Alteration of the Reduction Potential of the [4Fe-4S]^{2+/-} Cluster of Azotobacter vinelandii Ferredoxin I*  

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Kaisheng Chen§, Gareth J. Tilley§, Vandana Sridhar§, G. Sridhar Prasad¶, C. David Stout¶, Fraser A. Armstrong§, and Barbara K. Burgess¶

From the §Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697, the ¶Department of Chemistry, Oxford University, Oxford OX1 3QR, United Kingdom, and the ‡Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

The [4Fe-4S]^{2+/-} cluster of Azotobacter vinelandii ferredoxin I (FdI) has an unusually low reduction potential (E°) relative to other structurally similar ferredoxins. Previous attempts to raise that E° by modification of surface charged residues were unsuccessful. In this study mutants were designed to alter the E° by substitution of polar residues for nonpolar residues near the cluster and by modification of backbone amides. Three FdI variants, P21G, I40N, and I40Q, were purified and characterized, and electrochemical E° measurements show that all had altered E° relative to native FdI. For P21G FdI and I40Q FdI, the E° increased by +42 and +53 mV, respectively validating the importance of dipole orientation in control of E°. Protein Dipole Langevin Dipole calculations based on models for those variants accurately predicted the direction of the change in E° while overestimating the magnitude. For I40N FdI, initial calculations based on the model predicted a +168 mV change in E° while a −33 mV change was observed. The x-ray structure of that variant, which was determined to 2.8 Å, revealed a number of changes in backbone and side chain dipole orientation and in solvent accessibility, that were not predicted by the model and that were likely to influence E°. Subsequent Protein Dipole Langevin Dipole calculations (using the actual I40N x-ray structures) did quite accurately predict the observed change in E°.

Iron-sulfur ([Fe-S]) proteins contain clusters composed of iron and inorganic sulfide atoms ligated to the protein primarily by cysteine residues. They are ubiquitous, and have diverse functions ranging from electron transfer to regulation of gene expression (for recent reviews, see Refs. 1–7). In order to carry out these different functions, individual proteins can dramatically alter the reactivity of [Fe-S] clusters in a number of ways. For example, by adding or subtracting iron and sulfide atoms to the cluster type (1–7), by bridging a cluster between two subunits (8, 9), by introducing non-cysteine ligands (8, 10–12), by bridging an [Fe-S] cluster to another prosthetic group (e.g. Ref. 13), by grouping multiple clusters in a particular order as revealed by the recent hydrogenase structures (13–16) or by adding other metals or organic groups as occurs in the [Mo-7Fe-9S-homocitrate] FeMo cofactor sites of nitrogenase (17).

For this study it is especially important to note that, even without modification of [Fe-S] type and organization, proteins are still able to control the reactivities of the clusters they contain. For example, [4Fe-4S] clusters with four cysteine ligands utilize three different redox couples. The +3/+2 couple is used in a class of proteins designated the high potential iron proteins (18–20), the +2/+ couple is used in most ferredoxins and redox active enzymes (21), and the +0/+0 couple has been recently reported for the iron protein (Fe protein) of nitrogenase (22, 23). Even when a particular [4Fe-4S] redox couple has been selected the reactivity of these proteins can be extended further by protein modulation of the reduction potential (E°) of a particular redox couple. Thus, high potential iron proteins have potentials ranging from 90 to 450 mV (18–21, 24–29), while ferredoxins that contain structurally indistinguishable [4Fe-4S]^{2+/-} clusters have reduction potentials ranging from −280 to −715 mV in different native proteins (21, 30).

This study is focused on the question of how a protein could control the E° of a [4Fe-4S]^{2+/-} cluster that is ligated via a typical CysXXCysXXCys motif and one remote Cys ligand. Early studies of protein control of [4Fe-4S]^{2+/-} E° focused on three structurally characterized, related proteins. The [4Fe-4S]^{2+/-} cluster of Azotobacter vinelandii ferredoxin I (FdI) has an unusually low E° of −630 mV at pH 8 (31), while the analogous clusters in Peptostreptococcus asaccharolyticus ferredoxin (PaFd) and Clostridium acidurici ferredoxin (CaFd) have E° ≈ −430 mV (32). Thus the E° for the [4Fe-4S]^{2+/-} clusters contained within these proteins vary by over 200 mV. Early comparisons of the structures and sequences for these three proteins showed that the peptide folding around the analogous clusters is highly conserved with respect to the location of the four Cys ligands, the Cys dihedral angles, and the eight amide groups H-bonded to sulfur atoms of the cluster (33). These similarities have also been confirmed by the new 1.4-Å structures of A.fDfd (34, 35) and by the 0.95-Å structure of CaFd (36). Thus, these factors do not appear to be responsible for the observed differences in reduction potential among these proteins that all use the same [4Fe-4S]^{2+/-} couple.

Another long standing idea is that proteins might control

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The atomic coordinates and structure factors (code 1b0v) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† To whom correspondence should be addressed: Dept. of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697. Tel.: 949-824-4297; Fax: 949-824-8551; E-mail: bburgess@uci.edu.

1 The abbreviations used are: Fd, ferredoxin; MES, 4-morpholineethanesulfonic acid; TAPS, 3-[tris(hydroxymethyl)methylamino]propanesulfonic acid; PDLD, Protein Dipole Langevin Dipole; PDB, Protein Data Bank; ω, ohm(s).

2 The organism P. asaccharolyticus was formerly named Peptococcus aerogenes.
[4Fe-4S]^{2+/+} E^0 by introducing or removing charged residues. Thus, removing a negative surface charge near the cluster should make the cluster easier to reduce (raise E^0), while adding a negative charge should make it more difficult to reduce (lower E^0) (18, 37, 42). Indeed, the lower potential AvFdI does have more negatively charged residues near its [4Fe-4S]^{2+/+} cluster than CaFd or PaFd (43). This idea was attractive because it predicted that the formation of salt bridges between redox partners, when they bind to each other, might serve to raise the potential of the electron acceptor while lowering the potential of the electron donor, thus facilitating electron transfer (37). To test this idea a number of site-directed variants of the lower E^0 AvFdI were constructed by changing the negatively charged surface residues near its [4Fe-4S]^{2+/+} cluster to their neutral or positively charged counterparts in the higher E^0 PaFd (43). X-ray structures of the mutant proteins proved that the orientations of the residues were the same in the mutant AvFdIs as they were in native PaFd. Surprisingly, however, the E^0 of the AvFdI [4Fe-4S]^{2+/+} cluster was unaffected by these mutations (43). The conclusion from this study was that differences in surface charged residues were not responsible for the large differences in reduction potential observed for the [4Fe-4S]^{2+/+} clusters of AvFdI and PaFd.

Another factor that has been suggested to be important is the relative solvent accessibility of the [4Fe-4S]^{2+/+} clusters in the two classes of proteins with one group predicting that the higher E^0 of PaFd arises from the presence of buried water molecules (21, 44, 45). A recent 0.95-Å resolution x-ray structure of a protein in the PaFd class, however, failed to reveal the presence of any internal water molecules (36). Comparison of high resolution structures of AvFdI (34, 35) and CaFd (36) also failed to reveal any significant differences in the solvent accessibility of the homologous [4Fe-4S]^{2+/+} clusters contained within the two proteins.

In this study we use site-directed mutagenesis to manipulate side chain and backbone dipoles that are close to the [4Fe-4S]^{2+/+} cluster of AvFdI (but not directly H-bonded to the sulfur atoms of the cluster) in order to examine an additional recent proposal that these factors are of critical importance in protein control of E^0 (21, 40, 44, 45).

EXPERIMENTAL PROCEDURES

Mutagenesis of fdxA, and Expression and Purification of FdI Variants—For mutagenesis T4 DNA ligase and T4 polynucleotide kinase were obtained from Life Technologies, Inc., while all restriction enzymes were from New England Biolabs (Beverly, MA). The in vitro mutagenesis was performed as described previously (43) using a Mutagen M13 in vitro mutagenesis kit from Bio-Rad and the following oligonucleotides with the altered base(s) indicated in bold. The sequences are 5'-CCGGAGCAGTGGCGAGACTGCGCCTC-3' for I40Q, 5'-CCGGAGCAGTGGCGAGACTGCGCCTC-3' for I40N, and 5'-GGTTGAAGCTGCGGCTAGACTGTTTTTTT-3' for P21G. For all the mutants at Ile^{+4} position, oligonucleotides with a mixed sequence were used to generate the mutants: 5'-GGGGCGCAATCTCTGTCGCA(A/GC) CATCAGCGGACG-3'. The success of the mutagenesis was confirmed at the DNA level by dye-exchange DNA sequencing using the Sequenase version 2.0 DNA sequencing kit from Amersham Pharmacia Biotech. The overexpression of the FdI variants in their native background in A. vinelandii was carried out as described previously (46), except that the parent strain used for the overexpression was A. vinelandii LM100, a strain that does not synthesize native FdI, and electroporation (BTX TransPorator Plus electroversion system; BTX, Inc., San Diego, CA) was used instead of the triparental mating method in the transformation process.

Cell growth and the purification and triclinic crystallization of native FdI and FdI variants was carried out as described previously (43, 47). As a precautionary measure, the FdI variants were initially purified anaerobically in the presence of dithionite. The anaerobically purified fast protein liquid chromatography (MonoQ, with a linear gradient of 0.15–0.5 M NaCl in Tris-HCl, pH 8.0) and triclinic crystallization (43) were done aerobically.

Spectroscopy—For spectroscopic studies all samples were prepared anaerobically under argon in a Vacuum Atmospheres glove box (O_2 < 1 ppm). UV-visible absorption spectra were recorded using a Hewlett-Packard 8452 diode array UV-visible spectrophotometer. CD spectra were recorded using an Aviv model 620 spectropolarimeter. EPR spectra were obtained using a Bruker 300 E spectrometer, interfaced with an Oxford Instruments ESRI-9002 liquid helium continuous flow cryostat.

Reduction Potential Calculations—The E^0 values were calculated using the program POLARIS, which was developed by Warshel et al. in the Department of Chemistry at the University of Southern California. This program is now commercially available from A. Warshel’s group. In this study we used version 6.30 of this program on the sp2000 computer at the University of California, Irvine Office of Academic Computing. POLARIS was developed to calculate the free energies and electrostatic properties of molecules and macromolecules in solution using a Protein Dipole Langevin Dipole (FDLD) model. A description of how the calculations are done for [Fe-S] proteins is found in Ref. 21 and will only be briefly considered here. The calculations begin with a protein structure in a Protein Data Base (PDB) file format. In this case the starting point for calculations was the new 1.35-Å structure of the oxidized state of native FdI (34) (7FDI). A number of PDB files were then created from that structure by modeling the 134N, 134Q, I40N, I40Q, or P21G mutations using the Insight II package (MSI, San Diego). The program replaces the wild-type residue with a mutant residue after local energy minimization. The actual I40N x-ray structures were also used as a starting point for calculations where indicated (accession code 1b0v). Once a structure had been obtained in PDB format, the next step was to convert that structure to a form suitable for FDLD calculations using a program called PREPARE that is included in the POLARIS package. This procedure involved deletion of all unwanted atoms (in this case the crystallographically observed ordered water molecules) and addition of H atoms if necessary. In the case of side chains that are capable of free rotation, the relative (Coulombic) energies of four orientations were evaluated and the configuration of minimum energy was selected (21). For His residues, the relative energies of the N\textsubscript{\textalpha} and N\textsubscript{\textbeta}-protonated forms were evaluated similarly, and the configuration of minimum energy was chosen. Then, with the exceptions indicated below, all atoms were assigned charges including the atoms of the [Fe-S] cluster of interest, in this case the [4Fe-4S(Cys)\textsubscript{4}] cluster in its oxidized and reduced states (21). The iron and inorganic sulfide atoms of the other [3Fe-4S(Cys)\textsubscript{4}] cluster were treated as uncharged and assigned as zero. Additionally, the \beta-CH\textsubscript{2} and \alpha-CH moieties of all ligating Cys residues were treated as noncharged, while non-ligated Cys residues were treated as normal amino acids. All the ionizable residues were treated as uncharged (total charge of the residue is zero).

During the first part of the FDLD calculation, the Coulombic interactions of the [4Fe-4S(Cys)\textsubscript{4}] cluster, in its oxidized and reduced states, with all other protein atoms was calculated. This included charge-charge interactions and charge-induced dipole interactions. The next part of the calculations involved construction of a Langevin dipole grid representing water molecules around the protein, and the interactions between the grid dipoles and the cluster were calculated. In this case the grid filled a sphere of radius r_g = 25 Å and was composed of two sections, an inner section with 1-Å spacing in a 12-Å radius and an outer section with a 3-Å spacing. For the oxidized state of the cluster, the dipoles on the constructed grid were optimized by sampling a set of 30 grids to give the maximum energy, and then the optimized grid was used without reoptimization for the reduced cluster. The final step involved calculation of the interaction of the cluster charges with the bulk media more than 25 Å away from the cluster. In the actual calculation process, the FDLD calculation is not only carried out on the starting protein structure, but also on molecular dynamics generated at each step of the procedure. This is to take advantage that the average results based on a series of structures generated by molecular dynamics are more accurate than the single result from the x-ray crystal structure (21). The molecular dynamics simulations were done using a program called ENZYMIC which is attached to the POLARIS program. The molecular dynamics simulations were done at 300 K, generating a snapshot structure every 500 fs. A total of 50 structures
were generated for each protein and each of them was subjected to PDLD calculation. The final results were averaged (21).

Electrochemical Experiments—Purified water of resistivity ~ 18 MΩ cm (Millipore, Bedford, MA) was used in all experiments. The buffers MES, HEPES, and TAPS and the co-adsorbent neomycin sulfate were purchased from Sigma. A home-built electrochemical analyzer (EcoChemie, Utrecht, The Netherlands) was used to record DC voltammograms. The three-electrode configuration featuring all glass-cells has been described previously (48). The sample compartment (typically holding 500 µL) was maintained at 0 °C to optimize stability. All E° values are given with reference to the standard hydrogen electrode. The saturated calomel reference electrode was held at 22 °C which we have adopted as E° (saturated calomel reference electrode) = +243 mV versus the standard hydrogen electrode. E° values from cyclic voltammetry were calculated as the average of the anodic and the cathodic peak potentials, E° = \frac{1}{2}(\text{E}_a + \text{E}_c). The pyrolytic graphite “edge” electrode (surface area typically 0.18 cm²) was polished prior to each experiment with an aqueous alumina slurry (Buehler Micropolish, 1.0 µm) and then it was sonicated extensively to remove traces of Al₂O₃. All experiments were carried out under anaerobic conditions in a Vacuum Atmospheres glove box with an inert atmosphere of N₂ (<1 ppm).

Prior to the electrochemical experiments, all protein samples were checked for purity by running fast protein liquid chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden) with a Mono Q column equilibrated with 0.5 M Tris-HCl, pH 7.4, and a gradient from 0 to 1 M NaCl. Bulk electrolyte chromatography solutions contained 0.05–0.1 mM protein in 60 mM mixed buffer (15 mM HEPES, 15 mM MES, 15 mM TAPS, 15 mM acetate), with 0.1 M NaCl as supporting electrolyte and 4 mM neomycin. Neomycin stabilizes the protein-electrode interactions. For the investigation of pH dependence, protein solutions were dialyzed extensively against the buffered solution at the required pH, using an Amicon 8MC diafiltration unit equipped with a microvolume assembly and a YM-3 membrane.

Crystalization and Structure Determination of 14ON FdI—Due to the altered solubility properties of the 14ON, 14OQ, and P21G variants of FdI with respect to the native protein, it was not possible to crystallize these mutants using the conditions for native FdI (49) or seeding FdI with respect to the native protein, it was not possible to crystallize altered solubility properties of the I40N, I40Q, and P21G variants of FdI. For I40N FdI, these mutants using the conditions for native FdI (49) or seeding I40N FdI with respect to the native protein, it was not possible to crystallize.

The structure of I40N FdI was solved by molecular replacement using the structure of bulk solvent and anisotropic scaling corrections, resulting in an R-factor of 0.31 for all data to 3.0-Å resolution. The final results were averaged (21).

### Table I: Crystallographic data of 14ON

| Unit cell          | Space group | Cell parameters | Molecules/asymmetric unit |
|--------------------|-------------|-----------------|---------------------------|
|                    |             | a = 41.97 Å     |                           |
|                    |             | b = 55.26 Å     |                           |
|                    |             | c = 62.29 Å     |                           |
|                    |             | α = 78.1°       |                           |
|                    |             | β = 85.2°       |                           |
|                    |             | γ = 71.9°       |                           |
|                    |             |                  |                           |
|                    |             |                  |                           |
|                    |             |                  |                           |
|                    |             |                  |                           |

### RESULTS AND DISCUSSION

Selection of Mutants—Factors that have been proposed to influence E° of [4Fe-4S]2+/+ proteins, that have not been examined experimentally, are the orientation of the side chain and backbone dipeptide relative to the cluster and the solvent accessibility of the cluster. Warshel has developed a method for the quantitative modeling of these factors in proteins, which is known as the PDLD method (21, 40, 55–58). This method calculates the differences in free energies of the oxidized and reduced states of a redox-active prosthetic group, modeling the interaction of the group with protein and solvent. As considered in detail elsewhere, the results are expressed as the sum of four
terms (21, 40, 55–58). One term, $\Delta V_{\text{Cys4}}$, represents the Coulombic interactions of the atom charges from the [4Fe-4S]Cys$_4$ cluster in its oxidized and reduced states with the charges of the rest of the protein atoms. A previous study of low $E^\circ$ ferredoxins showed that this term is strongly influenced by backbone amides (21, 44). A second term, $\Delta V_{\text{polar}}$, represents the interaction of the [4Fe-4S]Cys$_4$ atom charges with the induced dipoles of the protein. The last two terms, $\Delta V_\text{Ind}$ and $\Delta V_{\text{polar}}$, respectively, represent the interactions of the [4Fe-4S]Cys$_4$ atom charges with the water molecules surrounding the protein, which are represented as Langevin dipoles, and with the rest of the aqueous solution which is represented as a continuum dielectric. It is important to emphasize at the outset that this method can be used to predict the variation of $E^\circ$ for the same type of group in different proteins but cannot be used to predict absolute $E^\circ$.

The PDLG method has been used successfully to model the $E^\circ$ of cytochrome c (57, 58), and most recently it has been applied to the study of [Fe-S] proteins (21, 44, 45). In that case, it was concluded that to a first approximation, the choice of redox couple made by a [4Fe-4S] cluster was largely influenced by the interaction of the cluster with the polar backbone amide groups and that modification of those amide groups is likely to lead to changes in $E^\circ$. Those computational studies further predicted that once the [4Fe-4S]$^{2+/+}$ couple was selected by the overall protein folding, further modification of the $E^\circ$ was unlikely to occur by changes in charged residues near the cluster (21, 44, 45). As discussed in the Introduction, this prediction is fully consistent with experimental evidence in the case of site-directed mutation of the [4Fe-4S]$^{2+/+}$ cluster region of AcFdII (43). Changes in $E^\circ$ of a protein-bound [4Fe-4S]$^{2+/+}$ cluster were predicted to be strongly influenced by the presence or absence of internal water molecules or by the interaction of the cluster with polar amino acid side chains (21, 44, 45).

In a recent review article, Stephens et al. analyzed nine site-directed variants of AcFdII using the PDLG method (21) and the results are summarized in Table II. X-ray structures are available for all of the proteins shown in Table II and these were used for the calculations. Overall, the results of this study were very encouraging with only minor differences between observed and PDLG calculated relative $E^\circ$ values. The average difference was 29 mV or 0.67 kcal. This led the authors of that review article to suggest us additional mutants that might be expected to alter the $E^\circ$ of the [4Fe-4S]$^{2+/+}$ cluster of AcFdII.

One of those mutants was designed to create a cavity in the protein that might accommodate a water molecule. We did not

| Table II | PDLG calculations of [4Fe-4S]$^{2+/+}$ $E^\circ$ |
|----------|----------------------------------|
| FdI variant | Calculated $E^\circ$ | Observed $E^\circ$ |
| Native | Reference | mV | Reference | mV |
| D23N | +20 | 0 |
| H35D | −30 | 0 |
| E38S | +16 | 0 |
| E46A | +17 | 0 |
| F24Y | +35 | 0 |
| F25I | −35 | −20 |
| C20A | −123 | −100 |
| C20S | −111 | −40 |
| C24A | +78 | +50 |

$^a$ All calculations were done with molecular dynamics except C20A as reported in Ref. 21. All $\Delta E^\circ$ values are versus the standard hydrogen electrode.

select that mutant, however, both because our experience suggested that cavity mutants were unstable and because more recent information suggests that internal waters are unlikely to be present in naturally occurring low potential ferredoxins. Thus, of the seven x-ray structures and three NMR structures that are now available for [4Fe-4S]$^{2+/+}$ containing naturally occurring ferredoxins, including one at 0.94 Å (36), none have internal water molecules. Internal water molecules have also not been observed for any of the FdI variants whose structures have been previously reported, nor were they introduced by any of the calculations reported herein.

Three of the suggested mutants involved replacing non-polar Ile residues near the [4Fe-4S]$^{2+/+}$ cluster of FdI with polar residues, either Glu or Asn (Fig. 1). One of those residues, Ile$^{44}$, is a fully buried hydrophobic residue with contacts to five other residues and was not selected because of probable (based on our experience) stability and folding problems. The position of the other two Ile residues, Ile$^{10}$ and Ile$^{44}$, relative to the [4Fe-4S]$^{2+/+}$ cluster, is shown in Fig. 2. Four mutants were constructed placing glutamine and asparagine at each of those two positions to give I34N, I34Q, I40N, and I40Q. A final set of six suggested mutants were designed to introduce potential changes in backbone amide orientation by replacing residues with Gly (Fig. 1). These residues were targeted because earlier studies had shown that the contribution of their amide groups to the $\Delta V_{\text{polar}}$ term exceeded 4.0 kcal/mol (44). Of these one, P21G, was selected because it was in a region of the protein that we had previously shown to tolerate several mutations without problems (43, 50, 59, 60) and because the amide N of this residue was already pointing toward the cluster in native FdI. In addition, the homologous residue had already been successfully mutated in another protein C. pasteurianum Fd to yield stable products (61, 62). The position of Pro$^{21}$ relative to the [4Fe-4S]$^{2+/+}$ cluster of AcFdII is also shown in Fig. 2.

**Initial Calculations on the Selected Mutants**—At the outset, all mutant structures were modeled using the available native oxidized FdI structure, at 1.35-Å resolution (34), as a template. After replacing the wild-type amino acid residue with the desired residue using Insight II, local energy minimization was carried out. As described under “Experimental

$^4$ The x-ray structures are as follows: A. vinelandii FdI (7FDI), two C. acidurici 2[4Fe-4S] ferredoxins (2FDN and IFCA), Bacillus thermoproteoides ferredoxin (2FXB), Desulfowloba africana ferredoxin (1FXX) (1DA1/NMR), P. asacharolyticus ferredoxin (1FDX), and Chromatium vinosum 2[4Fe-4S] ferredoxin (1BLU).

$^5$ The NMR structures are: Clostridium pasteurianum ferredoxin (1CLF), Bacillus schlegelii 7-Fe ferredoxin (1BC6), and Thermatoga maritima 4Fe ferredoxin (1ROF).
Procedures” and elsewhere (21), those structures (in PDB format) were then converted to a form suitable for PDLD calculations using the PREPARE program in the POLARIS package. PDLD calculations were then carried out on the 50 snapshot pictures generated by molecular dynamics, and the final results were averaged. Table III presents the results of these initial calculations.

For all five FdI variants, large positive changes in $E^O$ were predicted. The calculations in Table III suggest that the predicted changes in $E^O$ are not influenced by the interaction of the cluster with the bulk solvent ($\Delta V_{B}$) and none of the calculations suggested that water molecules would actually enter the protein. In three cases, I34N, I34Q and I40N, the $\Delta V_{B}$ term indicated that the clusters would become somewhat more accessible to solvent, an event that should cause the $E^O$ to become more negative. For I40Q the $\Delta V_{B}$ term was very similar to that of native FdI, whereas for all other variants the change was again expected to result in a decrease in $E^O$ with I40N predicted to have the biggest decrease. The dominating factor in the calculations for all of the variants, which overall were predicted to have large increases in $E^O$, is the $\Delta V_{Q}$ term, reflecting the importance of the orientation of positive end of the introduced side chain dipoles and/or the reorientation of the backbone amides relative to the cluster.

Purification and Characterization of the FdI Variants—Ile$^{24}$, Ile$^{49}$, and Pro$^{32}$ are all surface residues close to the [4Fe-4S]$^{2+}$ cluster (Fig. 2). Prior to construction of the FdI variants, modeling studies indicated that all changes could be easilyaccommodated without interfering with other residues. All of our AvFdI variants are expressed in their native backgrounds in A. vinelandii (46). In previous studies we had modified other surface residues and had not experienced any problems with stability such that the mutant proteins accumulated to the same levels in vivo as native FdI (e.g. 31, 43, 63). In contrast, two of the mutants, I34N and I34Q, were present in such low levels that they could not be purified. The other three mutants did accumulate to levels similar to those found for the native protein.

As described under “Experimental Procedures,” I40N, I40Q, and P21G FdI were successfully purified and their O$_2$ oxidized UV-visible absorption spectra were indistinguishable from that of native FdI (data not shown). Native FdI is an air-stable protein and the FdI variants also appeared to be air stable in their oxidized states. The addition of dithionite to I40N, I40Q, P21G, or native FdI results in partial bleaching of the spectrum (data not shown). For the native protein this is known to arise from the reduction of the [3Fe-4S]$^{+}$ cluster to the 0 oxidation state, because the $E^O$ of the [4Fe-4S]$^{2+}$ cluster is too low to be reduced by dithionite at neutral pH (31, 60). For I40N the result was indistinguishable from native FdI, however, for both I40Q and P21G the bleaching on reduction was more extensive than is seen for the native FdI. This result would be consistent with partial dithionite reduction of the [4Fe-4S]$^{2+}$ cluster caused by raising its $E^O$ in I40Q and P21G relative to native FdI, something that was predicted by the calculations shown in Table III.

To examine this issue further EPR experiments were carried out. In general, the presence of an oxidized [3Fe-4S]$^{+}$ cluster in any protein is easily identified by the appearance of a characteristic g = 2.01 EPR signal. The EPR spectra of oxidized I40N, I40Q, and P21G FdI were found to be both qualitatively and quantitatively indistinguishable from that of native FdI (data not shown). As is the case for native FdI, all three FdI variants were EPR-silent in the dithionite-reduced state in the perpendicular mode. This is consistent with the formation of [3Fe-4S]$^{0}$ but inconsistent with the reduction of [4Fe-4S]$^{2+}$ to the paramagnetic 1+ oxidation state, if that cluster is S = $\frac{1}{2}$. Thus, the bleachig observed in the UV-visible experiment is unlikely to arise from reduction of the [4Fe-4S]$^{2+}$ cluster and is more likely due to a lowered stability of the reduced protein. This possible instability of the reduced protein was confirmed by re-oxidation of the proteins by exposure to air. For I40N and native FdI the bleaching of the UV-visible spectrum was completely reversible, whereas for I40Q and P21G it was not. The cause for the relative instability of the reduced protein is currently unknown. The wavelength dependence and form of the CD spectra of oxidized and reduced native FdI and I40N, I40Q, and P21G were extremely similar (data not shown). In previous studies of C20A and C20S mutants, where the [4Fe-4S]$^{2+}$ cluster had undergone a ligand exchange and structural rearrangement, the CD spectra were dramatically different (50, 59). Thus, the CD data, combined with the UV-visible and EPR data, strongly suggest that the overall protein folding and the environment around the clusters has not been significantly perturbed in any of the altered proteins.

Reduction Potential Measurements—As was the case for all of the proteins shown in Table II, the [4Fe-4S]$^{2+}$/[4Fe-4S]$^{0}$ for I40N, I40Q, P21G, and native FdI were measured under identical conditions, at the same time, using direct electrochemical methods as described under “Experimental Procedures.” Results obtained at pH 7.0, for ferredoxin solutions containing 4 mM neomycin, are shown in Fig. 3. Two pairs of well-defined oxidation and reduction peaks are observed in each case; currents vary proportionally with (scan rate)$^{1/2}$ up to 20 mV s$^{-1}$, as expected for a reaction involving protein molecules diffusing to a planar electrode surface. The [4Fe-4S]$^{2+}$/[4Fe-4S]$^{0}$ is known to be weakly pH-dependent (31, 60), and the same pH dependence was observed for all of the variants examined herein. Table IV summarizes the data collected at pH 7.0 and compares the results to the $\Delta E^0$ values predicted from the PDLD calculations. For both I40Q and P21G, $E^0$ became more positive, as predicted by the calculations, but the magnitude of the change was substantially less than predicted. The big surprise in this study was that I40N whose [4Fe-4S]$^{2+}$ cluster was predicted to have an $E^0$ of 168 mV more positive than native FdI actually had an $E^0$ of 33 mV more negative.

Attempts to Bring the I40N Calculations Closer to the Observation—The $E^0$ information shown in Tables III and IV was available prior to the solution of the x-ray structure of I40N FdI. Therefore, the starting point for the initial calculations

<sup>6</sup> If the [4Fe-4S]$^{2+}$ cluster were partially reduced to the +1 level but was in an S = $\frac{1}{2}$ spin state, then it would probably not have been observed due to the broadness of that signal.
variants.

Conditions: 60 mM mixed buffer, 0.1 M NaCl, and 4 mM neomycin at 0 °C, pH 7.0, scan rate 5 mV/s.

Table IV

| FdI variant | ∆ν_{red} | ∆ν_{ox} | ∆ν | ∆ν_{rev} | Total ∆ν | Calculated ∆E° |
|-------------|----------|---------|-----|----------|-----------|----------------|
| Native      | 89.12    | 30.85   | 49.81| 31.20    | 200.97    | Reference      |
| I34N        | 98.67    | 28.03   | 48.06| 31.23    | 205.98    | +217           |
| I34Q        | 97.52    | 28.36   | 48.12| 31.21    | 205.21    | +184           |
| I40N        | 97.20    | 27.84   | 48.63| 31.20    | 204.85    | +168           |
| I40Q        | 92.55    | 30.53   | 49.40| 31.21    | 203.68    | +116           |
| P21G        | 94.29    | 28.13   | 49.31| 31.30    | 202.94    | +86            |

FIG. 3. Bulk solution cyclic voltammetry of native and FdI variants. Conditions: 60 mM mixed buffer, 0.1 M NaCl, and 4 mM neomycin at 0 °C, pH 7.0, scan rate 5 mV/s. Protein concentrations are in the range of 0.05–0.1 mM. Voltammograms measured on third cycle.

Table IV: Observed versus calculated ∆E° for FdI variants

| FdI variant | E° (pH 7.0) | Observed ∆E° | Calculated ∆E° |
|-------------|-------------|---------------|----------------|
| Native      | -619        | Reference     | Reference      |
| I40N        | -652        | -33           | +168           |
| I40Q        | -566        | +53           | +116           |
| P21G        | -577        | +42           | +86            |

was a model for I40N based on the native structure. As described above, in this procedure the PDB file for the 1.35-Å resolution structure of oxidized FdI was modified using the Insight II program by substitution of Ile by Asn. There are six allowable orientations for the Asn side chain generated by the program, and the program uses energy minimization to select the lowest energy orientation. This structure is then used as the starting point, and, as described under "Experimental Procedures" and elsewhere (21), it is then further modified, molecular dynamics is performed and finally the ∆E° calculations are completed. Because the critical factor in control of ∆E° in this case was expected to be the orientation of the positive ends of the side chain and backbone dipoles of the introduced Asn, we decided to fix the residue in different orientations at the outset rather than use the orientation selected by local energy minimization from the program.7 Fig. 4A shows the two extreme orientations. Orientation one has the side chain pointing toward the cluster and is the one chosen by energy minimization, while orientation six is the other extreme with the side chain pointing away from the cluster. As shown in Table V, the change in ∆E° predicted for orientation one was 168 mV more positive than that of native FdI whereas the prediction for orientation six was only 19 mV more positive, bringing the calculations in much closer agreement with the observation shown in Table IV. As shown in Table V, the large difference between the calculations for the two orientations arose from the ∆ν_{red} terms, with the model based on orientation six being in much closer agreement with the native protein.

X-ray Structure of I40N FdI—Because of the extreme difference between the theory and the experimental results, the I40N mutant was chosen as a target for crystallization. The Ile→Asn mutant of FdI proved difficult to crystallize, and once crystals were obtained they could not be frozen at 100 K without introducing severe mosaicity into the diffraction pattern. Consequently, data collection was limited to ambient temperature, resulting in a 2.8-Å resolution data set (Table I). Nevertheless, this resolution proved sufficient to define the orientation of the Asn side chain in all four independent copies of I40N FdI in the asymmetric unit (Fig. 5). These data revealed that four different orientations were chosen for the Asn side chain and Fig. 4 compares those orientations to those chosen in the models. As shown in Fig. 4, the actual structures did not correspond to either orientation one or six, both of which represent extreme cases. The actual orientations of the dipoles were all somewhere in the middle. Additionally, both our modeling and the PDLD program failed to predict the change in the backbone conformation of the protein as it was revealed by the x-ray structure.

In addition to the change in the orientation of the side chain at position 40, the unbiased electron density map revealed significant alteration in the conformation of the main chain at residues 40–41, as well as change in the conformation of the Asn side chain (see “Experimental Procedures”). A superposition of the four independent structures of I40N FdI onto native FdI is shown in Fig. 6. Overall, the structures are very similar with root-mean-square differences of only 0.21–0.25 Å for all 527 N, Cα, C, O, and Cβ atoms in the protein following least squares fit of each copy of I40N FdI (molecules A, B, C, and D) onto native FdI. However, displacements of 0.22–0.81 and 0.13–0.59 Å occur at the Cα atoms of Asn and Asp, respectively, while the side chains of these residues adopt a unique conformation.

7 The same treatment was not applied to I40Q, where 13 orientations of the side chain are possible. In that case, no x-ray structure is available so it is not clear if side chain orientation was the important factor or if, as occurred for I40N, other structural changes occurred.
conformation in each independent copy of the protein (Fig. 6).

In the P1 triclinic unit cell, the four molecules of I40N FdI pack as two asymmetrically related dimers (A-B and C-D); each dimer involves a Trp94-Trp94 stacking interaction between pseudo-two-fold related monomers. Consequently, each of the four copies of I40N FdI occupies a unique environment in the crystal lattice, accommodating the four unique conformations of Asn40-Asp41.

An explanation for this crystal packing arrangement is that the Ile40 side chain is also in contact with Pro21 and Val22. The Cα atom is 3.6 Å from Val22, and in molecule D the Nδ2 atom is 3.6 Å from Val22. Apparently, the range of conformations observed for Asn40-Asp41 reflects relatively unfavorable interactions with either of two nearby hydrophobic residues, and a lack of any other favorable new interaction. Although Asp41 displays variation in the position and orientation of the carboxyl group in the four independent structures, all four conformations allow two hydrogen bonds to the N terminus at Ala1, as observed in native FdI.

**PDLD Calculations Based on the I40N Structures**—Once the I40N structures were available, all four structures were used as a starting point for the PDLD calculations and the data are summarized in Table V. The average calculated \( \Delta E^0 \) for the four orientations is \(-1.8 \text{ mV} \), within 31 mV or 0.7 kcal of the observed \( \Delta E^0 \), with structures A and D being in very close agreement. In both of these cases, the calculations predicted a somewhat increased exposure to solvent as was suggested by the structures and also reflected the importance of the changes in the side chain and backbone amides that were observed. It should be noted that we do not know whether or not all four orientations are present in solution or if one is preferred. The electrochemical measurements for this mutant have an error of \( \pm 10 \text{ mV} \) (for a given conformation), so if more than one structure is present in solution they all must have \( E^0 \) values that are very close together.

**Conclusions**—The [4Fe-4S]^{2+/+} cluster of native AcFdI has an unusually low \( E^0 \) (31, 60). In previous studies we have shown that this \( E^0 \) is insensitive to changes that occur at the [3Fe-4S]^{1+/0} site of the protein, which is 6.7 Å away at its closest point of contact (e.g., Refs. 31 and 63). We have also shown that the \( E^0 \) is insensitive to modification of surface charged residues near the [4Fe-4S]^{2+/+} cluster (43). The reduction potential did become substantially more negative in two variants C20A FdI and C20S FdI that underwent [4Fe-4S]^{2+/+} ligand exchange, with a major change in ligand torsion angle, and an accompanying structural rearrangement (Table II) (50, 59). In this study we have extended the range of \( E^0 \) without such a structural rearrangement. I40Q and P21G exhibit \( E^0 \) 53 and 42 mV more positive than native FdI, respectively; while I40N has \( E^0 \) more negative by 33 mV. To date, the 12 FdI variants that have been constructed in the region of the [4Fe-4S]^{2+/+} cluster have a 153-mV range in \( E^0 \) (Tables II and IV). It should be noted that none of these mutations affected the reduction potential of the [3Fe-4S]^{1+/0} cluster eliminating the other cluster as an important factor in control of \( E^0 \).

In a recent review article (21), Stephens et al. examined nine FdI variants whose structures and \( E^0 \) were known (Table II) and found that their observed [4Fe-4S]^{2+/+} \( E^0 \) values were in close agreement with \( \Delta E^0 \) calculated using the PDLD methodology. This encouraged the approach of trying to predict the [4Fe-4S]^{2+/+} \( E^0 \) of FdI variants prior to their construction.

The greatest agreement between initial calculation and observation in this study was in the analysis of P21G FdI. This variant was purified and characterized spectroscopically, and appears to be structurally homologous to the native protein. P21G FdI was designed to alter the orientation of the backbone amide relative to the [4Fe-4S]^{2+/+} cluster. It was predicted to have a \( \Delta E^0 \) of 86 mV more positive than native FdI, whereas the
observed $\Delta E^0$ was 42 mV more positive. Thus, the direction of the change was accurately predicted while the magnitude was overestimated, in this case by 44 mV or 1 kcal. As shown in Table II for eight of the nine FdI variants examined previously, the calculated changes in $E^0$ were also larger than the observations on average by 29 mV or 0.67 kcal. A previous study of C. pasteurianum FdI showed that the substitution of the analogous Pro by Lys, Asn, Met, or Thr led to much smaller changes in $E^0$ (13 mV) (61, 62). The PDLD calculations suggest that adding one NH ... S interaction by substitution of Pro by Gly should increase $E^0$ by +86 mV. However, when we take into account the fact that the PDLD calculations appear to consistently overestimate $\Delta E^0$ (see above), that value is much closer to the observed $\Delta E^0$ of +42 mV. This in turn suggests that there has been essentially no conformational change in the backbone for P21G and that the amide dipole should be oriented as it is in the model.

I40Q was designed to change the [4Fe-4S]$^{2+/+}$ cluster $E^0$ by replacing a nonpolar residue with a polar residue near the cluster. The observed $\Delta E^0$ was 53 mV, whereas the predicted $\Delta E^0$ was 116 mV. In this case, the direction of the change was again accurately predicted and the magnitude was again an overestimate. Nonetheless, the magnitude of the change observed for this mutation is greater than has been observed for alteration of surface charged residues in any low potential ferredoxin (43, 61, 62), thus providing validation for the conclusion that [4Fe-4S]$^{2+/+}$ reduction potential can be controlled by the orientation of polar residues on the surface of the protein near the cluster. This in turn brings up the interesting possibility that conformational changes induced upon docking of electron transfer partners could cause reorientation of a surface polar residue in such a way as to alter the $E^0$ of one of the partners to facilitate electron transfer.

I40N was also designed to influence the $E^0$ of the [4Fe-4S]$^{2+/+}$ cluster by introducing a polar residue in the vicinity of the cluster. In this case, a very large positive $\Delta E^0$ was initially predicted (+168 mV) and a negative $\Delta E^0$ of −33 mV was observed. Further calculations showed that, even with molecular dynamics, the starting orientation of the polar residue had a very large effect on the outcome of the PDLD calculations. Thus, in cases where mutant x-ray structures are being modeled based on native structures, it appears to be important to test every allowable orientation rather than rely on the lowest energy orientation prior to beginning the $\Delta E^0$ calculations.

Although it was possible to bring the I40N calculations closer to the observation by modeling different orientations of the side chain, the actual structure of I40N FdI showed three features that could affect the $E^0$ of its [4Fe-4S]$^{2+/+}$ cluster in addition to the orientation of its side chain. First, the conformation of the adjacent charged residue, Asp$^{41}$, is affected (Fig. 6). Previous

### Table V

| I40N FdI | $\Delta\nu_{0}$ | $\Delta\nu_{02}$ | $\Delta\nu_{L}$ | $\Delta\nu_{N}$ | Total $\Delta\nu$ | Calculated $\Delta E^0$ |
|----------|----------------|----------------|----------------|---------------|-----------------|---------------------|
| Model (1) | 97.20 | 27.84 | 48.63 | 31.20 | 204.85 | +168 |
| Model (6) | 88.45 | 31.45 | 50.34 | 31.17 | 201.39 | +18 |
| Struct. A | 93.83 | 28.10 | 47.69 | 31.11 | 200.74 | −10 |
| Struct. B | 90.15 | 30.26 | 49.32 | 31.11 | 201.11 | +6 |
| Struct. C | 90.94 | 28.49 | 48.89 | 31.06 | 200.23 | −32 |

$\Delta\nu$ is in kcal, $E^0$ in mV.
studies have shown that the presence versus absence of adjacent charged groups on the FdI protein surface had very little effect on $E^{0*}$ (43), so it is unlikely that this change causes the observed $\sim$33 mV change. Second, the conformational changes resulting from the mutation include significant changes in the main chain at residues 40 and 41, and this in turn affects two favorable NH...S interactions in native FdI. In particular, the residue 40 amide to S4 distance changes from 3.46 Å in native FdI to 3.4, 3.4, 3.0, and 3.5 Å in molecules A, B, C, and D, respectively. In addition, the linearity of the interaction is reduced in molecules B and D. At the same time, the residue 41 amide to Cys$^{39}$ Sy distance changes from 3.4 Å to 3.7, 3.4, 3.9, and 3.3 Å in molecules A, B, C, and D, respectively, while the linearity of the interaction is significantly reduced for molecule C. Together, these changes indicate both more and less favorable interactions, but are overall relatively small. Consequently, their effects upon the $E^{0*}$ may cancel out. Third, the solvent exposure of the cluster is increased in the I40N mutant. Although the Asn$^{40}$ side chain is positioned, on average, as the Ile$^{40}$ side chain (Fig. 2), it is considerably less bulky (C$_2$N$_1$O$_1$H$_4$ versus C$_4$H$_9$). Solvent accessibility to the cluster will also be directly model in advance a structure for a FdI variant, even failure in this case was in the ability of InsightII to accurately determine the difference in $E^{0*}$ versus $E^{0*}$. Studies have shown that the presence of FdI. Further, the individual components of the calculation is likely to be critically dependent upon determining the main chain angles of the protein are affected and the lack of a single, discrete conformation for I40N FdI. Consequently, the main chain angles of the protein are affected and this has a major effect on the position of the introduced side chain. This may be a general problem with small [Fe-S] proteins whose structures are dominated by the clusters they contain, and points out that in these cases data interpretation is likely to be critically dependent upon determining actual x-ray structures of protein variants.

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