity to the FAD prosthetic group of the enzyme, as would be expected if ferredoxin were to be positioned at a docking site on FNR suitable for efficient electron transfer between the two proteins in the complex (Karplus, P.A., personal communication). The minor cross link involved the α-amino group of the isoleucine that was reported to be at the amino terminus of FNR by Zanetti et al. [177]. However, Karplus et al. have reported that isoleucine is the second rather than the first amino acid in FNR, with the true amino terminus being a pyroglutamyl residue [130]. As mentioned above, there have been reports from several laboratories that multiple forms of FNR may arise from proteolysis and this may have occurred with the FNR preparation utilized by Zanetti and co-workers for the cross-linking experiments. In fact, Zanetti et al. reported that their FNR preparation also contained some enzyme with a pyroglutamyl residue at the N-terminus [177] and it is thus likely that the major form in their preparation, with isoleucine at the N-terminus, arose from proteolysis of the true N-terminal pyroglutamyl residue (Zanetti, G., personal communication). Thus, the minor cross-link to the α-amino group of isoleucine may not represent a

point of physiologically significant interaction between ferredoxin and FNR.

Although cross-linking experiments have not implicated other regions near the N-terminus of FNR in binding ferredoxin, recent measurements on FNR which had been subjected to limited proteolysis with either trypsin or chymotrypsin [178] and on a genetically engineered, truncated form of spinach FNR expressed in *E. coli* [134a], suggest that the FNR region from Lys-22 to Phe-32 is likely to be involved in ferredoxin binding. It was proposed that within this putative ferredoxin-binding region of FNR, the amino acids from Val-29 to Phe-32 play a more important role in binding ferredoxin than do the amino acids from Lys-22 to Thr-28 [178]. The region of spinach FNR from Ile-27 to Asn-49 is highly conserved in all four FNR's for which sequences are available, with 11 completely conserved residues and 2 conservative substitutions in a stretch of 19 amino acids (see Table II). This high degree of conservation and the presence of three positively charged lysine residues in this region, are consistent with its functioning as part of the binding domain for the negatively charged ferredoxin.

In addition to the presence of lysine residues at the FNR binding site for ferredoxin, it appears possible that at least one FNR arginine residue is also involved in electrostatic complex formation with ferredoxin. Treatment of the spinach FNR with the arginine-modifying reagent 2,3-butanedione resulted in 70% inactivation of the enzyme when two arginine residues were modified [163]. The enzyme could be partially protected against this inactivation by $NADP^+$ (see above) and could also be protected against butanedione inactivation by ferredoxin [163]. These results were interpreted in terms of the presence of one arginine residue at the $NADP^+$-binding site (see above) and a second arginine residue at the ferredoxin-binding site of FNR. The precise location of the arginine at the ferredoxin-binding site is not yet clear. However, there is an arginine at position 93 in the spinach enzyme that is conserved in all four known FNR sequences (Table II). This arginine is part of a well-conserved, positively charged region of FNR that also contains the two lysine residues implicated in ferredoxin binding by cross-linking experiments (see above). NMR experiments using the $Cr(CN)_6^{3-}$ anion as a probe have implicated a highly positive region, such as that from residues 85 to 93, in binding ferredoxin [184]. It has also been concluded, based on the protective effect of ferredoxin against inactivation by phenylglyoxal, that an arginine residue is present at the ferredoxin-binding site of the FNR isolated from the cyanobacterium *A. variabilis* [164]. However, chemical modification studies of *Bumilleriopsis filiformis* FNR showed no effect of arginine modification by butanedione on the ferredoxin-binding capability of the enzyme [153]. It may thus be possible that the ferredoxin-binding site of some, but not all, FNR's contain arginine.

In addition to the likely presence of positively charged lysine and arginine residues at the ferredoxin-binding site of FNR, some evidence suggests that aromatic amino acids [174,185] and cysteine [186] may also be present at interaction interface between these two proteins. Complex formation between the two proteins results in the quenching of FNR tryptophan fluorescence by approx. 50% and titration of this quenching as a function of ferredoxin concentration gives a K_d for ferredoxin in good agreement with that calculated from other data [174]. Six tryptophan residues are strictly conserved in the three known higher plant FNR sequences [130,132–134], with five of these also conserved in the *Spirulina* sp. FNR sequence [131]. This degree of conservation suggests that tryptophan residues may play some essential role in the function of the enzyme and the quenching results suggest that two of these tryptophans may be at or near the ferredoxin-binding site [174]. Solvent perturbation measurements indicate that complex formation between FNR and ferredoxin results in a decreased exposure of tryptophan to the solvent [185], consistent with tryptophan being present at the interaction domain between the two proteins. The solvent perturbation data also suggest that the interaction domains on one or both of the proteins may include tyrosine residues [185].

Spinach FNR has been reported to contain three thiols and a disulfide bridging the cysteines at positions 132 and 137 [130]. The two cysteines that contribute to this disulfide bridge are conserved in all four of the currently known FNR sequences [130–134]. However, there is some evidence that the disulfide bride may result from cysteine oxidation during the denaturation of the enzyme for sequencing [187]. The recent 0.22 resolution structure indicates that the native enzyme does not contain a disulfide (Karplus, P.A., personal communication). Treatment of membrane-bound spinach FNR with iodosobenzoate, a reagent known to oxidize vicinal dithiols, inhibited the ferredoxin-dependent $NADP^+$ reduction catalyzed by the enzyme, but had little or no effect on the ability of the enzyme to catalyze ferredoxin-independent electron flow from NADPH to ferricyanide [186]. The loss of two FNR sulfhydryl groups that resulted from treatment with iodobenzoate also interfered with the ability of the enzyme to form a complex with ferredoxin [186]. Furthermore, the loss of enzymatic activity could be partially prevented by pre-addition of ferredoxin [186]. These results suggest that a pair of cysteine residues may be present at or near the ferredoxin-binding site of FNR. Whether one of these cysteines is the same cysteine that appears to be involved in the binding of the FAD prosthetic group to the enzyme [156,188,189] has not yet been determined. Deprotonation of a cysteine

residue with a pK near 8.0 results in the conversion of FNR from a less active to a more active form [190], but whether this cysteine is also involved in ferredoxin binding is not yet clear.

The solvent perturbation studies cited above indicate that certain amino acid side chains which are exposed to the solvent in the isolated proteins become less accessible to solvent upon complex formation between the two proteins. Evidence also exists that complex formation between ferredoxin and FNR affects the exposure of the prosthetic groups of both proteins to the solvent [149,191]. The *o*-phenanthroline complex of the rapidly relaxing paramagnetic rare earth ion Dy^{3+} broadens the EPR signal of reduced spinach ferredoxin through dipolar interactions [191]. The extent of broadening was substantially decreased if FNR were present under conditions known to favor complex formation between the two proteins, indicating that the [2Fe-2S] cluster of reduced ferredoxin is less exposed to the positively charged, hydrophilic probe (and thus less exposed to the solvent) in the complex than in ferredoxin alone. The negatively charged EDTA complex of Dy^{3+} was shown to produce a substantial broadening [149] of the neutral semiquinone EPR signal that results [192] when NADPH is added to FNR under aerobic conditions. This broadening was partially reversed by the addition of oxidized ferredoxin in amounts equimolar to FNR [149]. As this free radical EPR signal is thought to arise from FADH semiquinone [192], these results suggest that the FAD group of FNR, like the [2Fe-2S] cluster of ferredoxin, becomes less solvent exposed as a result of complex formation between the two proteins [149]. The relative ineffectiveness of the *o*-phenanthroline complex of Dy^{3+} in broadening the FNR semiquinone EPR signal and the relative ineffectiveness of the EDTA complex of Dy^{3+} in broadening the EPR signal of ferredoxin were interpreted in terms of a negatively charged region surrounding the [2Fe-2S] cluster of ferredoxin and a positively charged region surrounding the FAD group of FNR [149]. A laser flash photolysis kinetic study of the reduction of ferredoxin, FNR and of the ferredoxin/FNR complex by 5-deazariboflavin semiquinone also demonstrated that complex formation between the two proteins renders the [2Fe-2S] cluster of ferredoxin less accessible but showed no effect of complex formation on the reactivity of the FAD group of FNR with the 5-deazariboflavin semiquinone [191].

IV-D. Oxidation-reduction properties and mechanism

A detailed discussion of the kinetics of the reactions catalyzed by FNR falls outside the scope of a review article that concentrates on the structure, substrate-binding and oxidation-reduction properties of the enzyme. However, questions about the role of the FAD semiquinone oxidation state of FNR in the reduction of $NADP^+$ catalyzed by the enzyme and about possible effects of ferredoxin–FNR interactions on the redox properties of the proteins are in fact related to some of the redox properties of FNR discussed above. Furthermore, the question of whether the FNR/ferredoxin complex, characterized in measurements involving the isolated purified proteins, actually functions during $NADP^+$ reduction in chloroplasts is also germane to the main subject of this review. Readers interested in a more detailed discussion of the likely mechanism(s) of FNR-catalyzed reactions should consult Refs. 125 and 193 for reviews.

As mentioned above, differences in the protein : protein binding affinities that accompany changes in the

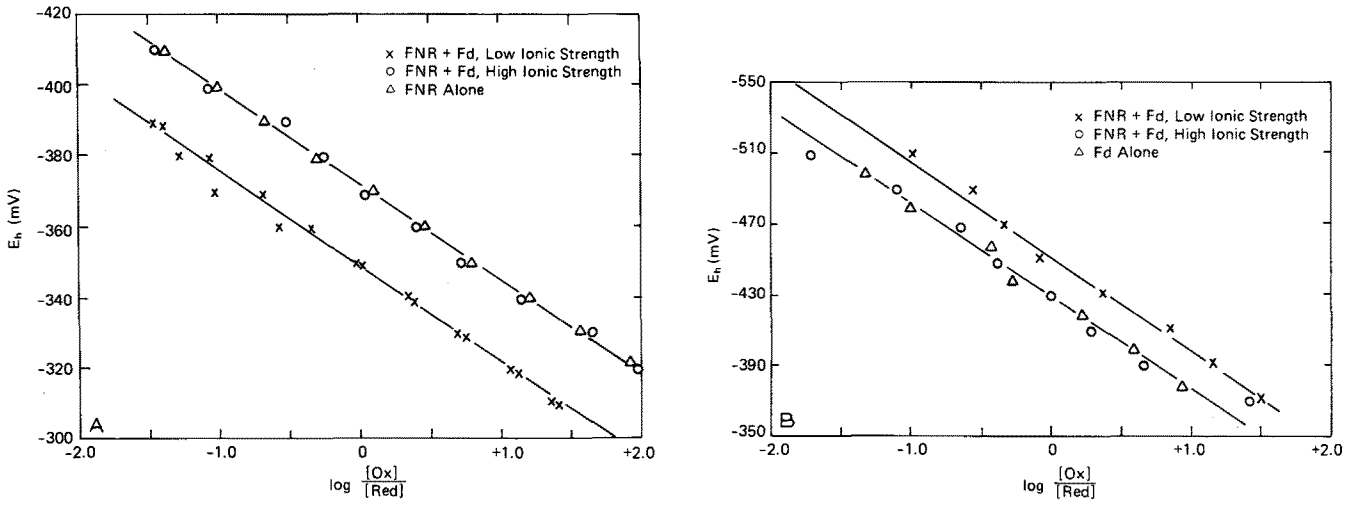

Fig. 9. Oxidation-reduction titrations of the ferredoxin/FNR complex. (A) The oxidation state of the FAD group of FNR was monitored by following changes in the absorbance spectrum of FNR at different ambient potentials. (B) The oxidation state of the [2Fe-2S] cluster of ferredoxin was monitored by following changes in the circular dichroism spectrum of ferredoxin at different ambient potentials. From Ref. 157.

oxidation states of ferredoxin and FNR affect the E_m values of both proteins [157,180]. Fig. 9A, taken from work performed in our laboratory, shows the results of an oxidation-reduction titration of the FAD group of FNR in the presence and absence of ferredoxin. The E_m value for the FAD/$FADH_2$ couple becomes 23 mV more positive under conditions where ferredoxin is complexed to the enzyme [157]. At high ionic strength, where dissociation of the ferredoxin/FNR complex is favored, the presence of ferredoxin has no effect of the E_m value of FNR. Fig. 9B shows that the E_m value for the [2Fe-2S] cluster of ferredoxin becomes 22 mV more negative on complex formation with FNR [157]. Results that agree qualitatively with those obtained in our laboratory were reported by Batie and Kamin [180]. However, these workers reported a considerably larger shift in the E_m value for ferredoxin, from -420 mV for ferredoxin itself to -500 mV for ferredoxin bound to FNR [180]. The reasons for the differences in the magnitude of ΔE_m observed by the two groups are not completely clear. However, this discrepancy may have arisen from differences in the ionic strength and pH at which the oxidation-reduction titrations were carried out, as the protein : protein binding affinities are known to be affected by both these parameters (see above). In any event, the results obtained in both laboratories indicate that complex formation increases the thermodynamic driving force for electron transfer in the physiological important direction, i.e., from ferredoxin to FNR. A similar improvement in the thermodynamic feasibility of electron transfer in the physiological direction resulting from complex formation between the enzyme and its pyridine nucleotide substrate was described above.

There is good agreement between E_m values measured in several laboratories for the FAD/$FADH_2$ couple of spinach FNR. The earliest measurements, by Keirns and Wang [156], gave values of -360 mV at pH 7.0 and -390 mV at pH 8.0, Batie and Kamin [155] reported a value of -376 mV at pH 8.0 and work in our laboratory [157] gave a value of -372 mV at pH 7.3. In contrast, there is disagreement over the E_m values for the two one-electron couples involving the semiquinone state. Keirns and Wang reported E_m values (at pH 7.0) of -320 mV and -400 mV for the FAD/$FAD^{\cdot}$ and $FAD^{\cdot}$/$FADH_2$ couples, respectively, based on titrations of the enzyme with NADPH [156]. However, Batie and Kamin have pointed out that if the E_m value for the oxidized FAD/FAD semiquinone couple were in fact 80 mV more positive than that of the semiquinone/$FADH_2$ couple, one would expect to observe some semiquinone during equilibrium oxidation-reduction titrations using reductants other than NADPH [155]. The lack of significant amounts of observable FAD semiquinone during such titrations [155–157] allows one to calculate that the E_m value for the oxidized FAD/FAD semiquinone couple is likely to be at least 90 mV more negative than that of the semiquinone/$FADH_2$ couple [155]. One possible source of uncertainty in these measurements is that absorbance features that probably arise from a charge-transfer complex between NADPH and FNR may have been mistakenly attributed to a FAD semiquinone [155].

Despite the uncertainty about the E_m values of FNR redox couples involving the FAD semiquinone state, this state almost certainly plays a role in the enzyme-catalyzed reduction of $NADP^+$. The FAD semiquinone state of FNR has been detected by EPR spectroscopy [149,192,194] and by optical absorbance measurements [191,195,196]. Both the optical and EPR spectra indicate that the semiquinone is the neutral rather than the anionic form [149,191,192,194,195], so that one proton is taken up during the one-electron reduction of the FAD group of FNR. As ferredoxin is a one-electron carrier and there is only a single ferredoxin-binding site for ferredoxin on FNR, it is difficult to imagine mechanisms for electron transfer from reduced ferredoxin to the FAD group of the enzyme that do not involve at least the transient formation of the FAD semiquinone state. It has been known since some of the earliest studies on FNR-catalyzed electron transfer from ferredoxin to $NADP^+$ that reduced ferredoxin can completely reduce the FAD group of FNR [196]. However, this net two-electron reduction, measured on a slow time-scale, could have resulted from two sequential one-electron transfers from two reduced ferredoxins. Flash photolysis experiments in which ferredoxin was rapidly reduced by the 5-deazariboflavin radical, allowed the measurement of the kinetic parameters for electron flow from reduced ferredoxin to FNR that produced the neutral $FADH^{\cdot}$ semiquinone state of the enzyme [191]. At pH 7.0 and an ionic strength of 310 mM, the reaction was second-order ($k = 1.5 \cdot 10^8\ M^{-1} \cdot s^{-1}$). At high concentrations of FNR, the rate of electron transfer from reduced ferredoxin to FNR became independent of FNR concentration, with a limiting first-order rate constant of 4000 s^{-1} [191]. It was not possible to measure the kinetics of electron transfer from reduced ferredoxin to FNR at low ionic strengths, where the binding of the two proteins is tight. Under these conditions, the shielding of the [2Fe-2S] group of ferredoxin from access to the 5-deazariboflavin semiquinone in solution that occurs in the FNR/ferredoxin complex (see above) results in initial reduction of FNR rather than of ferredoxin. Evidence from flash photolysis experiments using intact cells of the green alga *Chlorella pyrenoidosa* suggests that the FAD semiquinone state of FNR is also formed during the reduction of the enzyme by reduced ferredoxin in vivo [198–200].

It has not yet been firmly established whether the reduction of $NADP^+$ by the reduced enzyme involves

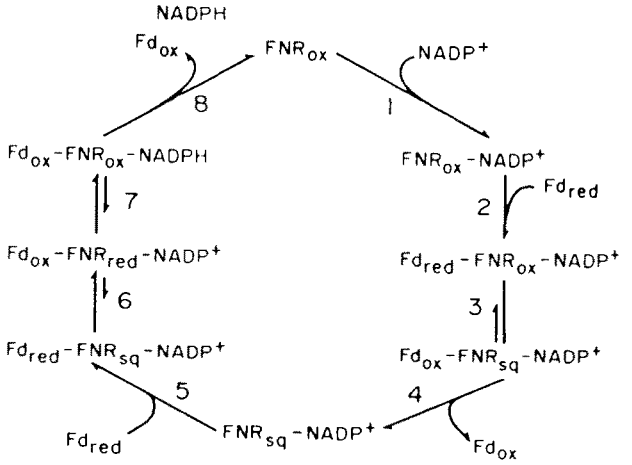

Fig. 10. Scheme for the mechanism of the FNR-catalyzed reduction of $NADP^+$ by ferredoxin. Fd_{ox} and Fd_{red} refer to the oxidized and reduced forms of ferredoxin, respectively. FNR_{ox}, FNR_{sq} and FNR_{red} refer to FNR with an oxidized FAD, a one-electron-reduced FAD semiquinone and a fully reduced FAD group, respectively. From Ref. 197.

the FAD semiquinone state of FNR. The E_m value for the one-electron reduction of NAD^+ to $NAD^\cdot$ in aqueous solution has been estimated, using pulse radiolysis techniques, to be between -920 [201] and -940 mV [202], suggesting that a one-electron reduction of $NADP^+$ by either the $FADH^\cdot$ semiquinone or $FADH_2$ states of FNR would be thermodynamically impossible. However, the environment of $NADP^+$ at its binding site on FNR may differ considerably from that in aqueous solution. If this were to be the case, the E_m value for the $NADP^+/NADP^\cdot$ couple at the pyridine nucleotide binding site of the enzyme could differ substantially from that in aqueous solution. Furthermore, favorable enzyme/substrate binding energy may stabilize the one-electron reduced $NADP^\cdot$ at the pyridine nucleotide binding site of FNR. While it thus may be possible that pyridine nucleotide reduction proceeds through two sequential one electron steps, thermodynamic considerations make it seem more likely that the FAD group of the enzyme is first fully reduced and that the $FADH_2$ then reduces $NADP^+$ in a concerted two-electron step (Fig. 10).

In addition to questions concerning the role of the FAD semiquinone state in the mechanism of the enzyme, the role in the electron transfer reaction of the binary and ternary complexes described above must be addressed. The evidence given above for the existence of these complexes involves measurements that have been made under static conditions (i.e., conditions that do not involve turnover or electron transfer) and it is important to document the role of these complexes under turnover conditions. The effects of ionic strength [170,191] and chemical modification of either ferredoxin or FNR [125,29,183] on the kinetics of electron transfer reactions catalyzed by FNR suggest that an electrostatically stabilized complex between the two proteins does in fact play a role in the reaction mechanism. Furthermore, kinetic studies suggest that the ternary complex between the enzyme, ferredoxin and $NADP^+$ also participates in electron transfer from reduced ferredoxin to $NADP^+$ catalyzed by both the *S. platensis* and spinach enzymes [197,203]. Rapid kinetic studies conducted with spinach FNR indicate that $NADP^+$ binding, leading to ternary complex formation, increased the rate of electron transfer from reduced ferredoxin to FNR by facilitating the dissociation of inhibitory, oxidized ferredoxin from FNR [197]. As the rate of electron transfer from reduced ferredoxin to FNR can also be increased by the binding of a non-reducible $NADP^+$ analog [197], this effect is more likely to arise from the negative cooperativity between the ferredoxin and pyridine nucleotide binding sites in the ternary complex than from the electron accepting capacity of $NADP^+$. Thus the negative cooperativity between the ferredoxin and pyridine nucleotide binding sites on FNR, detected in equilibrium binding measurements (see subsection IV-B), appears to play an important role in the enzyme mechanism. The effect of pyridine nucleotide binding on the triplet lifetime of an eosin probe attached to the putative ferredoxin-binding site of FNR provides additional evidence for cooperative interactions between the two separate substrate-binding sites on the enzyme [147,204]. The rapid kinetic measurements with spinach FNR, and steady-state kinetic investigations of the *S. platensis* enzyme [203], support a mechanism involving a ternary complex with an ordered addition of substrates to FNR (Fig. 10, Ref. 197).

Evidence is also available in support of a role for the FNR/ferredoxin complex during $NADP^+$ photoreduction in vivo. The effects of pH and ionic strength on the K_m for ferredoxin during $NADP^+$ photoreduction by thylakoid membranes are quite similar to those determined for the K_d for ferredoxin in the electrostatically-stabilized complex formed between the two purified proteins [205]. There also appear to be some additional effects of the chloroplast membrane surface potential on the interaction of membrane-bound FNR with its substrates [185,205], but the interactions between the two proteins in situ do not appear to differ greatly from those observed with the purified proteins. Binding of FNR that had been covalently modified to incorporate a triplet probe by treatment with eosin isothiocyanate to thylakoid membranes has also been used to investigate interactions between FNR and ferredoxin in situ [204]. In the absence of ferredoxin, the rotational motion of FNR in the thylakoid membrane is quite rapid (the rotational diffusion correlation time is < 10 μs at 10 °C) but addition of ferredoxin greatly decreases the rotational motion, increasing the correlation time to 40 μs [204].

The results of studies with eosin-modified FNR that has been reconstituted into thylakoid membranes have resulted in a proposal that ferredoxin may mediate formation of a ternary complex with FNR and the Photosystem I reaction center [204]. Formation of such a ternary complex would be possible only if the binding site on ferredoxin for the Photosystem I reaction center differed from the site on ferredoxin involved in interaction with FNR. It is not clear how this possibility can easily be reconciled with the inability of the cross-linked ferredoxin/reaction center adduct to photoreduce $NADP^+$ (see above) and with other data from cross-linking experiments that suggest ferredoxin cross-linked to the Photosystem I reaction center cannot be reduced via FNR by NADPH [206]. Obviously, additional experiments will be required to clarify the question of whether the same domain on ferredoxin is involved in both its oxidation and its reduction. In this regard, detailed information on the identity of specific amino acids on ferredoxin involved in the docking of ferredoxin to the Photosystem I *psaD* subunit will be particularly useful so that a comparison can be made with the amino acids known to be involved in binding to FNR.

V. Nitrite reductase and glutamate synthase

V-A. Nitrogen assimilation in plants

The assimilation of nitrogen in plants and algae, which are incapable of independent nitrogen fixation, involves the reduction of nitrate to nitrite and the subsequent reduction of nitrite to ammonia, followed by the ATP-dependent incorporation of ammonia into glutamate to form glutamine. This early phase of nitrogen assimilation is completed by the reductive conversion of glutamine plus 2-oxoglutarate to two molecules of glutamate. This pathway (Fig. 11, Ref. 207) involves two ferredoxin-dependent steps: the 6-electron reduction of nitrite to ammonia catalyzed by ferredoxin: nitrite oxidoreductase (EC 1.7.7.1, hereafter referred to as nitrite reductase) and the 2-electron reductive conversion of 2-oxoglutarate plus glutamine to glutamate catalyzed by glutamate synthase (EC 1.4.7.1). The conversion of glutamate plus ammonia to glutamine, catalyzed by glutamine synthetase, does not involve any change in the oxidation state of nitrogen. The two-electron reduction of nitrate to nitrite in plants and algae, catalyzed by nitrate reductase, utilizes reduced pyridine nucleotide, rather than reduced ferredoxin, as the electron donor [207]. Although much less is known about the structures and mechanisms of nitrite reductase and glutamate synthase than about that of FNR, evidence is emerging that these two soluble enzymes, located in the chloroplast stroma, both contain ferredoxin-binding sites that may be quite similar to that of FNR.

V-B. Nitrite reductase

It is clear that the six-electron reduction of nitrite to ammonia catalyzed by nitrite reductase utilizes reduced ferredoxin as the electron donor for this process in vivo [207]. In both spinach and green algae, immunocytochemical techniques have confirmed earlier conclusions that the ferredoxin-dependent enzyme is localized in chloroplasts [207a,b], where ferredoxin is also localized. Since the early observation that reduced viologen dyes are excellent non-physiological electron donors for the reaction catalyzed by nitrite reductase [208], most puri-

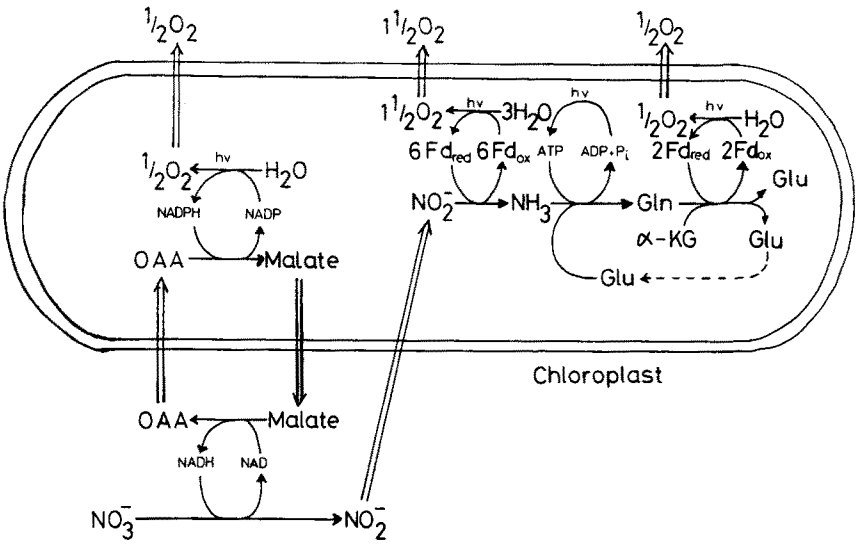

Fig. 11. Nitrate assimilation in photosynthetic eukaryotes. Fd_{ox} and Fd_{red} refer, respectively, to the oxidized and reduced forms of ferredoxin. OAA, Glu, Gln and α-KG refer to oxaloacetate, glutamate, glutamine and 2-oxoglutarate, respectively. The requirement for light to photoreduce ferredoxin is indicated by *hν*.

fication protocols for the enzyme have utilized reduced methyl viologen rather than reduced ferredoxin as the electron donor for enzyme assays [209–217]. This has resulted in some controversy as to whether or not there are significant differences between 'viologen-linked' nitrite reductases and 'native' or 'ferredoxin-linked' nitrite reductases [209–217]. Many of the details of this controversy lie outside the scope of this review, but the subject will be covered briefly insofar as it relates to the likely mode of interaction between the enzyme and ferredoxin. The assumption will be made that there is only a single form of nitrite reductase present in chloroplasts and that the different properties of the enzyme reported by different groups, particularly with respect to subunit composition and prosthetic group content, result from alterations of the enzyme during purification. However, the possibility that more than one form of nitrite reductase is present in vivo, while speculative, cannot be eliminated on the basis of currently available data.

V-B.1. Prosthetic group content

Assimilatory nitrite reductases have been isolated from a number of higher plants [209–215,217–225], algae [216,226–227a] and cyanobacteria [228–230]. These enzymes contain siroheme (Fig. 12) as a prosthetic group. There is some uncertainty as to the siroheme content of the enzyme, arising in part from the fact that chemical analysis for siroheme does not produce 100% recovery of the chromophore and also from the possibility that this non-covalently bound heme may be partially lost during purification of the enzyme [218,231]. The best estimate available for monomeric forms of the enzyme appears to be that there is one siroheme present per enzyme molecule [214]. There is, however, one report that a heterodimeric preparation of the enzyme may contain two sirohemes [232]. Oxidation-reduction titrations of the enzyme, using either EPR [225,233,234] or optical [217] features to monitor the oxidation state of the siroheme, yield different estimates for the E_m value in different preparations of the enzyme. Titrations, using EPR detection, of monomeric forms of the squash [234] and barley [225] enzymes and of a preparation of the spinach enzyme for

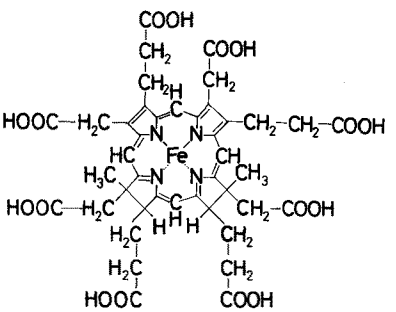

Fig. 12. The structure of siroheme. From Ref. 230a.

which the molecular weight was not determined [233], yielded E_m values of −120, +80 and −50 mV, respectively. A titration, using optical absorbance changes to monitor the oxidation state of the siroheme, yielded E_m values of −30 and −305 mV for monomeric and heterodimeric preparations, respectively, of the spinach enzyme [217]. The latter value is similar to the E_m = −340 mV value reported [235] for the siroheme prosthetic group of *E. coli* NADPH : sulfite oxidoreductase (EC 1.8.1.2, hereafter referred to as *E. coli* sulfite reductase).

In addition to siroheme, nitrite reductase contains an iron-sulfur cluster that is detectable by EPR spectroscopy [225,231–234,236]. Non-heme iron and inorganic sulfide content measurements indicate the presence of 4 non-heme irons and 4 inorganic sulfides per siroheme in spinach nitrite reductase [210,214,231]. EPR spectra of the reduced enzyme in 80% dimethylsulfoxide suggest that the enzyme contains a single [4Fe-4S] cluster, rather than two [2Fe-2S] clusters [231]. Oxidation-reduction titrations on the [4Fe-4S] cluster have not been performed with unambiguously heterodimeric preparations of the enzyme, but a pH-dependent E_m value has been determined for the monomeric squash enzyme (E_m values of −570, −615 and −660 mV were measured at pH values of 8.1, 8.7 and 9.8, respectively [234]) and an E_m value of −517 mV has been estimated for the barley enzyme at pH 7.5 [225]. The E_m value of the [4Fe-4S] cluster of a preparation of the spinach enzyme for which the molecular weight was not determined was estimated to be −550 mV at pH 9.0 [233].

Spinach, squash and barley nitrite reductases resemble spinach ferredoxin : sulfite oxidoreductase (EC 1.8.7.1, hereafter referred to as spinach sulfite reductase) and the hemoprotein subunit of *E. coli* sulfite reductase in prosthetic group content [237,238]. The two spinach enzymes resemble each other further in that both catalyze the ferredoxin- or methyl viologen-dependent reduction of nitrite to ammonia, sulfite to sulfide and hydroxylamine to ammonia [238]. Mössbauer [239] and EPR [240] studies of the *E. coli* sulfite reductase and EPR studies of spinach sulfite reductase [241] provided evidence for strong magnetic coupling, probably through a common bridging ligand, between the siroheme and the [4Fe-4S] cluster in both enzymes. Mössbauer [239], EPR and optical spectroscopic evidence [242] for a similar magnetic interaction between the [4Fe-4S] cluster and siroheme prosthetic groups of spinach nitrite reductase has also been obtained. X-ray data on the hemoprotein subunit of the *E. coli* sulfite reductase indicate that the iron of the siroheme and one of the non-heme irons of the [4Fe-4S] cluster are separated by only 0.44 nm and that the two irons appear to be bridged by a single amino acid, possibly a sulfur from one of the cluster cysteines [243]. There is a second site of interaction between the two prosthetic groups,

with one of the inorganic sulfides of the cluster making Van der Waals contact with an edge of the siroheme macrocycle [243]. In view of the many other similarities between nitrite and sulfite reductases, a similar arrangement of the prosthetic groups of chloroplast nitrite reductases should be considered likely.

V-B.2. Subunit composition

As indicated above, there has been controversy over the subunit composition of chloroplast nitrite reductases. Most groups working with the enzyme have reported that it is a monomeric protein, with a molecular mass between 61 and 63 kDa, depending on the species from which the enzyme was isolated [214,215,218–220,223–225]. However, two groups have reported that the spinach enzyme contains two subunits, of 61 and 24 kDa, respectively [209–213,217,232], and nitrite reductase isolated from the green alga *Chlamydomonas reinhardtii* was reported to contain a 63 kDa and a 25 kDa subunit [216]. The nitrite reductase isolated from etiolated bean shoots was also reported to be a heterodimer, with the two subunits having molecular masses of 66 and 35 kDa [222]. In the case of the spinach heterodimer, it was reported that both the siroheme and [4Fe-4S] prosthetic groups are located on the larger, 61 or 63 kDa, subunit [211,213,232]. Treatment of the spinach or *C. reinhardtii* enzymes to remove the smaller subunit had little or no effect on the ability of the larger subunit to catalyze nitrite reduction with reduced methyl viologen serving as the electron donor but did result in a large decrease in activity when reduced ferredoxin was supplied as the electron donor [211,213,216,217]. These results were interpreted in terms of a role for the smaller subunit in ferredoxin binding [211,213,216,217] and, indeed, the 24 kDa subunit of the spinach enzyme was shown to contain a relatively high-affinity ferredoxin-binding site [213]. The small subunit of the spinach enzyme was, by itself, devoid of catalytic activity, consistent with its lack of electron-carrying prosthetic groups [211,213,217]. The possible role of the small subunit and the question of whether the enzyme actually contains a second subunit will be discussed in more detail below.

The genes for two plant, ferredoxin-dependent nitrite reductases have been cloned and sequenced and the amino acid sequences for the spinach and maize enzymes have been deduced from these gene sequences [244,245]. It should be pointed out that these sequences are for the 63 kDa subunit and, in fact, no genetic evidence has yet been obtained for the existence of the small subunit as an intrinsic constituent of any ferredoxin-dependent nitrite reductase. Transcription of both the spinach [244] and maize [245] genes, and also of the genes coding for nitrite reductase in wheat [224], pea [246,247] and barley [248] depends on the presence of nitrate. Light appears to further stimulate gene expression [246,247]. As would be expected in the case of a protein encoded by a nuclear gene, synthesized on cytoplasmic ribosomes and subsequently transported across the chloroplast envelope into the stromal space, nitrite reductase is synthesized as a larger, precursor form and subsequently the N-terminal leader sequence is cleaved [224,244–247]. The mature spinach enzyme, which contains 562 amino acids and a deduced molecular mass of 62 883 Da, is derived by cleavage of a 32-amino-acid-long N-terminal extension [244]. The spinach and maize enzymes are 66% homologous at the amino acid level and 86% similar when conservative amino acid substitutions are taken into account [245]. Although these nitrite reductase sequences have not allowed any assignments to be made of amino acids likely to be involved in siroheme-binding, four conserved cysteines (located at positions 473, 479, 514 and 518 in the spinach sequence) that are likely to serve as ligands to the [4Fe-4S] cluster have been identified [244,245]. N-terminal amino acid analysis of the purified spinach enzyme (Refs. 214, 244, and also Hilliard, N., Hirasawa, M., Knaff, D.B. and Shaw, R.W., unpublished observations) revealed some heterogeneity that probably arises from proteolysis of the enzyme during isolation.

V-B.3. Nitrite binding

A large body of evidence has been obtained identifying the siroheme group of the enzyme as the site of nitrite binding. Addition of nitrite [209,211,215,218, 232,234,236] or of other ligands that act as inhibitors of the enzyme and are known to bind to hemes (e.g., cyanide [210,218,232,236] or CO [218]) results in perturbation of the siroheme optical absorbance spectrum. Addition of nitrite [218,225,232,236,249,250] or cyanide [210,218,232,236] to the oxidized enzyme eliminates or substantially diminishes the EPR signals characteristic of high-spin ferric heme. Features characteristic of low-spin, six-coordinate heme have been observed in the EPR spectrum of the cyanide complex of nitrite reductase [232]. Resonance Raman spectra of the enzyme and of its cyanide complex are also consistent with the conclusion that binding of this ligand produces a transition from high-spin to low-spin heme [251]. EPR investigations of the enzyme/nitrite complex have failed to detect any low-spin signals that can be unambiguously attributed to this complex (see Refs. 249 and 250 for a detailed discussion of this phenomenon). Features indicating the presence of a small amount of low-spin siroheme have been observed in the EPR spectrum of the resting spinach and barley enzymes [225,232,249]. There is a recent report that addition of nitrite to the barley enzyme resulted in a broadening of this low-spin, minor species [225] but this phenomenon was not observed with the spinach enzyme [249].

Nitrite reductase can also bind NO and the NO/fer-

rous siroheme adduct has been detected by observation of its characteristic EPR signal [210,211,225,231,234, 236,252]. Analysis of differences in the hyperfine structure observed in the EPR spectra of the ^{14}NO and ^{15}NO ferrous siroheme/adducts for the enzyme confirms that siroheme is the binding site for this ligand [225,252]. Quantitation of the EPR signal arising from the NO complex indicates that 1 mole of NO can be bound per mole of siroheme [231]. Nitrite reductase also binds hydroxylamine, which can be reduced by the enzyme and has been proposed as a possible intermediate formed during the reduction of nitrite to ammonia [210,218]. Perturbation of the optical absorbance spectrum of the enzyme indicates that the siroheme provides the binding site for this ligand [210,218]. Perturbations in the optical and EPR spectra of the enzyme by sulfite, which like nitrite can undergo a six-electron reduction catalyzed by the enzyme, support the conclusion that this ligand also binds to the siroheme group of nitrite reductase [210,218,249].

The high-spin EPR signal observed with the oxidized, resting enzyme has been quantitated and accounts for most of the siroheme present in the enzyme [232,249]. Although the resonance Raman spectrum of the resting enzyme has been interpreted in terms of six-coordinate, high-spin siroheme [251], the EPR spectrum of resting nitrite reductase is equally consistent with the siroheme iron either being five-coordinate or being coordinated to a weakly bound sixth ligand. Interpretation of the resonance Raman spectra of nitrite reductase is somewhat difficult because of the lack of Raman data for model siroheme compounds of unambiguously known spin states [251]. In the case of the siroheme in the structurally similar *E. coli* sulfite reductase, the siroheme iron has been shown by ENDOR spectroscopy to be five-coordinate [253]. If the siroheme iron in chloroplast nitrite reductases is five-coordinate or contains a weakly bound sixth axial ligand, binding of nitrite to this site should be facile. Binding of nitrite would be further facilitated if, as appears to be the case in the *E. coli* sulfite reductase [243], the siroheme is located close to the surface of the protein and the vacant sixth coordination position on iron is freely accessible to the solvent.

Equilibrium dialysis measurements [231,232] and titrations of the spectral perturbations caused by the addition of nitrite [215] indicate a 1 : 1 ratio between the number of binding sites for nitrite on spinach nitrite reductase and the siroheme content of the enzyme. The spectral perturbations that result from nitrite binding to oxidized nitrite reductase have been used to quantitatively measure the binding affinity of the spinach enzyme for this substrate [215,232,254]. The binding data reported by Mikami and Ida could be fitted to an equation describing a single binding isotherm with K_d = 10 μM [215], identical within the experimental uncertainties to the 11 μM value reported earlier by Hirasawa et al. [254]. The 10 μM value reported by Mikami and Ida for K_d was identical to the 10 μM value observed as the K_m value for nitrite in kinetic assays with reduced ferredoxin serving as the electron donor [215]. Of interest is the oservation that the apparent K_m for nitrite depends on the electron donor used during the assay, with the value being considerably higher, 110 μM, when reduced ferredoxin was replaced by reduced methyl viologen [214,215]. This effect of electron donor on the K_m for the reducible substrate suggests the possibility that the separate binding sites for nitrite and ferredoxin may exhibit cooperativity, as is the case for the two substrate-binding sites in FNR (see above). Measurements of the effect of nitrite on the affinity of nitrite reductase for ferredoxin [213] also support the idea of cooperativity between the two substrate-binding sites on the enzyme (see below). A series of measurements in our laboratory, made on the heterodimeric preparation of the enzyme, indicated that binding of both nitrite and cyanide showed cooperativity, with Hill coefficients of 1.6–1.8 and 2.3–2.8, respectively, observed for the binding of the two ligands [232]. Plots of enzyme activity versus nitrite concentration were sigmoidal, with a Hill coefficient of 2.4 [232]. These data were interpreted in terms of cooperativity in nitrite binding between two siroheme groups [232]. However, more recent N-terminal amino acid analyses of the heterodimeric preparation indicate that heterogeneity in the enzyme preparation may provide an alterative explanation for non-hyperbolic kinetic and binding profiles (Hilliard, N., Hirasawa, M., Knaff, D.B. and Shaw, R.W., unpublished observations).

Nitrite binding to the enzyme may cause conformational changes in the enzyme, as suggested by changes produced in the CD spectrum that accompany nitrite binding to the enzyme. These changes occur both in the visible region [255], where they presumably reflect changes in the environment of the siroheme, and in the ultraviolet region (Hirasawa, M. and Knaff, D.B., unpublished observations). In the latter case, the changes in the CD that occur on nitrite binding presumably reflect changes in the secondary structure of the enzyme. The presence of relatively low concentrations of nitrite specifically protects the enzyme from denaturation at low ionic strength [256], an observation that is also consistent with the occurrence of nitrite-induced conformational changes in the enzyme. Nitrite binding also causes changes in absorbance in the region from 250 to 300 nm [232,254], indicating the substrate binding to nitrite reductase may perturb the environement of one or more aromatic amino acids.

V-B.4. Ferredoxin binding

The first evidence supporting electrostatically stabilized complex formation between ferredoxin and nitrite

reductase came from observations that the spinach enzyme could bind to a ferredoxin-Sepahrose affinity column at low ionic strength and could be eluted from the affinity column at high ionic strength [209,211,213,214, 257–259]. Investigations in our laboratory and in that of Kamin initially suggested that the monomeric, 63 kDa form of the enzyme could not bind to a ferredoxin-Sepharose affinity column [213,260], while the heterodimeric preparation of the enzyme [213] and the 24 kDa subunit itself [213] did bind to the affinity column. These results, and the observation that loss of the small subunit from either spinach or *C. reinhardtii* nitrite reductase specifically inhibited the ferredoxin-dependent activity of the enzymes with little effect on the methyl viologen-dependent activity [211,213,216,217], lead to the proposal that the small subunit of nitrite reductase contained the major binding site for ferredoxin bound on the enzyme. Although reconstitution experiments designed to increase the rate of ferredoxin-dependent reduction of nitrite by adding the small subunit to the prosthetic group-containing large subunit proved unsuccessful (Hirasawa, M. and Knaff, D.B., unpublished observations), additional support for the hypothesis that the 24 kDa subunit contained a ferredoxin-binding site came from spectral perturbation measurements showing that the small subunit could bind ferredoxin at low ionic strength with $K_d = 4.4$ μM [213].

In recent years it has become clear that 61 to 63 kDa, monomeric forms of nitrite reductase, prepared from a number of different higher plants, exhibit high activity with ferredoxin serving as the electron donor and bind to ferredoxin-Sepharose affinity columns [214,215,224, 225,238,256,261]. Furthermore, spectral perturbation measurements in our laboratory showed that, although removing the small subunit resulted in a decrease in the affinity of the enzyme for ferredoxin (K_d increased from 0.63 to 11 μM), the large subunit is capable of binding ferredoxin [217]. It was thus proposed that, while optimum ferredoxin binding requires the presence of the small subunit, at least a portion of the ferredoxin-binding site resides on the large subunit [217]. Additional evidence for the presence of a ferredoxin-binding site on the large subunit came from observations that ferredoxin protects a monomeric form of the enzyme against denaturation at low ionic strengths with an apparent K_d of 1 μM and that ferredoxin can be cross-linked to monomeric nitrite reductase in the presence of EDC [256]. It must be mentioned that three recent preparations of nitrite reductase in our laboratory, using protocols that had previously resulted in unambiguously dimeric preparations of the enzyme, produced a monomeric, 63 kDa form of the enzyme with high ferredoxin-dependent activity. No evidence for the presence of the 24 kDa subunit was found in the final purification stages of these preparations (Hilliard, N., Hirasawa, M., Knaff, D.B. and Shaw, R.W., unpublished observations). The arguments for the existence of a second nitrite reductase subunit, involved in ferredoxin binding, are thus currently under critical reexamination in our laboratory.

Additional evidence for electrostatic complex formation between ferredoxin and nitrite reductase has been obtained from the observation that the two proteins co-migrate during gel-filtration chromatography at low ionic strength but not at high ionic strength [262]. Alterations in the CD spectra also have been observed on mixing of the two proteins at low but not high ionic strength (Ref. 255, Hirasawa, M. and Knaff, D.B., unpublished observations). Perturbations of absorbance spectra in the visible region have been observed on addition of ferredoxin to both heterodimeric [213] and monomeric [215] preparations of the spinach enzyme, providing further evidence for complex formation between the two proteins. Although the difference spectra reported for ferredoxin binding to spinach nitrite reductase were different for the two different preparations of the enzyme, similar K_d values, 0.63 μM [213] and 1.0 μM [215], for ferredoxin were obtained in these studies. The fact that these spectral perturbations were observed only at low ionic strength [213,215], provides additional support for electrostatic stabilization of the complex. Spectral perturbation measurements have also been utilized to demonstrate formation of a ternary complex between the enzyme, nitrite and ferredoxin, establishing unequivocally that the enzyme contains distinct binding sites for its two substrates [213]. As in the case of FNR, there appears to be negative cooperativity between the two sites, with the K_d for ferredoxin binding increasing from 0.63 μM for nitrite reductase alone to 11 μm for the enzyme/nitrite complex [213].

As mentioned above, chemical modification of three or four carboxyl groups on ferredoxin to remove the negative charges leads to a decreased affinity of ferredoxin for FNR. A similar effect of modification of ferredoxin carboxyl groups has been observed for the interaction of ferredoxin with nitrite reductase [262]. The K_d determined for binding the modified ferredoxin to spinach nitrite reductase was considerably larger, 100 μM, than that determined for unmodified, native ferredoxin [262]. Modification of ferredoxin carboxyl groups by EDC, after ferredoxin lysine groups were first methylated by treatment with dimethylamine borane to prevent EDC-induced dimerization of ferredoxin, also resulted in a decreased affinity of ferredoxin for spinach nitrite reductase [256]. These observations are consistent with the hypothesis that ferredoxin may have a common binding site, defined by a group of negatively charged amino acids, for a number of ferredoxin-dependent enzymes [262]. Additional evidence in support of this hypothesis, in so far as FNR and nitrite reductase are concerned, has been obtained from both cross-linking

[256] and immunological [263] experiments. Cross-linking studies in which EDC was added to mixtures of ferredoxin, nitrite reductase and FNR produced no ternary cross-linked complexes, but instead resulted only in formation of binary complexes with ferredoxin cross-linked to either FNR or to nitrite reductase [256]. The apparent competition observed in the cross linking experiments (i.e., the amount of FNR cross-linked to ferredoxin decreased as the amount of nitrite reductase present was increased and vice versa) are consistent with a common binding site, or overlapping binding sites, for the two enzymes on ferredoxin [256]. In the immunological studies, it was shown that antibodies raised in either rabbit or mouse against spinach FNR recognized spinach nitrite reductase, demonstrating that these two chloroplast, ferredoxin-dependent enzymes have at least one antigenic determinant in common [263]. The observation that the antibodies against FNR inhibited the enzymatic activity of nitrite reductase to a much greater extent when reduced ferredoxin served as the electron donor than when reduced methyl viologen supplied electrons suggests that the common antigenic determinants are at the ferredoxin-binding sites of the two enzymes [263]. Evidence will be presented below that the ferredoxin-binding site of a third chloroplast, ferredoxin-dependent enzyme, glutamate synthase, contains a ferredoxin-binding site that is structurally related to those of FNR and nitrite reductase.

V-C. Glutamate synthase

Ferredoxin-dependent glutamate synthase (EC 1.4.7.1), which catalyzes the following reaction:

$$\text{glutamine} + \text{2-oxoglutarate} + 2\text{ferredoxin}_{\text{Red}} \rightarrow 2\text{glutamate} + 2\text{ferredoxin}_{\text{Ox}}$$

is located in the chloroplasts of higher plants and algae and differs from the NADH-dependent enzyme (EC 1.4.1.14), which is known to be present in some non-green plant tissues [264,265]. There are also early reports of glutamate synthases in pea roots [266] and developing soybean cotyledons [267] that could utilize either NADH or reduced ferredoxin as electron donors. Ferredoxin-dependent forms of the enzyme have been identified in a number of higher plants including pea [268,269], field bean [270], corn [271], spinach [272,273], rice [274,275] and tomato [276,277]. The enzyme has also been isolated from the green alga *C. reinhardtii* [278]. Both the NADH-dependent and the ferredoxin-dependent enzyme were found to be present in etiolated pea shoots [269]. During greening of the pea shoots, the amount of NADH-dependent enzyme remained constant, while the amount of ferredoxin-dependent enzyme increased along with increasing chlorophyll content [279,280]. This developmental pattern, which has also been observed in barley [281], suggests that a regulatory relationship exists between expression of the gene coding for ferredoxin-dependent glutamate synthase and the presence of the photochemical apparatus needed to generate the photoreduced ferredoxin required for the enzyme to function. Recent immunocytochemical studies on tomato [282] have confirmed earlier data from cell fractionation studies [268,279,279a,b] which indicated that ferredoxin-dependent glutamate synthase is localized in the chloroplast, as might be expected in view of the localization of ferredoxin in this organelle. Spinach and *C. reinhardtii* glutamate synthases, both of which have been purified to homogeneity, are the best characterized of these ferredoxin-dependent enzymes in terms of what is known about their ability to interact with ferredoxin. The remaining portion of this section will thus concentrate on these two glutamate synthases.

V-C.1. Peptide composition and prosthetic group content

Both the spinach and *C. reinhardtii* enzymes are monomeric proteins. The molecular mass of the *C. reinhardtii* enzyme was estimated as 144 kDa from a combination of Stokes radius and sedimentation coefficient data, estimated as 144.5 kDa from electrophoresis data under non-denaturing conditions and as 151 kDa from electrophoresis data obtained under denaturing conditions [278]. The molecular mass of the spinach enzyme was estimated to b 140 kDa from gel-filtration data and to be between 150 and 170 kDa from electrophoresis data obtained under denaturing conditions [273,286]. Molecular masses between 145 and 180 kDa have been estimated for the bean [270] and corn [271] enzymes. Recent Western blot experiments in our laboratory, using a monospecific antibody raised against spinach glutamate synthase, indicate that the pea enzyme is antigenically related to the spinach enzyme and has a very similar molecular mass (Hirasawa, M., Nalbantoglu, B., Nguyen, H. and Knaff, D.B., unpublished observations). In contrast, rice glutamate synthase was reported to have a molecular mass of 224 kDa and to be composed of two possibly identical subunits with 115 kDa [274]. Antibodies raised against the *C. reinhardtii* enzyme were effective in inhibiting both the ferredoxin-dependent and methyl viologen-dependent activities of extracts from a number of green algae but had little effect on extracts prepared from spinach and other higher plants or from cyanobacteria [283]. This observation indicates that, although *C. reinhardtii* and spinach glutamate synthases may resemble each other in some ways (see below), enough differences are present between the two enzymes to render them antigenically distinct.

Spectra of spinach and *C. reinhardtii* glutamate synthases established that they are both flavin-containing

enzymes [273,278]. Chemical analyses indicate that both enzymes contain one FAD and one FMN [273,284]. Both enzymes also contain non-heme iron and inorganic sulfide, suggesting the presence of one or more iron-sulfur clusters. Analysis of the *C. reinhardtii* enzyme yielded values of 3 mol of iron and 4 mol of sulfide per mol of enzyme [284], while values of 4 mol of iron and 4 mol of sulfide per mol of enzyme were reported for spinach glutamate synthase [273]. No EPR signals were observed at 6 K with samples of the *C. reinhardtii* enzyme that had been preincubated under strongly reducing conditions [284], conditions under which both [2Fe-2S] or [4Fe-4S] clusters should be reduced and would be expected to exhibit EPR signals. From this observation and the iron content of the enzyme, it was concluded that *C. reinhardtii* glutamate synthase probably contains a [3Fe-xS] cluster [284]. However, although the oxidized forms of [3Fe-xS] clusters are known to exhibit characteristic, relatively sharp EPR signals [285], no such signals were observed with samples of the *C. reinhardtii* enzyme [284]. As there are a number of reasons why EPR signals from [2Fe-2S] or [4Fe-4S] clusters present in the enzyme might not have been observed, it would seem prudent to reserve judgement as to the nature of the iron-sulfur cluster(s) in *C. reinhardtii* glutamate synthase until additional spectroscopic evidence is obtained. EPR measurements have not yet been performed on spinach glutamate synthase, but circular dichroism measurements were consistent with the presence of two [2Fe-2S] clusters in the spinach enzyme [263]. However, as circular dichroism spectra do not allow unambiguous identification of iron-sulfur clusters, additional spectroscopic measurements must be conducted before any firm conclusions can be drawn as to the nature of the iron-sulfur cluster(s) in the spinach enzyme. No information is yet available about E_m values for any of the prosthetic groups in either the spinach or *C. reinhardtii* enzyme. It should be pointed out that the rice leaf enzyme contains neither flavin nor non-heme iron, nor inorganic sulfide [274]. Thus the rice enzyme differs from other leaf glutamate synthases in terms of prosthetic group content as well as subunit composition.

V-C.2. Ferredoxin binding

Complex formation between spinach glutamate synthase and ferredoxin at low ionic strength has been demonstrated by spectral perturbation measurements [262], co-chromatography during gel filtration [262], changes in circular dichroism spectra [263] and cross-linking [263,286]. No evidence for interaction between the two proteins could be observed at high ionic strengths. The ionic strength dependence of the interaction between spinach glutamate synthase and ferredoxin suggests that the glutamate synthase/ferredoxin complex, like those between ferredoxin and FNR or nitrite reductase, is stabilized by electrostatic forces. Titrations of the absorbance changes [262] and CD changes [263] caused by adding ferredoxin to glutamate synthase produced data that fit single binding isotherms with K_d values of 9 μM and 14.5 μM, respectively. These two values for K_d agree to within the experimental uncertainties present in the measurements. Membrane ultrafiltration binding assays [263] and cross-linking data [286] suggest that the ferredoxin to glutamate synthase stoichiometry in the complex is 2 : 1. Modification of 3 to 4 carboxyl groups on spinach ferredoxin, a treatment that decreased the ability of ferredoxin to bind both FNR and nitrite reductase (see above), also decreased the ability of ferrodoxin to bind glutamate synthase [262]. Titrations of optical spectral perturbations indicated that this modification of ferredoxin increased the K_d for binding by glutamate synthase from 14.5 μM to 100 μM [262]. Modification of ferredoxin carboxyl groups also increased the K_m for ferredoxin from 2 μM to 84 μM [262]. These observations suggest that ferredoxin supplies the negatively charged groups involved in complex formation with spinach glutamate synthase. Furthermore, the similar effects of ferredoxin modification on its ability to bind to FNR, nitrite reductase and glutamate synthase are consistent with the hypothesis that ferredoxin has a common, negatively-charged binding site for all three of these chloroplast enzymes.

Additional evidence for similar modes of interaction between ferredoxin and these three ferredoxin-dependent, chloroplast enzymes comes from the observation that rabbit and mouse antibodies against spinach FNR can also recognize spinach nitrite reductase (see above) and glutamate synthase [263]. As is the case for nitrite reductase, the antibody raised against FNR inhibits glutamate synthase to a considerable greater extent if reduced ferredoxin serves as the electron donor than if reduced methyl viologen serves as the source of electrons [263]. These observations have been interpreted in terms of antigenically related determinants at the ferredoxin-binding sites of all three enzymes [263]. In the case of spinach glutamate synthase, evidence in support of this hypothesis comes from recent observations on the enzymatically active, EDC cross-linked complex between ferredoxin and glutamate synthase. Although antibodies raised against FNR recognize glutamate synthase itself, they were unable to recognize the cross-linked complex, implying that when ferredoxin binds to glutamate synthase the site on glutamate synthase recognized by the antibody against FNR is no longer accessible to the antibody [286].

Ferredoxin-binding studies have not yet been carried out with *C. reinhardtii* glutamate synthase. However, the observation that this enzyme binds to a ferredoxin-Sepharose affinity column at low ionic strength and is eluted at high ionic strength [278] provides evidence for electrostatic interaction between the two proteins. Im-

munological experiments indicate that glutamate synthase and nitrite reductase isolated from this green alga have at least one antigenically similar determinant in common [283,287]. Antibody raised against *C. reinhardtii* glutamate synthase was much more effective in inhibiting the reaction catalyzed by *C. reinhardtii* nitrite reductase when reduced ferredoxin served as the electron donor than when reduced methyl viologen served as the electron donor [287]. Furthermore, ferredoxin was able to protect *C. reinhardtii* nitrite reductase against immunoprecipitation by antibodies raised against glutamate synthase [287]. These observations, taken together, are most readily interpreted in terms of the antigenically similar determinants on the two *C. reinhardtii,* ferredoxin-dependent enzymes being located at the ferredoxin-binding sites [287].

VI. Ferredoxin: thioredoxin reductase

In recent years it has become clear that a number of reactions in metabolic pathways that are localized in chloroplasts are regulated through changes in the oxidation state of regulatory dithiol/disulfide groups on chloroplast enzymes [288–290]. These reactions are mediated by thioredoxin, which is reduced in a reaction catalyzed by the enzyme ferredoxin: thioredoxin reductase (hereafter abbreviated FTR), utilizing reduced ferredoxin as the electron donor. Thioredoxin subsequently reduces a disulfide to two cysteine thiols on the regulated enzymes (Fig. 13). Thioredoxins are low-molecular-weight proteins that exist in two oxidation states that differ only by the presence of a disulfide (cystine) in oxidized thioredoxin instead of a dithiol (two cysteines) in reduced thioredoxin [291–293]. Thioredoxins play a number of roles in non-photosynthetic organisms, for example serving as a reductant in the conversion of ribonucleotides to deoxyribonucleotides and as a subunit of a phage DNA polymerase [291–293]. A description of these functions of thioredoxins lies outside the scope of this article, but interested readers should consult Refs. 291–293 for excellent reviews.

In photosynthetic organisms, thioredoxins carry out many of the same functions as in non-photosynthetic organisms and also serve as a key component in the

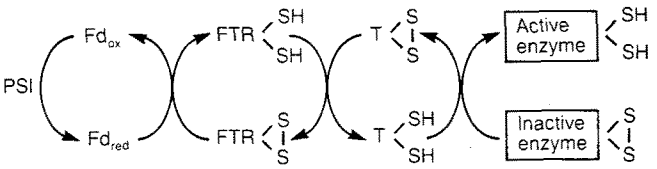

Fig. 13. Scheme for thioredoxin-dependent regulation of chloroplast enzymes. PSI represents the components of the Photosystem I reaction center involved in ferredoxin photoreduction. Fd, FTR and T denote ferredoxin, ferredoxin: thioredoxin reductase and thioredoxin, respectively. From Ref. 290.

regulation of carbon metabolism and of the chloroplast CF_1 ATP synthase. The discussion of plant thioredoxins presented below will be confined to these regulatory functions. The discussion of thioredoxin reduction will be confined to the reactions that lead from reduced ferredoxin to thioredoxin via FTR and will not deal with the cytosolic system in which thioredoxin *h* is reduced by NADPH in a reaction catalyzed by a 68 kDa flavoprotein reductase [294]. As might be expected, the thioredoxins involved in the activation of chloroplast enzymes, thioredoxins *f* and *m*, appear to be localized in the chloroplast [295]. In the case of spinach thioredoxin *f*, it has been established that the protein is coded for by a nuclear gene and is synthesized as a precursor larger than the mature protein [296]. It has been established that the N-terminal leader sequence of this thioredoxin is subsequently cleaved after passage across the chloroplast envelope, but there is some uncertainty as to the exact location of the cleavage site [296]. Before describing what is known about FTR and its interaction with ferredoxin, brief descriptions of the properties of thioredoxins and the regulatory sites on their target enzymes will be presented.

VI-A. Thioredoxin

Two different types of thioredoxin are found in the chloroplasts of higher plants and algae and are classified as either *m*- or *f*-type thioredoxins on the basis of their preferential ability to activate either $NADP^+$-dependent malate dehydrogenase or fructose-1,6-bisphosphatase, respectively [288]. Thioredoxin *f* is capable of an extremely rapid activation of fructose-1,6-bisphosphatase and is also capable of activating $NADP^+$-dependent malate dehydrogenase. Thioredoxin *m* effectively activates malate dehydrogenase but cannot activate fructose-1,6-bisphosphatase [295]. Amino acid sequences have been determined for both thioredoxin *f* [296] and thioredoxin *mb* [297] from spinach and for a thioredoxin *m* from *C. reinhardtii* [298]. The *m*-type thioredoxins from these two photosynthetic eukaryotes exhibit considerable sequence homology with thioredoxins from cyanobacteria [297,298]. The amino acid sequence of spinach thioredoxin *f* is, surprisingly, more closely related to mammalian and avian thioredoxins than to those of *m*-type thioredoxins from photosynthetic organisms [296]. Spinach and *C. reinhardtii* *m*-type thioredoxins exhibit 47% and 41% sequence homology, respectively, with *E. coli* thioredoxin [297,298]. Spinach thioredoxin *f* shows considerably less sequence homology, 24%, with the *E. coli* protein, but does contain the well-conserved active site sequence (...Cys-Gly-Pro-Cys...) found in *E. coli* thioredoxin and in the *m*-type thioredoxins [296]. Both spinach thioredoxins *m* and *f* can effectively replace *E. coli* thioredoxin in producing an active phage T7 DNA

polymerase [299]. *E. coli* thioredoxin can replace spinach thioredoxin *m* in activating $NADP^+$-dependent malate dehydrogenase but cannot replace spinach ferredoxin thioredoxin *f* as an activator for fructose-1,6-bisphosphatase [300,301]. The results of these activity studies indicate that there are likely to be both significant similarities and differences in the structures of the two spinach thioredoxins and that of *E. coli* thioredoxin.

A 0.28 nm resolution X-ray structure of oxidized *E. coli* thioredoxin has been available for some time [302] and recently the structure has been refined to 0.17 nm resolution [303]. The region around the active site disulfide forms a protrusion on the surface of the protein. There are several hydrophobic residues in the vicinity of the disulfide bond of *E. coli* thioredoxin that have been implicated in the interaction of thioredoxin with other proteins [304]. This conserved hydrophobic 'patch' is also present in spinach thioredoxin *f* [296]. However, it may not be involved in the interaction between thioredoxin *f* and its target enzyme, fructose-1,6-bisphosphatase, as *E. coli* thioredoxin cannot substitute for thioredoxin *f* in activating this enzyme (see above). No structural information on reduced *E. coli* thioredoxin is available from X-ray diffraction studies, but NMR studies indicate that the overall structures of the oxidized and reduced proteins are quite similar [305–307]. There are, however, significant local conformational changes in the region near the active site [305–307]. The availability of an increasing number of thioredoxin sequences, of structural data from NMR studies on thioredoxins other than the *E. coli* protein [308] and of information from reactivity studies with hybrid thioredoxins [309] and with thioredoxin mutants [310] constructed by site-directed mutagenesis should lead to rapid progress in our understanding of how thioredoxins interact with other proteins.

VI-B. Reduction of target enzymes

In vitro studies with purified components have established that the activation of chloroplast enzymes by reduced thioredoxin involves the reduction of one regulatory disulfide bond per subunit on each enzyme, with the concomitant oxidation of reduced thioredoxin [311–317]. Recent experiments using a membrane-permeable, thiol-labeling reagent have established that a similar series of events occur in intact chloroplasts [318]. There have been relatively few determinations of the E_m values of these sulfhydryl groups, but those for the active site dithiol/disulfide in corn thioredoxin *m* and corn $NADP^+$-dependent malate dehydrogenase have been estimated to be -300 and -330 mV, respectively [319]. An early estimate of the E_m value for yeast thioredoxin yielded a more positive value, -240 mV, than that reported for corn thioredoxin [320]. The reduction of at least some of the target enzymes by thioredoxin appears to involve complex formation between the proteins [321–323]. Amino acid sequences are now available for the regulatory sites of three soluble, chloroplast enzymes: fructose-1,6-bisphosphatase [312, 323a], $NADP^+$-dependent malate dehydrogenase [311] and phosphoribulokinase [324]. In the case of the first two enzymes, the regulatory site is separated from the active site [318,323], while in the case of phosphoribulokinase, one of the regulatory cysteines is also part of the active site [318,324,325]. The regulatory cysteines on the chloroplast CF_1 ATP synthase [326] have also been identified [327,328].

VI-C. FTR subunit and prosthetic group content

FTR has been purified to homogeneity from spinach, corn and the cyanobacterium *Nostoc muscorum* [329]. In all three cases, the enzyme has a molecular mass of approx. 30 kDa and is composed of two different kinds of subunit [329]. In terms of molecular mass and antigenic properties, one of the subunits, of 13 kDa, appears to be similar in all three enzymes, while the second subunit (16, 15 and 7 kDa in spinach, corn and *N. muscorum,* respectively) is more variable [329]. The amino acid compositions and N-terminal amino acid sequences of spinach FTR have recently been determined [329a]. The catalytically active disulfide, which becomes reduced to two cysteine thiols when the enzyme reacts with reduced ferredoxin, is located on the similar, 13 kDa subunit [318,329,330]. These two cysteines on the 13 kDa subunit become reoxidized to a disulfide when FTR reduces thioredoxin [318,330].

FTR contains 4 non-heme iron and 4 inorganic sulfides and the absorbance spectra of the spinach, corn, and *N. muscorum* proteins are all consistent with the presence of at least one iron-sulfur cluster [329]. It is likely that FTR is identical to the protein earlier called 'ferralterin' [329]. If this is the case, studies with ferralterin had previously established that the iron-sulfur cluster of the protein is EPR-silent under reducing conditions and exhibits a rhombic EPR signal at low temperatures (g values of 2.10, 2.05 and 2.00 and 2.09, 2.04 and 1.98 were observed for the spinach and *N. muscorum* proteins, respectively) when the protein is oxidized [331]. A value of $+410$ mV was estimated for the E_m of the *N. muscorum* enzyme [331]. These results are consistent with the cluster being either a 'HiPIP-type' [4Fe-4S] or a [3Fe-xS] cluster [285]. Circular dichroism spectra of spinach FTR are most consistent with the presence of an iron-sulfur cluster of the [4Fe-4S] type [332], but additional spectroscopic measurements will be required before the nature of the iron-sulfur cluster can be decided unambiguously. Although it has been demonstrated that the iron-sulfur cluster in FTR can be photoreduced by illuminated chloroplast membranes [331], the role of this cluster in the reaction catalyzed by FTR is unclear.

VI-D. Ferredoxin binding

Although few details are known about the interaction of FTR and thioredoxin, a picture is beginning to emerge of how FTR interacts with its other substrate, ferredoxin. Early studies suggested that FTR could form a tight complex with ferredoxin (see Ref. 329 for a discussion of these observations) and it was known that the enzyme would bind to a ferredoxin-Sepharose affinity column at low ionic strength and could be eluted from the affinity column at high ionic strength [333]. Recently, complex formation between spinach FTR and ferredoxin at low ionic strength has been demonstrated directly by monitoring the perturbations in both absorbance and circular dichroism spectra that occur when the two proteins are mixed and by co-chromatography of the two proteins during gel-filtration chromatography [332]. No evidence for any interactions between FTR and ferredoxin could be observed at high ionic strength [332]. Thus, as in the cases of ferredoxin complexes with FNR, nitrite reductase and glutamate synthase, the FTR/ferredoxin complex appears to be stabilized by electrostatic forces. The apparent molecular mass of the complex, as estimated by gel-filtration chromatography, and titrations of the spectral perturbations caused by adding ferredoxin to FTR, suggest that the stoichiometry of ferredoxin to FTR in the complex is 1 : 1 [332]. The binding between the two proteins at low ionic strength was so tight that an accurate value for K_d could not be determined, but an upper limit of 100 nM was estimated [332].

Chemical modification of three or four carboxyl residues on ferredoxin had no measurable effect on either the binding of ferredoxin to FTR or on the K_m of the enzyme for ferredoxin [332]. A 30% decrease in the maximum velocity of the reaction was observed when modified ferredoxin replaced native ferredoxin in an assay system coupling FTR to spinach thioredoxin *m* and corn $NADP^+$-dependent malate dehydrogenase [332]. This is in striking contrast to the effects of this modification of ferredoxin on its ability to interact with FNR, nitrite reductase and glutamate synthase (see above). The absence of any detectable effect of this ferredoxin modification on the FTR/ferredoxin complex may result from the presence of an interaction site on ferredoxin for FTR that differs from the putative, common interaction site for FNR, nitrite reductase and glutamate synthase. Alternatively, the binding between FTR and ferredoxin may be so tight that the effect of decreases in binding affinity resulting from the modification of ferredoxin are too small to detect.

VII. Conclusion

The information supplied above should give the reader a feeling for the considerable progress that has been made in recent years in elucidating the mechanism by which ferredoxin becomes photoreduced during oxygenic photosynthesis and how the reduced ferredoxin is productively reoxidized by a number of ferredoxin-dependent enzymes. Fig. 14 summarizes the central role of ferredoxin in distributing electrons derived from the photosynthetic oxidation of water to several of these enzymes. The limitations of space were such that this article could not discuss all of the ferredoxin-dependent enzymes that have been investigated. It should be mentioned that for sulfite reductase, one of the chloroplast enzymes shown in Fig. 14 but not discussed in this review, evidence has been obtained for electrostatic complex formation between ferredoxin and this ferredoxin-dependent enzyme [334]. The cyanobacterium *Plectonema boryanum* contains a novel, ferredoxin-dependent nitrate reductase [335] and there is some evidence from affinity chromatography that suggests that cyanobacterial nitrate reductases may form complexes with ferredoxin [336]. Newly discovered, ferredoxin-dependent enzymes, such as a spinach choline monooxygenase [337], have yet to be investigated in terms of their capacity to interact with ferredoxin. The opportunity to characterize ferredoxin binding by these enzymes and the availability of tools to characterize in detail the ferredoxin-binding sites of the enzymes discussed in this review would appear to make the immediate future in this area of research truly exciting.

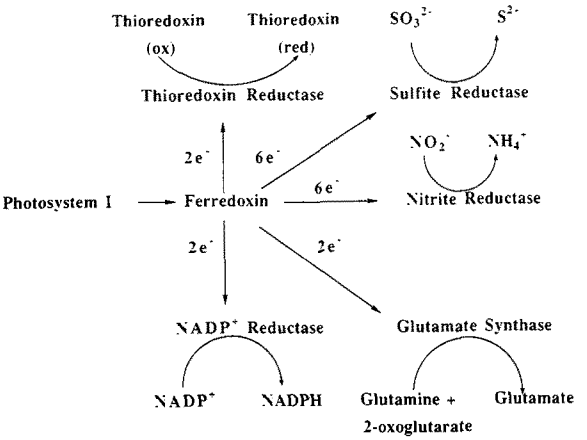

Fig. 14. The role of ferredoxin as an electron donor in chloroplasts.

Acknowledgements

The authors would like to thank Prof. John Biggins, Dan Davis, John Golbeck, Andrew Karplus, Richard Malkin, Peter Schürmann, Robert Shaw and Giuliana Zanetti and Ms. April Zilber for their helpful comments and/or providing copies of manuscripts in advance of publication. Research in the authors' laboratory was supported, in part, by grants from the U.S. Department of Agriculture and the Robert A. Welch Foundation.

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