In plants, phytochromobilin synthase (HY2) synthesizes the open chain tetrapyrrole chlorophore for light-sensing phytochromes. It catalyzes the double bond reduction of a heme-derived tetrapyrrole intermediate biliverdin IXα (BV) at the A-ring diene system. HY2 is a member of ferredoxin-dependent bilin reductases (FDBRs), which require ferredoxins (Fds) as the electron donors for double bond reductions. In this study, we investigated the interaction mechanism of FDBRs and Fds by using HY2 and Fd from Arabidopsis thaliana as model proteins. We found that one of the six Arabidopsis Fds, AtFd2, was the preferred electron donor for HY2. HY2 and AtFd2 formed a heterodimeric complex that was stabilized by chemical cross-linking. Surface-charged residues on HY2 and AtFd2 were important in the protein-protein interaction as well as BV reduction activity of HY2. These surface residues are close to the iron-sulfur center of Fd and the HY2 active site, implying that the interaction promotes direct electron transfer from the Fd to HY2-bound BV. In addition, the C12 propionate group of BV is important for HY2-catalyzed BV reduction. A possible role for this functional group is to mediate the electron transfer by interacting directly with AtFd2. Together, our biochemical data suggest a docking mechanism for HY2:BV and AtFd2.

Photosynthetic organisms absorb light as their energy source. To enable them to harvest and sense light, these organisms use tetrapyrrole molecules as light-absorbing chromophores. Open chain tetrapyrroles can function as the chromophores of light-sensing phytochromes and light-harvesting phycobilisomes. To synthesize tetrapyrrole chlorophores, the heme is first linearized by heme oxygenase into the open-chain tetrapyrrole intermediate, biliverdin IXα (BV). BV is subsequently reduced by different ferredoxin-dependent bilin reductases (FDBRs) with distinct double bond specificities. In cyanobacteria, red algae, and cryptomonads, BV is converted into chromophores of light-harvesting phycobiliproteins (1). Two enzymes 15,16-dihydrobiliverdin:ferredoxin oxidoreductase (PebA; EC 1.3.7.2) and phycocerythrobilin (PCB):ferredoxin oxidoreductase (PebB; EC 1.3.7.3) catalyze double bond reductions first on the C15-C16 methine bridge, then on the A-ring of BV to yield PCB. A recently identified Pcb synthase (PcbS) can itself catalyze the two double bond reductions to yield PCB (2). Another single enzyme phycocyanobilin (PCB):ferredoxin oxidoreductase (PcyA; EC 1.3.7.5) catalyzes two double bond reductions of the D-ring vinyl group and the A-ring diene system to yield PCB (3). In plants, phytochromobilin (PΦB) synthase HY2 (LONG HYPOCOTYL 2; EC 1.3.7.4) is the FDBR that produces the PΦB chromophore for light-sensing phytochromes (4).

Biochemical and structural studies have revealed several universal properties of FDBRs. First, transient radical intermediates are present in catalytic reactions of FDBRs. Two organic radical intermediates have been detected in the two double bond reduction steps of PcyA (5). Also, we have recently reported that an organic radical species is involved in HY2-catalyzed A-ring 2,3,3′,3″-diene reduction (6). A radical mechanism for this HY2 reaction has been proposed (Fig. 1). These data strongly support the existence of a universal radical mechanism for FDBR-catalyzed BV reduction. Second, structures of several FDBRs and mutagenesis data indicate FDBRs share a similar α-β-α sandwich fold, but their double bond specificities are decided by the location of different proton-donating residues and waters in their active sites (7–9). Third, all FDBRs require the donation of electrons from reduced ferredoxins (Fds) to reduce double bonds (10–12). The plant-type [2Fe-2S] Fds have been shown to be the major electron donor. However, there is only limited information on how FDBRs interact with Fds for electron transfer.

In photosynthetic organisms, Fds function not only in electron transfer system of photosynthesis but also in the redox reactions of several oxidoreductases, such as sulfite reductase, nitrate reductase, glutamate synthase, and ferredoxin:thioredoxin reductase. Studies of these Fd-dependent enzymes indicate they interact with their Fd partners mainly through electrostatic interactions (13–17). In Arabidopsis, the PΦB synthase HY2 has been shown to reside in the plastid, where it meets the substrate BV and electron donor Fd (4). Six Fd isoforms have been identified in Arabidopsis. Four of them are plastid-type [2Fe-2S] Fds, and the remaining two are Fd-like proteins. Two of the four plastid-type Fds, AtFd1, and AtFd2, were predicted to be typical leaf-type Fds (18). Although these proteins...
Fds share high similarity, their functions could be diverse because of different expression patterns, abundance, subcellular localizations, redox potentials, and specificities to redox enzymes.

It has been proposed that PcyA and PebS interact with ferredoxins through the basic patches on the surface near the substrate binding sites (7–9). However, there is no experimental evidence to support this hypothesis. The HY2 homology model also shows similar characteristics to the PcyA and PebS structures (supplemental Fig. S1) (6). Therefore, we approached the study of the interaction of HY2 and Fd through a combination of enzyme assay, site-directed mutagenesis, and chemical cross-linking. Our results suggest that HY2 utilizes AtFd2 as the main electron donor. HY2 and Fd interact in a 1:1 ratio, mainly through charged residues on each side. Similar heterodimerization is also present in other FDBRs like PcyA, PebA, PebB, and PebS. We also found that the C12 propionate group of BV is important for HY2-catalyzed BV reduction. These findings then allowed us to propose a docking mechanism for HY2 and AtFd2.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were American Chemical Society grade or better unless otherwise specified. NADPH (cat. no. N-1630), ferredoxin: NADP⁺ oxidoreductase (FNR; cat. no. F-0628), glucose oxidase (cat. no. G6125), catalase (cat. no. C-40), bovine serum albumin (BSA; cat. no. 100–018), 1-ethyl-3-(3-dimethylaminopropylcarbodiimide (EDC), and 3-[2-carboxyethyl]-N-[2-hydroxy-3-phosphonylmethoxypropyl]sulfosuccinimide (Sulfo-NHS) were purchased from Sigma-Aldrich. The Superdex 75 gel filtration matrix and Sep-Pak Light cartridges were purchased from J.T. Baker. HPLC-grade acetone, acetonitrile, and formic acid were obtained from Waters (cat. no. WAT023501). BCA protein assay reagents were purchased from Pierce (cat. no. 23228). Biliverdin IXα was either obtained from Frontier Scientific, Inc. or prepared as described previously (19). BV monoamides were gifts from Prof. J. Clark Lagarias.

**Protein Expression, Purification, and Site-directed Mutagenesis**—HY2 and site-directed mutant proteins were expressed and purified as described previously (6). For expressing PcyA from *Synechocystis* sp. PCC6803 (PcyA_SYNY3), PebA and PebB from *Synechococcus* sp. WH8020 (PebA_SYNPY and PebB_SYNPY), and PebS from cyanophage myovirus P-SSM2, coding regions of these bilin reductases were subcloned into pTYB12 expression vectors (New England Biolabs). The proteins were expressed and purified with the similar method for HY2.

For expressing recombinant ferredoxins from *Arabidopsis thaliana*, the six AtFd coding regions without the predicted N-terminal transit peptide (or targeting signal) were subcloned into the *Escherichia coli* (*E. coli*) expression vector pET42b (Novagen) to construct pET42b-*mAtdfs*. The stop codons of each *mAtdf* coding sequence were retained to produce tag-free proteins. Cultures of *E. coli* strain BL21 containing pET42b-*mAtdfs* were generated to express tag-free AtFds. The bacterial cells were grown at 37 °C in 500-ml batches of NZCYM medium (5 g/liter NaCl, 5 g/liter yeast extract, 1 g/liter casamino acid, and 2 g/liter MgSO₄·7H₂O, pH 7.5) containing Fe(NH₄)₂ citrate (12 mg/liter) and ampicillin (100 µg/ml) to an *A*₆₀₀ of 0.6–1.0. Cultures were induced by the addition of 0.1 mM isopropyl thio-β-galactoside and incubated overnight at 16 °C, and subsequently harvested by centrifugation. The tag-free AtFds were purified first with a HiTrap DEAE FF ion-exchange column according to instructions supplied by the manufacturer (GE Healthcare), and then further purified with a Superdex 75 size exclusion column pre-equilibrated with TK buffer (25 mM TES-KOH pH 8.5 and 100 mM KCl). All site-directed mutants were generated in pET42b-*mAtdfs* using the QuikChange site-directed mutagenesis kit (Stratagene). Mutant *mAtdf* proteins were expressed and purified with a method similar to wild type. Recombinant *Synechococcus* sp. PCC7002 Fd (SynFd) was purified as described previously (20). Purified Fds were dialyzed against TK buffer (25 mM TES-KOH, pH 8.5 and 100 mM KCl). The protein concentration of Fds solutions was determined by absorption at 420 nm (using a molar extinction coefficient of 9.7 mM⁻¹·cm⁻¹) and BCA methods. All purified proteins were flash-frozen in liquid nitrogen and stored at −80 °C prior to use.

**Enzyme Assay and Spectroscopic Analysis**—Steady-state bilin reductase assays were performed similarly to those described previously with minor modifications (3). Steady-state assay
Electrostatic Interaction of HY2 and AtFd2

conditions consisted of 25 mM TES-KOH, pH 8.5, 100 mM KCl, 0.025 units/ml FNR, 1 μM (determined by spectroscopy) wild-type or mutantFd, 100 μM bovine serum albumin, 5 μM BV, 0.1 μM wild-type, or mutant HY2, 25 units/ml glucose oxidase, 100 mM glucose, and 25 units/ml catalase. Excess NADPH (i.e. 30 μM) was added to initiate catalysis. Reaction mixtures were incubated at 30 °C for 30 min (determined to be within the linear range for mHY2 activity). Crude bilins were extracted with C18 Sep-Pak light cartridge and subsequently evaporated to dryness using a SpeedVac concentrator. HPLC analysis was performed as described previously (5).

Ultraviolet-visible absorption spectroscopic measurements were performed as described previously (6). For measuring the UV-visible spectra of BV complexes of wild-type HY2 and site-directed mutants, equimolar solutions (10 μM) of BV, and enzymes were mixed and incubated at 25 °C for 5 min prior to the measurement. To compare the efficiency of BV binding between wild-type HY2 and site-directed mutants, the long- to short wavelength absorption ratios \( A_{650}/A_{380} \) of enzyme-BV complexes were measured. Ratios for free and wild-type HY2-bound BV were set as 0 and 100%, respectively.

Chemical Cross-linking and Protein Electrophoresis—To label the Fd with EDC, 200 μM (determined by BCA method) of Fd proteins in 50 mM phosphate buffer at pH 6.5 were incubated with 3.2 mM EDC and 6.4 mM Sulfo-NHS for 2 min at room temperature. An equal volume of 20 μM mHY2-BV complexes in 50 mM phosphate buffer at pH 8.0 were immediately mixed with EDC-labeled Fd and incubated for 10 min at room temperature. The cross-linking reaction was stopped either by adding SDS-PAGE sample buffer or 50 mM Tris-HCl, pH 8.0. Protein samples with sample buffer were boiled at 95 °C for 10 min and analyzed by SDS-PAGE on 4 to 12% NuPAGE gels (Invitrogen). Gels were stained with Coomassie Blue for visualizing the protein bands. The intensities of protein bands were quantified using the program ImageJ (NIH).

Structure Simulation and Modeling—The homology model of HY2:BV complex was generated as described previously (6). The structure of maize Fd extracted from the Fd:FNR complex was used as a template to generate the homology model of HY2:BV complex. The structure of maize Fd extracted from the Fd:FNR complex was generated as described previously (6). This structure was used as a template to generate the homology model of HY2:BV complex. As described previously (6), the resulting model for AtFd2 was manually edited in Coot to lessen steric clashes and then subjected to energy minimization in Discovery Studio 2.0 (21).

To generate the docking model for HY2 and mAtFd2, the ZDOCK program in Discovery Studio 2.0 program package was used. A total of 54,000 docked poses were generated. The top 100 poses were categorized into 16 clusters, and the top pose in cluster 3 (ranked 18 out of 54,000 docked poses), which fitted with our mutagenesis data were selected for further analysis.

RESULTS

AtFd2 Is the Major Reductant for HY2—We first checked which Arabidopsis Fd is the preferred electron donor for HY2. In all, there are six Fd genes in A. thaliana. All Fd proteins were predicted to have a transit peptide or targeting signal in their N-terminal regions. Therefore, mature proteins of all six Fds were expressed, purified, and tested for their ability to function as the electron donor of HY2. All purified AtFds showed typical absorption of plant-type Fds in oxidized form (data not shown). Absorption at 420 nm from iron-sulfur clusters was used to quantify the concentration of Fd for bilin reductase assay. Saturated amounts of Fds and FNR were included in the reaction. The result showed that AtFd2 transfers electrons for HY2 reaction more efficiently than other AtFds (Table 1). Although both AtFd1 and AtFd2 have been shown to be the leaf-type and plastid-localized Fds with almost 90% sequence identity and similar redox potential, AtFd1 was still less effective at supporting HY2 catalysis. Based on the expression level of different AtFds previously reported by Hanke et al. (18), we believe that AtFd2 is the major electron donor for HY2 in Arabidopsis.

Homology Models of AtFd2 and HY2 Reveal the Possible Interaction Mechanism—For successfully transferring electrons to HY2-bound BV, AtFd2 needs to dock on the surface of protein. Structures of both PcyA and PebS show a basic area composed of conserved, positive-charged residues on the surface of the BV-binding pocket (supplemental Fig. S1) (7–9). A similar characteristic can also be observed in the HY2 homology model we previously generated (Fig. 2, top panel) (6). This area is a potential docking site for the acidic Fd protein. We also generated a homology model of AtFd2 according to a multiple sequence alignment of Fds and the structure of mature maize Fd, which is 77% identical to AtFd2 in primary sequence (supplemental Fig. S2B) (16). Interestingly, as shown in the bottom panel of Fig. 2, a surface area on AtFd2 contributed by conserved acidic residues fits nicely with the basic area of HY2. Corresponding residues of the acidic area in Fds from other plant species have also been predicted to be important for charge interaction with FNR (13, 16). The electrostatic interaction could potentially bring the iron-sulfur cluster and BV close enough together for electron transfer. Based on these observations, we then mapped the interaction mechanism between AtFd2 and HY2 in more detail.

Dimerization of Fds and FDBRs Indicates the Interaction Involves Salt Bridges—The interaction of Fd and HY2 may involve intermolecular salt bridges in the binding interface, which could be formed between adjacent carboxyl and amine residues. We therefore tested this hypothesis by the chemical cross-linking technique using EDC, a zero-length cross-linker that can generate an amide bond from two adjacent carboxyl and amine groups. A similar method has been successfully used in generating the heterodimer of Fd and FNR (22, 23). It also has been used for identifying the interactions of ferredoxins with nitrite reductase and glutamate synthase in Chlamydomonas.

| Proteins | Relative activity | % of AtFd2 |
|----------|------------------|------------|
| AtFd1    | 76.8             |           |
| AtFd2    | 100.0            |           |
| AtFd3    | 43.8             |           |
| AtFd4    | 6.3              |           |
| AtFd1d   | 7.5              |           |
| AtFd2    | 6.7              |           |

TABLE 1 Relative activities of Arabidopsis Fds for HY2 catalysis

Steady state bilin reductase assays were performed as described under “Experimental Procedures.” Integrated peak areas of Z2/3E-Pfd reaction products from HPLC profiles were determined as a percentage of AtFd2 activity.
To enhance the cross-linking efficiency, a stabilizing compound for the EDC reaction, Sulfo-NHS, was included in this study. In a time course experiment, a protein band with a molecular mass of about 45 kDa was observed after cross-linking AtFd2 and HY2 for 5 min (Fig. 3, lanes 2 and 8). The size of the protein complex is in good agreement with the calculated molecular mass of an AtFd2:HY2 heterodimer (43,485 Da). The native molecular mass of AtFd2:HY2 complex was also determined by size-exclusion chromatography (supplemental Fig. S3). A relative molecular mass of 40.9 kDa was deduced, supporting our size prediction from SDS-PAGE. The heterodimerization was further confirmed by mass spectrometry-based protein identification (data not shown). Furthermore, cross-linking of AtFd2 and HY2 only produced heterodimers following the time. The addition of BV resulted in condensed protein bands without generating more heterodimer complexes, suggesting that the bound BV increases the specificity of Fd docking but not the affinity (Fig. 3, lanes 8–13). These results strongly suggest that the interaction between HY2 and Fd at least involves salt bridges between carboxyl and amino residues on each side.

We then tested the heterodimerization of HY2 with six AtFds. As shown in Fig. 4A, all AtFds can interact with HY2 in a 1:1 ratio. The interaction of Fd and HY2 is specific because replacing Fd with Arabidopsis heme oxygenase 1 (HO1) did not generate any cross-linked product. More heterodimer formation was observed in the reactions of AtFd1 and AtFd2 and less in AtFd3, AtFd4, AtFd-l-1, and AtFd-l-2. The ability of each AtFd to interact with HY2 correlated with their activity in the HY2 reaction (Table 1), indicating that the affinity of AtFds and HY2 is the determinant for BV reduction activity. The leaf-type Arabidopsis Fds, especially AtFd2, are the preferred redox partner for HY2.

A recent study by Okada (26) reported that cyanobacterial PcyA and Fd formed a complex in a 1:2 ratio. A long space-arm, amine-reactive cross-linker bis-(sulfo succinimidyl) suberate (BS3) was used in his experiments. Because our use of the zero space-arm cross-linker EDC in the Fd-HY2 reaction gave a result clearly different from his study, we therefore performed the EDC cross-linking experiment on cyanobacterial FDBRs and Fd and found that all bilin reductases including cyanophage PebS formed heterodimers with Fds. (Fig. 4B). The affinity of PebA_SYNPY for SynFd was not only higher than that of PcyA_SYNY3, but also PebB from the same species (Fig. 4B, lanes 7–9). More interestingly, the cyanophage PebS also interacted strongly with SynFd (Fig. 4B, lane 10). An unknown cross-linked complex was observed in the PebS reaction. These data strongly support the hypothesis that FDBRs interact with Fds in a 1:1 ratio and also suggest the formation of salt bridges is universal for the interactions between FDBRs and their partner Fds.

**Conserved Charged Residues on the Surface of HY2 and AtFd2 Are Involved in Protein-Protein Interaction**—We further investigated details of the interaction mechanism using HY2 and AtFd2 as model proteins. The electrostatic interaction of HY2 and AtFd2 requires charged residues on each side. HY2 resi-
Electrostatic Interactions of HY2 and AtFd2

Several conserved residues in the acidic surface area or close to the iron-sulfur cluster of AtFd2 were also selected for site-directed mutagenesis. The ability of AtFd2 mutant proteins to function as the electron donor for HY2 was analyzed by bilin reductase assay. Among the residues selected, mutations of Glu-81, Arg-92, and Asp-112 residues decreased AtFd2 activity (Table 2). The corresponding residues in maize Fd have all been shown to form intermolecular salt bridges with FNR (16). This result implies that the docking mechanism of AtFd2 to HY2 protein may be similar to that of Fd-FNR binding.

The dimerization of AtFd2 and HY2 can also be analyzed by the EDC cross-linking method. We therefore compared the dimerization ability of point mutants of HY2 and AtFd2. Fig. 5A shows the amount of heterodimers produced from wild-type AtFd2 and HY2 mutant proteins. Mutants with decreased reductase activity all generated fewer heterodimers after cross-linking (Table 2). The correlation of enzymatic activity and heterodimer formation reveals that these residues are critical in protein-protein interaction. A double mutation of the Lys-263 residue increased the HY2 activity 3-fold. So far, this is the first point mutation identified in FDBRs, which can improve enzymatic activity.

Table 2: Relative activities of HY2 and AtFd2 wild type and site-directed mutants

| Proteins | Relative activity | BV binding |
|----------|------------------|------------|
| HY2      |                  |            |
| WT       | 100              | 100        |
| E110Q    | 321.7            | 87.2       |
| K183Q    | 24.6             | 92.7       |
| E187Q    | 20.3             | 98.1       |
| R200Q    | 12.5             | 94.7       |
| K255Q    | 11.7             | 105.0      |
| H259Q    | 123.4            | 100.8      |
| K263Q    | 25.8             | 97.2       |
| R264Q    | 18.9             | 112.9      |
| R200Q/R264Q | 11.9 | 103.6 |
| AtFd2    |                  |            |
| WT       | 100              |            |
| D78N     | 107.2            |            |
| E81Q     | 69.0             |            |
| E82Q     | 116.0            |            |
| Y89F     | 83.6             |            |
| R92Q     | 8.2              |            |
| D112N    | 47.0             |            |

Steady state bilin reductase assays were performed as described under “Experimental Procedures.” Integrated peak areas of 3Z/3E-P reaction products from HPLC profiles were determined as a percentage of wild-type HY2 and AtFd2 activity. To compare the efficiency of BV binding between wild type and site-directed mutants, the long-to-short wavelength absorption ratios of enzyme-BV complexes were measured. Ratios for free and wild-type HY2-bound BV were set as 0 and 100%, respectively.
Electrostatic Interaction of HY2 and AtFd2

Heterodimerization of AtFd2 and HY2 point mutants. EDC cross-linking were performed as described under “Experimental Procedures.” A, EDC cross-linking of HY2 mutants with wild-type AtFd2. Solutions containing 20 μM HY2 point mutants complex with BV and 200 μM AtFd2 proteins were incubated in the presence of EDC and sulfo-NHS. Reactions were stopped by adding SDS-PAGE sample buffer and boiling immediately for subsequent SDS-PAGE analysis. Each lane was loaded with 3.5 μg of HY2 and 10.9 μg of AtFd2 proteins. B, EDC cross-linking of AtFd2 mutants with wild-type HY2. Solutions containing 20 μM wild-type HY2:BV complex and 200 μM AtFd2 point mutants were incubated in the presence of EDC and sulfo-NHS. Reactions were stopped by adding SDS-PAGE sample buffer and boiling immediately for subsequent SDS-PAGE analysis. Each lane was loaded with 3.5 μg of HY2 and 10.9 μg of AtFd2 proteins. The band intensities of cross-linked complexes were quantified by the program ImageJ and are labeled below the bands. Band intensities from wild-type reactions were set as 100.

Electrostatic Interaction of HY2 and AtFd2

Electrostatic interaction is an important role in the electron transfer reaction. It is possible that electrons are directly accepted by the BV molecule from Fd without traveling through other pathways. From the known structures of FDBRs and our HY2 model, one possible entry site for electrons is the solvent-exposed propionate groups of protein-bound BV (supplemental Fig. S1) (7, 9). To test this hypothesis, we used two BV analogs, BV C8 monoamide (BV-8amide), and BV C12 monoamide (BV-12amide), as the substrates for HY2 activity assay (Fig. 6). In these analogs, the ionizable carboxyl group on either C8 or C12 propionate side chain of BV was converted into a neutral amide group. Both BV analogs retained similar ability to bind HY2 (data not shown). We then replaced BV with BV-8amide and BV-12amide, respectively, as substrates of HY2 in bilin reductase activity assay. HY2 catalyzed the reduction of BV-8amide normally compared with the BV reaction (Fig. 6, top and middle panels). However, BV-12amide was a poor substrate for HY2 with less than 35% being reduced (Fig. 6, bottom panel). The un-reacted BV and its analogs in reaction mixtures were nonspecifically reduced by reduced Fds, therefore, could not be found in HPLC profiles. The result indicates the C12 propionate group of BV is functionally important in the double bond reduction step catalyzed by HY2. Ionization of the carboxyl group on the C12 propionate side chain may play an important role in the electron transfer reaction.

DISCUSSION

FDBRs are a family of enzymes that catalyze the reduction of BV via diverse double bond specificities. For reducing double bonds, FDBRs require electrons to be transferred from Fd proteins. This study examined the interaction mechanism of FDBRs and Fds by using HY2 and Fd from A. thaliana as model proteins. We found that AtFd2 is the preferred electron donor for HY2. HY2 and AtFd2 formed a heterodimer after chemical cross-linking. Similar phenomena were also identified in cyanobacterial and cyanophage FDBRs. Several charged residues on HY2 and AtFd2 were important in both heterodimerization and BV reduction activity. These results suggest that electrostatic interactions between FDBRs and their redox partners are required for enzymatic activities. Interestingly, these residues are all located on the surface area close to the Fd redox center or HY2 active site, suggesting that the interaction may promote direct electron transfer from Fd to HY2-bound BV molecule. We also found the C12 propionate group of BV is important for HY2-catalyzed BV reduction. A possible role for this functional group is to mediate the electron transfer by interacting directly with AtFd2. A combination of all our biochemical data suggested a docking model for HY2:BV and AtFd2. The mechanistic implications of this suggested protein-protein interaction are discussed below.

Mechanistic Prediction of AtFd2 and HY2 Interaction—We generated the docking structure of HY2:BV-AtFd2 complex using the homology models of HY2 and AtFd2 for simulation (see “Experimental Procedures”). The important surface-charged residues we found on each protein were used to examine the docking models. Fig. 7A shows the best-fit docking pose we obtained so far. In the model, AtFd2 contacts HY2 on the surface of the BV-binding pocket. As shown in Fig. 7B, the interaction is stabilized by several salt bridges between the two molecules. Most of the important surface-charged residues we identified in this study are components of these intermolecular salt bridges, such as the residue pairs Arg-263–Glu-81, Arg-200—Asp-112, and Lys-255—Asp-73 from HY2 and AtFd2, respectively. Several residues on HY2 were shown to be important for enzymatic activity and interaction with AtFd2, but were not found to be directly involved in the electrostatic interaction for the docking. It is possible that these residues function after the conformations of HY2 and AtFd2 are changed upon binding, further enhancing the affinity of the two proteins.

Such a conformation change may also induce the breaking of intramolecular bonding and the formation of intermolecular bonding. One possible candidate is the Arg-92 residue on AtFd2. The corresponding residue to Arg-92 in spinach Fd forms an intramolecular salt bridge with a conserved glutamate residue (Glu-81 on AtFd2) (27). In the structure of the maize Fd:FNR complex, both Fd residues form intermolecular salt bridges with FNR upon binding (16). In our docking model, the Glu-81 residue on Fd is paired with Arg-263 of HY2 upon inter-
action (Fig. 7B). The side chain of Arg-92 then points into the active site and potentially interacts with BV. Together with our activity data from BV analogs, we hypothesize that Arg-92 interacts with the carboxyl group on C12 propionate side chain during Fd docking. This interaction positions the Fd molecule precisely, and facilitates the electron transfer from the iron-sulfur center to the HY2-bound BV molecule. Alternatively, the salt bridge could neutralize the charges and allow the electron to be transferred directly through bonding of Arg-92 and the C12 propionate group.

Hydrophobic interactions and hydrogen bonding also play important roles in protein-protein interaction. Although we mainly focused on charged residues in this study, an interesting feature we observed from the docking model is the location of nonpolar and hydrogen-bonding residues in the binding interface (supplemental Fig. S4). These residues, from both AtFd2 and HY2, are distributed at the edge of the contact area, forming intermolecular hydrophobic interactions and hydrogen bonding. This characteristic could produce a hydrophobic environment in the central region of docking interface to stabilize the electron transfer reaction. A similar property has also been identified in the contact region of Fd:FNR complex (16). Further experimental testing is required to support this hypothesis.

In summary, we propose that Fd binds to HY2 primarily through electrostatic interactions. Binding affinity is further enhanced by hydrophobic interactions and hydrogen bonding in the contact interface. Conformational change of both proteins upon binding induces the formation of intermolecular bonding, which further anchors the Fd. The docked AtFd2 molecule is precisely oriented by the salt bridge between Arg-92 on AtFd2 and the C12 propionate side chain on HY2-bound BV. The electron transfer process mediated by the salt bridge is stabilized by the hydrophobic environment in the central area of the docking interface. The electron is then transferred to the BV backbone, delocalized on the conjugated double bond system, eventually stopping at the double bond reduction site with adjacent proton donors. Fd docking must be transient to allow the next round of electron transfer from another Fd molecule. The Fd:HY2 complex eventually dissociates due to lower affinity induced by conformational changes of both proteins.

Conformational changes could result from the protonation of BV and the oxidation of the iron-sulfur center on Fd (5, 28). This proposed Fd-HY2 interaction not only can accelerate the electron transfer, but also generate a more hydrophobic environment to stabilize the transient radical intermediate produced during BV reduction. This hypothesis would be better answered by further mutagenesis and protein-protein interaction experiments.

**Reducant Specificity of FDBRs**—FDBRs depend on Fds as the reductant for converting BV into bilin pigments (12). As FDBRs have evolved diverse activities, Fds may also be specialized for individual enzymes. Four Fd genes can be found in the genomes of *Synechocystis* and *Synechococcus*. The encoded Fd proteins share high sequence identity (more than 50%) and possibly have similar structural folds. However, we found the Fd from *Synechococcus* sp. PCC7002 has lower affinity to PcyA from *Synechocystis* sp. PCC6803 compared with that of *Synechococcus* FDBRs, indicating Fd recognition by FDBRs is partially species specific.

**FIGURE 6.** HPLC analysis of HY2-catalyzed reduction of BV and its analogs. Steady-state bilin reductase assays were performed as described under “Experimental Procedures.” 5 μM BV, BV-8amide, and BV-12amide were used as the substrates of HY2. Bilin pigments were extracted after reactions were stopped, and analyzed by reverse-phase HPLC. Elution positions of BV, 3Z-PΦB, 3E-PΦB, 3Z-PΦB-8amide, 3E-PΦB-8amide, 3Z-PΦB-12amide, and 3E-PΦB-12amide are indicated by arrows.
specific (Fig. 4B). Furthermore, even though from the same species, PebA and PebB also have different affinities to their Fd partner from the relative species. This result reveals that even Fds in single species have become optimized for specific Fd-dependent enzymes during evolution.

On the other hand, it is also likely that FDBRs are specialized for recognizing conserved Fds. PebS was recently identified in the genome of the cyanophage myovirus P-SSM2, which infects Prochlorococcus as the host (2). PebS together with its redox-partner PetF, another plant-type [2Fe-2S] Fd encoded by the P-SSM2 genome, are expressed in the early induction stage during cyanophage infection. It has been proposed that expression of the single enzyme PebS replaces the two separated enzymes PebA and PebB in host cells to improve the phage fitness (2). This could be beneficial to the viral reproduction. One interesting phenomenon we observed is the high affinity of PebS for SynFd (Fig. 4B), although SynFd is not the physiological partner for PebS. We propose that early induction of both pebS and petF during infection is the strategy for the virus to ensure an efficient control of host systems in the early stage. However, under high selection pressure, cyanophage PebS is evolved into a protein with high affinity and low specificity for any available redox partners in its hosts. It may thus take advantage of host Fds as electron donors for producing PEB in the late stage of viral infection, eventually altering host systems for the later actions, such as increasing the efficiency of viral replication. It will be interesting to compare binding affinities of phage-encoded and host Fds to PebS.

A recent study proposed that PcyA and Fd from Thermosynechococcus elongatus BP-1 interact in a 1:2 ratio (26). The conclusion was based on the molecular weight of the cross-linked PcyA:Fd complex calculated from SDS-PAGE. However, it has been shown that acidic Fds migrate more slowly in SDS-PAGE due to a reduced amount of bound SDS (29), which may lead to miscalculation of the molecular weight of the complex. Also, the long space-arm cross-linker used in the study may induce complex formation from some nonspecific interaction. Our data from the EDC cross-linking used here suggest the formation of heterodimers of Fds and FDBRs. The dimerization is further supported by the data from size-exclusion chromatography (Fig. S3). We believe that interaction in a 1:1 ratio of Fd to FDBR is the physiological condition for FDBR catalysis. We
cannot rule out the possibility that there might be alternative mechanisms in different organisms. As we also found in the PebS cross-linking reaction, a high molecular mass complex (∼63 kDa) was produced (Fig. 4B, lane 10). Further studies on the interaction mechanism for PebS and its redox partner as well as FDBRs from different organisms are necessary.

Functions of Fds in Arabidopsis—Although other protein reductants such as flavodoxins can partially provide reducing power, plant-type [2Fe-2S] Fds are known to be the preferred reductant for FDBRs (3). Many photosynthetic organisms contain more than one copy of Fd genes encoding [2Fe-2S] Fds. There are a total of six Fd isoforms in A. thaliana. Four of them, namely AtFd1, AtFd2, AtFd3, and AtFd4, are predicted to be localized in the plastid. The remaining two Fds, AtFdl-1, and AtFdl-2, are uncharacterized ferredoxin-like proteins. The physical properties and functions of the four plastid-type AtFds have been well characterized (18). AtFd1 and AtFd2 are photosynthetic Fds mainly expressed in the leaves, whereas AtFd3 is a non-photosynthetic Fd localized in the roots. AtFd4 is evolutionarily distant from other plastid-type AtFds, but shares low similarity with root-type Fds. The differential expression of AtFds raises an interesting question: how HY2 receive electrons under non-photosynthetic conditions, for example in the root tissue? HY2 gene is expressed in almost all plant tissues and every developmental stage (Genevestigator). However, HY2 is a very low abundance enzyme in plants (11). It could be that the amount of root-type Fds, like AtFd3, is enough to provide the reducing power for HY2 in roots.

Our biochemical data suggest that the photosynthetic AtFds donate electrons for HY2 catalysis more efficiently than non-photosynthetic Fds. Interestingly, both AtFd1 and AtFd2 have high sequence identity (87% in mature region) and similar redox potential (−425 and −433 mV, respectively). They interact with FNR with similar affinity (18). In our case, both AtFd1 and AtFd2 also show similar binding affinity to HY2, but with different activity in electron donation (Fig. 4A and Table 1). We propose that AtFd2 is the physiological electron donor for HY2 because it shows higher activity and comprises around 90% of the leaf Fds in Arabidopsis under standard growth conditions. We cannot rule out the possibility that AtFd1 may be able to replace AtFd2 function to some degree, although only less than 10% of leaf Fds are AtFd1. It is still not clear that why AtFd1 is less effective at donating electrons under our in vitro assay conditions, as it is almost identical to AtFd2. The few variations on primary sequences of both proteins may cause the difference. Further investigation is being carried out to answer this question.

Previous data from reduction of AtFd2 proteins by RNA interference experiments have implied that AtFd2 primarily functions in linear electron flow in photosynthesis (30). Although the concentration of Fd in chloroplasts is relatively high compared with other Fd-dependent enzymes, the majority of AtFd2 reduced by photosystem I possibly interacts with the predominately membrane-bound FNR for electron transfer (31–33). We also found that AtFd2 has much higher binding affinity to FNR than HY2 (data not shown). As photosynthetic electron flow is tightly controlled, the amount of reduced AtFd2 partitioned with other redox enzymes in chloroplasts could be limited. It is possible that Fd-dependent enzymes like HY2 interact with stromal Fds reduced from the pool of oxidized Fds by soluble, NADPH-bound FNR (33). This could be important for P4P production in the dark when the photosynthesis is abolished. Alternatively, HY2 as well as other redox enzymes could be compartmentalized to the surface of thylakoid membrane to more efficiently accept electrons from reduced Fds. Identification of the suborganelle localization of HY2 is underway.

Future Studies—Although we have proposed a docking model for HY2 and AtFd2, it will be better illustrated by the crystal structure of HY2:BV-AtFd2 complex. The crystallography study is in progress. More mutagenesis data are also required to support our predicted docking mechanism. We believe that by combining mutagenesis with other protein-protein interaction technique, such as Isothermal Titration Calorimetry, details of the mechanism of electron transfer from Fd to FDBRs can be better understood. Differential localization of HY2 in sub-organelle compartments will be another important research topic in understanding the regulation of tetrapyrrole biosynthesis. Such knowledge offers the possibility of modulating the HY2 activity as well as phytochrome functions in plants.

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