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Introduction of a [4Fe-4S (S-cys)4]$^{+1,+2}$ iron–sulfur center into a four-α helix protein using design parameters from the domain of the F_X cluster in the Photosystem I reaction center

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Abstract

We describe the insertion of an iron–sulfur center into a designed four α-helix model protein. The model protein was re-engineered by introducing four cysteine ligands required for the coordination of the mulinucleate cluster into positions in the main-chain directly analogous to the domain predicted to ligand the interpeptide [4Fe-4S (S-cys)4] cluster, F_X, from PsaA and PsaB of the Photosystem I reaction center. This was achieved by inserting the sequence, CDGPGRGGTC, which is conserved in PsaA and PsaB, into interhelical loops 1 and 3 of the four α-helix model. The holoprotein was characterized spectroscopically after insertion of the iron–sulfur center in vitro. EPR spectra confirmed the cluster is a [4Fe-4S] type, indicating that the cysteine thiolate ligands were positioned as designed. The midpoint potential of the iron–sulfur center in the model holoprotein was determined via redox titration and shown to be −422 mV (pH 8.3, n = 1). The results support proposals advanced for the structure of the domain of the [4Fe-4S] F_X cluster in Photosystem I based upon sequence predictions and molecular modeling. We suggest that the lower potential of the F_X cluster is most likely due to factors in the protein environment of F_X rather than the identity of the residues proximal to the coordinating ligands.

Keywords: four-helix bundle protein; iron–sulfur center; Photosystem I reaction center; protein design

Protein engineering and de novo design has emerged as a powerful experimental approach for testing ideas relating to protein folding and tertiary structures for the coordination of metals and redox centers. Ultimately, it may be possible to design structures capable of increased catalysis and range of chemical reactivity, and with greater stability. Recently, important advances have been made in the construction of model multiheme proteins (maquettes) utilizing features of naturally occurring oxidation–reduction proteins (Robertson et al., 1994). Their approaches have been extended and now include the successful incorporation of photoactive cofactors related to photosynthetic reaction center donors and, in a preliminary report, an iron–sulfur center into such maquettes (Gibney et al., 1995).

The objective of the study here was to synthesize a protein scaffold designed to incorporate a [4Fe-4S] cluster primarily to augment our ongoing investigations on the structure and function of the PS I reaction center, but also with the motivation that these approaches will provide important insights in the study of electron transfer processes and, ultimately, for the future engineering of new devices for solar energy conversion.

High-resolution structures have been published for several [4Fe-4S] proteins and about 50 predicted primary structures have been obtained based upon DNA sequencing (Beinhert, 1990). All the [4Fe-4S] proteins except two, the Fe-protein of nitrogenase (Georgiadis et al., 1992) and the F_X cluster of PS I (Golbeck & Cornelius, 1986), have intrapeptide coordination of the cluster(s). Fukuyama et al. (1988) suggested an evolutionary path whereby all such modern mono- and dicluster bacterial ferredoxins evolved from a 26-residue peptide by gene duplication followed by various deletions and insertions. The predominant arrangement of cysteine thiolate ligands for coordination of the clusters is **CxxCxxC** and a distal **CP**, the position of which depends upon the specific protein fold. Gibney et al. (1995) adopted this particular motif for cluster coordination in the loop of a 67-residue helix-loop-helix for their [4Fe-4S] protein design, which also contained histidines for heme ligation.

In contrast to the intrapeptide **CxxCxxC—CP** coordination motif of the ferredoxins, the iron–sulfur center F_X of PS I shows interpeptide coordination of the cluster whose cysteine ligands are

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Abbreviations: DTT, dithiothreitol; FeS, iron–sulfur; IPTG, isopropyl β-D-thiogalactopyranoside; PS I, Photosystem I; Tris, tris[hydroxymethyl] aminomethane.

contributed equally by two polypeptide subunits of the PsaA/PsaB heterodimer (Golbeck & Cornelius, 1986). A high-resolution crystal structure for PS I is not yet available, so we proposed a working hypothesis for the organization of this domain based on sequence predictions and molecular modeling, and also suggested a mechanism for the interaction of the core structure bearing F_X with the subunit, PsaC, which carries the terminal electron transfer centers, F_A/F_B (Rodday et al., 1993). Close protein–protein interaction between the PsaA/PsaB core and the PsaC subunit is most likely required for efficient forward electron transfer between F_X and the centers F_A and F_B (Sigfridsson et al., 1995, and recent references therein).

The working model we proposed for the F_X domain comprises a transmembrane four-α helix bundle formed by the predicted helices VIII and IX of the PsaA/PsaB core analogous to the LD, LE, MD, ME cofactor cavity of the bacterial reaction center (Diesenhofer et al., 1985). The four cysteine ligands for coordination of the F_X cluster reside on two identical interhelical loops, PCDGPGRGGTC, between helices VIII and IX of each PS I core subunit, and we suggested that the loops at the apex of the four-α helix bundle could form a surface-exposed cavity and contribute in the binding of the PsaC subunit (see Figure 1 of Rodday et al., 1993). The working model has been supported experimentally by chemical modification procedures and site-directed mutagenesis of key residues in the F_X loops of the PsaA/PsaB core subunits (Rodday et al., 1993, 1994, 1995; Hallahan et al., 1995) and PsaC (Biggins et al., 1995; Rodday et al., 1996).

The primary goal of the study here was to produce a stable protein scaffold optimized for incorporation of the F_X motif of PS I to coordinate a [4Fe-4S (S-cys)4] cluster. The model will be used for subsequent investigations on the redox center, such as the effect of protein environment on the electrochemistry of the cluster, and installation of additional cofactors relevant to photo-electron transfer processes using design changes facilitated by molecular cloning. The second goal, to be reported elesewhere, was to test directly the mechanism we have proposed for the protein–protein interaction between the PsaC subunit and the F_X domain in PS I by conducting binding studies using the protein model containing the putative binding site and PsaC. The putative binding site will be re-engineered, if necessary, and the spectroscopic experiments needed for characterization of the protein–protein interaction will be performed on the model more readily than on the pigmented native PS I.

In preliminary molecular modeling studies, several naturally occurring four-α helix bundle proteins were surveyed to determine if the design objectives could be achieved by protein re-engineering. Cytochromes b_{562} and cytochrome c' were found to be excellent candidates, but α4, a four-helix structure designed de novo by DeGrado and coworkers to test folding predictions (Ho & DeGrado, 1987; Regan & DeGrado, 1988; DeGrado et al., 1989; Regan et al., 1994), was judged to be better for the positioning of the cysteine ligands. α4 is a heat stable structure and can be produced readily and biologically in 100 mg to gram yields via expression of a gene constructed with convenient restriction sites already incorporated in the interhelical loop regions for design modifications (Regan & DeGrado, 1988). The α4 protein is also well-characterized biochemically, and has been exploited previously for the insertion of a metal center (Regan & Clarke, 1990). Therefore, α4 was modified by inserting the 10-residue sequence, CDGPGRGGTC, corresponding to the F_X domain of PS I as replacements for interhelical loops 1 and 3. This arrangement placed four cysteine ligands for coordination of the FeS cluster at the apex of the four-α helix structure in positions directly analogous to those for F_X in the PS I reaction center (Krauss et al., 1993). The main-chain direction of the two interhelical loops was chosen arbitrarily to be antiparallel in the overall tertiary structure.

The design changes of α4 were expedited by molecular cloning of the α4 gene constructed de novo by Regan and DeGrado (1988) and the resulting variant, hereafter referred to as α4-FeS, was expressed and produced in high yield in *Escherichia coli*.

Prior to committing to the re-engineering of α4, a small quantity of the α2-FeS variant (N-helix-PCDGPGRGGTC-helix) was synthesized using solid-phase methods. The peptide was shown to dimerize, as expected (Regan & DeGrado, 1988), and an FeS cluster was inserted as evidenced from the absorbance spectrum (data not shown). Thus, the interpeptide coordination was apparently successful, but the mechanism of dimerization and cluster assembly remained ambiguous. Although a four-α helix bundle most likely formed, the possibility of a linear configuration, whereby the cluster joined the two peptides end to end, could not be ruled out. The single-chain design finally adopted here, although not precisely analogous to the interpeptide arrangement of the F_X cluster in PS I, does guarantee that the final conformation will be a four-α helix bundle with the cluster coordinated by ligands in adjacent interhelical loops, which are the unique elements of the F_X binding domain in situ. The presence of loop number 2 at a position distal to the four cysteine ligands on loops 1 and 3 is unlikely to affect the environment of the FeS cluster.

We report that a [4Fe-4S] cubane iron–sulfur center of low potential has been inserted successfully into the re-engineered α4 polypeptide in vitro, indicating that the primary design goal was achieved.

Results

The modified four-α helix protein, α4-FeS, was produced readily in yields of ca. 250 mg/10 L batch culture using the bacterial expression system and purification protocol described. Figure 1 is a model showing the predicted positions of the four cysteine ligands required for coordination of the [4Fe-4S (S-cys)4] cluster. The α4-FeS holoprotein, formed by biochemical insertion of the FeS center into the expressed product in vitro, was characterized by optical and EPR spectroscopy to confirm the identity of the cluster.

The absorbance spectrum of α4-FeS in the visible range (Fig. 2) is characteristic of many bacterial ferredoxins and shows a maximum at 408 nm for the oxidized form and a decrease in absorbance upon reduction. We observed that the FeS center in the α4-FeS holoprotein was degraded oxidatively by exposure to air and the 408 nm absorbance could not be regenerated by re-reduction of the sample.

The oxidation–reduction potential of α4-FeS was measured by anaerobic titration of the protein using methyl viologen and benzyl viologen as mediators, by following the extent of oxidation–reduction spectrophotometrically at ambient temperature (Fig. 3). The absorbance change was measured at 430 nm rather than at the 408 nm maximum to eliminate the contribution of benzyl viologen to the signal. The midpoint potential was observed to be -422 mV (pH 8.3, $n = 1$), which is more positive than the value -705 mV determined for the F_X cluster (Chamorovsky & Cammack, 1982). However, the midpoint potential of α4-FeS is well within the range of midpoint potentials of iron–sulfur proteins containing [4Fe-

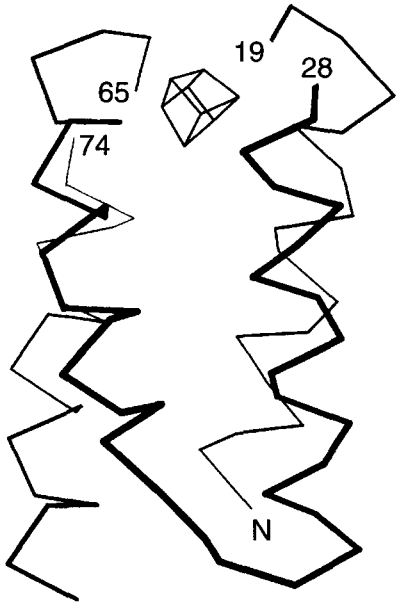

Fig. 1. Model of α4-FeS. The C_α main-chain coordinates are based on rigorous molecular modeling of the α4 prototype. The model shows the predicted positions of the four cysteine ligands and the [4Fe-4S] cluster at the apex of the four helix structure. The ligands are numbered based upon the amino acid sequence of the polypeptide after introduction of the inserts, which were re-engineered into the protein as shown in Figure 7. The orientation of the distorted cubane cluster is arbitrary because the assignment of specific cysteine ligands cannot be made at this stage. Loops 1 and 3 between C19 and C28, and between C65 and C74, respectively, are omitted for clarity. See Rodday et al. (1993) for a molecular model of the loops and Biggins et al. (1995) for predictions concerning the interaction of the loops with the PsaC subunit of PS I.

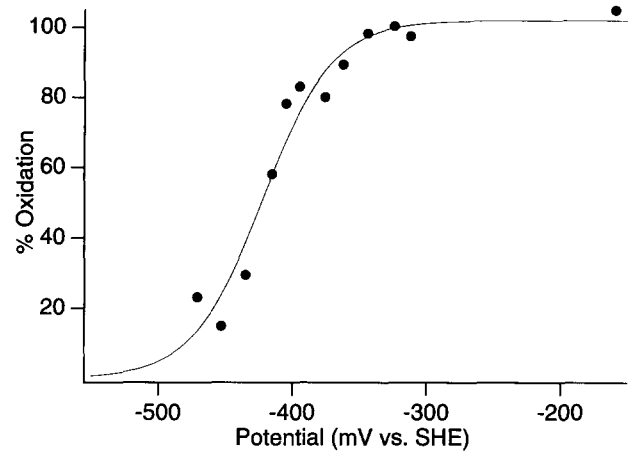

Fig. 3. Potentiometric titration of α4-FeS. A function described by the Nernst equation (——) was fit to the data (points). The % oxidation was monitored by measuring the absorbance at 430 nm following stepwise additions of sodium hydrosulfite.

$4S]^{+1,+2}$ clusters (100 mV to −700 mV), as reviewed recently by Johnson (1994), and similar to those of bacterial ferredoxins that range from −374 mV to −500 mV (Moura et al., 1994). The observation that the midpoint potential of α4-FeS is closer to those of ferredoxins rather than that of F_X may be because the solvation state of the cluster in the de novo designed protein here is more similar to the relatively solvent-exposed clusters of ferredoxins than to F_X, which is predicted to be inaccessible to solvent (Krauss et al., 1993).

The α4-FeS holoprotein was investigated by EPR spectroscopy at cryogenic temperature to determine the cluster identity of the FeS center. Figure 4 presents spectra in the $g = 2$ region for the oxidized and reduced forms of the protein. The reduced form is characterized by a rhombic spectrum with apparent g values of 2.053, 1.936, and 1.910 typical of a $[4Fe\text{-}4S]^{1+}$ cluster. The oxidized spectrum reveals the presence of a very low concentration of [3Fe-4S] clusters, and wider sweeps of 500 mT were undertaken to determine the possible presence of high spin signals. A small inflection at 170 mT was detected indicative of Fe products, most likely arising as artifacts of reconstitution of the protein, and a

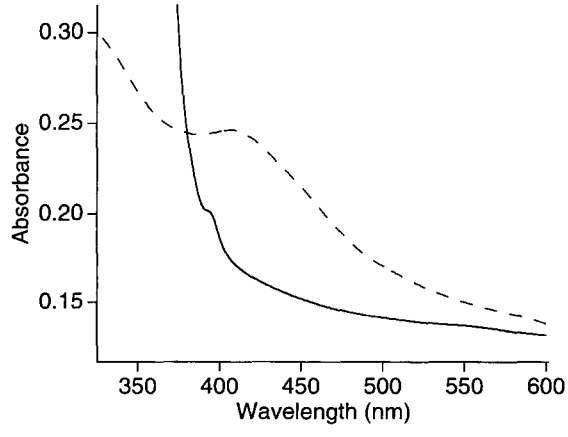

Fig. 2. Absorbance spectra of α4-FeS after insertion of the FeS cluster. The spectrum of the oxidized (- - - -) holoprotein shows a maximum at 408 nm. The protein was reduced (——) using sodium hydrosulfite.

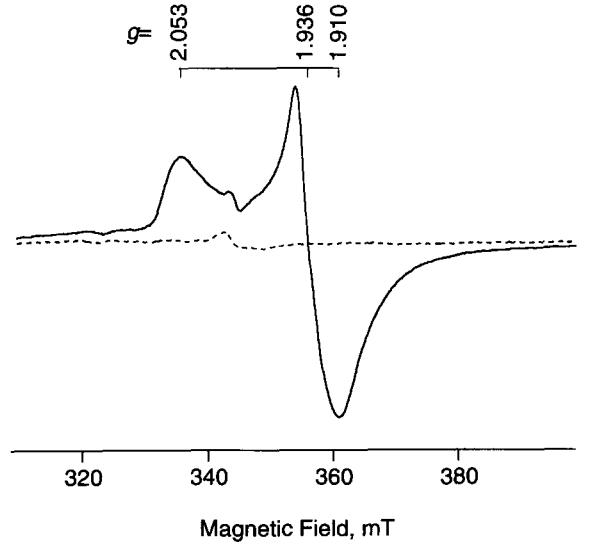

Fig. 4. EPR spectra of the oxidized (- - - -) and hydrosulfite reduced (——) forms of α4-FeS. EPR conditions were: 20 mW microwave power, 18 K temperature and 10 G modulation amplitude.

minor high spin signal of undetermined origin was noted at 150 mT (not shown). The temperature dependence of the EPR signal originating from the reduced FeS center showed an optimum of 18 K (Fig. 5) and power saturation data is shown in Figure 6. Although it is not possible to determine reliably the fraction of [4Fe-4S] clusters assembled correctly on a protein basis because of other iron-containing contaminants, the results indicate that the predominant metal center was a [4Fe-4S] cluster, confirming that all four cysteine ligands necessary for coordination of the redox center were positioned correctly in the protein design.

Discussion

The results here verify the successful insertion of a [4Fe-4S (S-cys)4] cluster into the de novo designed four α-helix bundle, α4. The heat stability of the design allowed convenient manipulation of the protein, which was exploited in the initial steps of our purification protocol and simplified the insertion of the FeS center in vitro and removal of non-protein contaminants.

We suggest that coordination of the [4Fe-4S (S-cys)4] cluster into this protein backbone supports the predictions that have been made regarding the organization of the four cysteine ligands for the native F_X cluster in the PS I core (Golbeck, 1993; Rodday et al., 1993, 1994). However, we also suggest that the specific intercysteinyl sequence we used for modification of loops 1 and 3 of α4, patterned after the F_X domain, is not essential for cluster insertion per se, and possibly two or three connecting glycine residues would suffice. Experiments now in progress directed toward ascertaining the second design goal will reveal whether the actual loops from the F_X domain we have installed in α4-FeS contribute to PsaC binding.

Table 1 compares the EPR properties and midpoint potential of α4-FeS with those of other iron–sulfur proteins with [2Fe-2S], [3Fe-4S], and [4Fe-4S] cluster types. The g values, line widths, and temperature dependence of the EPR spectrum of the FeS center in the α4-FeS holoprotein are typical of [4Fe-4S] ferredoxin clusters, suggesting that the α4-FeS holoprotein contains a [4Fe-4S] cluster (for recent reviews, see Beinhert, 1990; Johnson, 1994; Moura et al., 1994). Interestingly, the spectrum of bona fide F_X is somewhat atypical with regard to these parameters, so several differences exist between the F_X spectrum and that of α4-FeS. The

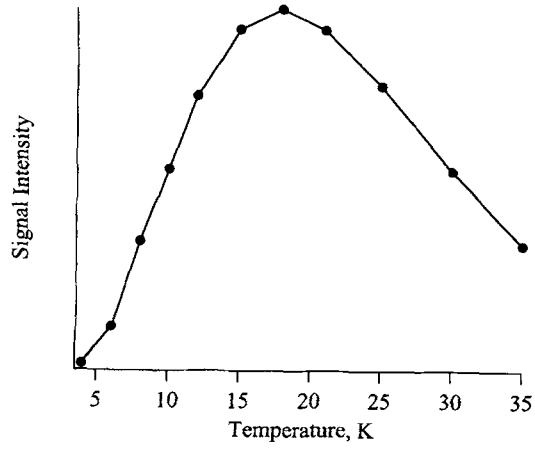

Fig. 5. Temperature dependence of the EPR signal of the reduced form of α4-FeS.

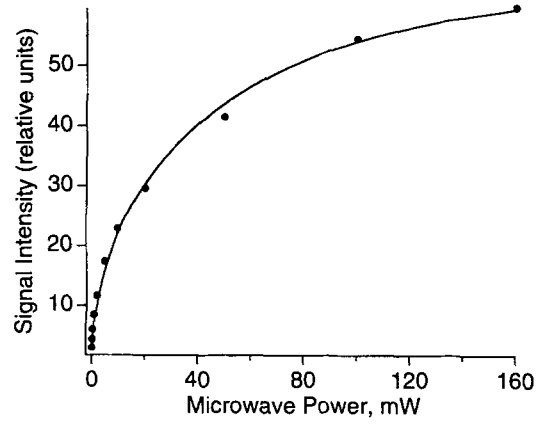

Fig. 6. Power saturation of the EPR signal of reduced α4-FeS at 18 K. Other details as in Materials and methods.

line,widths of the F_X spectrum are broad and the g anisotropy is distributed over an unusually large range, as shown by the g values of 2.08, 1.88, and 1.78 (Evans et al., 1976), whereas α4-FeS has relatively narrow line widths and apparent g values of 2.053, 1.936, and 1.910. The temperature optimum of the F_X spectrum is 10 K (Evans et al., 1976), compared to 18 K for the α4-FeS spectrum here. These differences indicate that the EPR properties of F_X are influenced by factors other than the sequence of the two loops containing the ligands to the cluster.

The redox titration of α4-FeS showed that, although the observed midpoint potential of the FeS cluster (−422 mV) was in the range of iron–sulfur proteins with [4Fe-4S] centers, it was considerably more positive than the F_X cluster in situ (−705 mV), which is one of the most electronegative FeS centers measured (Evans et al., 1974; Chamorovsky & Cammack, 1982). Although the intercysteinyl loops we inserted to create the α4-FeS variant were identical to those in the F_X domain of PS I, the results indicate that additional features in the protein environment, such as water solvent access and local H-bonding (Backes et al., 1991), maybe more important than the specific identity of residues immediately proximal to the cluster ligands in influencing the electrochemistry of the redox center. Langen et al. (1992) compared the measured redox potentials of four [4Fe-4S] proteins that had been characterized crystallographically with the potentials derived from a microscopic electrostatic model, which considers the protein and water environment (protein dipoles Langevin dipoles). Their calculations were in excellent agreement with the values obtained experimen-

Table 1. *Comparison of the EPR properties and midpoint potential of α4-FeS with those of iron–sulfur proteins with other cluster types*

Iron–sulfur protein	$g_x + g_y + g_z/3$	Optimum temperature (K)	Midpoint potential (mV)
[2Fe-2S] proteins	>2.00	<80	+300 to −460
[3Fe-4S] proteins	1.96–2.02	25–100	+70 to −420
[4Fe-4S] proteins	1.94–2.00	<30	+100 to −705
α4-FeS design	1.97	18	−422

tally, and demonstrated the contribution of both the access of solvent water to the vicinity of the cluster (e.g., between the ferredoxins of *Azotobacter vinelandii* and *Peptococcus aerogenes*) and the presence and orientation of amide groups for H-bonding in the cluster environment (e.g., between *Chromatium vinosum* HiPIP and *P. aerogenes* ferredoxins). These insights should be considered for future design changes in α4-FeS that may be directed toward changing the midpoint potential of the cluster.

Successful construction of an iron–sulfur protein using design parameters based upon the predicted architecture of the F_X domain in PS I provides support for the coordination motif of the F_X cluster in situ and extends the inventory of model proteins containing redox centers relevant to biological energy conversion.

Materials and methods

Molecular cloning

A culture of *E. coli* DH5-I lac T7 harboring the α4 gene on a pET 3c vector was kindly provided by Dr. Karyn T. O'Neil and Dr. W.F. DeGrado. The amino acid sequence of the design and the codon assignments chosen for construction of the gene are shown in Figure 7 (Regan & DeGrado, 1988). Modification of the gene required the deletion of RR in loops 1 and 3 of the structure and replacement by the F_X sequence, CDGPGRGGTC, in each case. The cloning strategy for the loop 1 change was digestion of the vector using *Apa* I and *Xho* I, followed by ligation of the double-stranded construct, INSERT [*Apa* I–*Xho* I] (Fig. 7). For modification of loop 3, the vector was digested first with *Sac* II and the resulting 3′ overhang removed using the 3′ → 5′ exonuclease activity of T4 polymerase. The vector was then digested using *Sac* I, followed by ligation of the construct, INSERT [*Sac* II–*Sac* I] (Fig. 7). Finally, the pET 3c vector with the α4-FeS gene was established in the *E. coli* expression host BL21(DE3) for protein production.

Production and purification of α4-FeS

E. coli BL21(DE3) pET 3c [*α4-FeS*] was grown in 10 L batch culture at 37 °C in a Microferm 14 fermentor (New Brunswick Scientific Co., Edison, New Jersey) in LB medium containing ampicillin (50 μg/mL). At A_{600} = 0.5, the cells were induced using IPTG (0.4 mM) and protein overproduction was continued for 5–6 h. The cells were harvested, washed with 50 mM Tris-Cl, pH 8.3, and suspended in the same buffer containing DNase at 10 μg/mL, 2% 2-mercaptoethanol, and stored frozen. The α4-FeS protein was purified in three steps starting by heat treatment of the cells at 85 °C for 10 min, followed by slow cooling with stirring to room temperature, and centrifugation at 15,000 × *g* for 20 min.

```
    HELIX 1                                                    LOOP 1
met gly glu leu glu glu leu leu lys lys leu lys glu leu leu lys gly pro arg arg
ATG GGT GAA CTG GAA GAG CTG CTT AAG AAA CTT AAA GAA CTT CTG AAG GGC CCG CGT CGT
                                                          Apa 1

    HELIX 2                                                    LOOP 2
    gly glu leu glu glu leu leu lys lys leu lys glu leu leu lys gly pro arg arg
    GGC GAA CTC GAG GAA CTG CTG AAA AAA CTG AAG GAG CTG CTT AAA GGT CCT AGG CGT
             Xho 1

    HELIX 3                                                    LOOP 3
    gly glu leu glu glu leu leu lys lys leu lys glu leu leu lys gly pro arg arg
    GGT GAG CTG GAG GAG CTG CTC AAG AAG CTT AAA GAA TTA CTG AAA GGT CCG CGG CGT
                                                          Sac 11

    HELIX 4
    gly glu leu glu glu leu leu lys lys leu lys glu leu leu lys gly end
    GGC GAG CTC GAA GAA CTG CTT AAG AAA TTG AAA GAA CTT TTG AAG GGC TAG
         Sac 1
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                19                                  28
                cys asp gly pro gly arg gly gly thr cys
        5'-CG TGC GAT GGC CCG GGT CGC GGC GGT ACC TGC GGC GAA C
    3'-CCG GGC ACG CTA CCG GGC CCA GCG CCG CCA TGG ACG CCG CTT GAG CT

                    INSERT [Apa 1-Xho 1] for LOOP 1
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                65                                  74
                cys asp gly pro gly arg gly gly thr cys
          5'-G TGC GAT GGC CCG GGT CGC GGC GGT ACC TGC GGC GAG CT
          3'-C ACG CTA CCG GGC CCA GCG CCG CCA TGG ACG CCG C

                    INSERT [Sac 11-Sac 1] for LOOP 3
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Fig. 7. Re-engineering of α4 by molecular cloning. The amino acid sequence and codon assignments used for the construction of the gene are shown (Regan & DeGrado, 1988). The two arginines in loops 1 and 3 were operationally deleted by insertion of the two double-stranded inserts shown between the restriction sites as underscored. The inserts (codon usage optimized for expression in *E. coli*) are labeled to highlight the four cysteine ligands 19 and 28 for loop 1, and 65 and 74 for loop 3 of the variant, α4-FeS. The proline residues in loops 1 and 3 were retained in α4-FeS because they are present in the native F_X domain, have been shown to stabilize [4Fe-4S] clusters (Quinkal et al., 1994), and would also assist in termination of helices 1 and 3.

The supernatant was heat-treated further at 95 °C for 20 min, followed by slow cooling and centrifugation as before. The supernatant was then fractionated using ammonium sulfate and the fraction precipitating between 50 and 70% saturation was retained and dissolved in 50 mM Tris-Cl, pH 8.3, 2% 2-mercaptoethanol containing 10 mM EDTA to chelate metal contaminants. Residual salt was removed by dialysis against the same buffer in the absence of EDTA. Although the protein was stable at room temperature, it was stored frozen to minimize oxidation of the thiols.

Insertion of the iron–sulfur cluster into the α4-FeS apoprotein

Ten milligrams α4-FeS protein were adjusted to 0.2 mg/mL for insertion of the FeS cluster using procedures developed by Golbeck and coworkers (Zhao et al., 1990; Li et al., 1991). All manipulations were performed in an anaerobic chamber. The following reagents (all in final concentrations) were added to the protein at 30-min intervals: 72 mM 2-mercaptoethanol; $FeCl_3$, 150 μM; Na_2S, 150 μM, and the preparation was incubated at ambient temperature overnight. The protein was then concentrated over an Amicon YM 10 membrane to a volume of ca 3 mL, and then passed down a Sephadex G-25 column to remove the residual cluster insertion reagents and other non-protein contaminants. The α4-FeS holoprotein was stored anaerobically.

Redox titration

Absorption spectrophotometry was conducted using a Cary Model I Bio or a SLM Aminco 3000 spectrophotometer. The oxidation–reduction potential of the α4-FeS protein was determined optically at 430 nm by titration of the protein in an anaerobic cell using benzyl viologen (1,1′-dibenzyl-4,4′-bipyridinium, $\xi^\circ = -350$ mV) to 33 mM and methyl viologen (1,1′-dimethyl-4,4′-bipyridinium, $\xi^\circ = -440$ mV) to 17 mM as mediators. The potential was adjusted by the addition of small aliquots of 100 mM sodium hydrosulfite in 1 M glycine buffer, pH 10.0. Potentials were measured in a laboratory-constructed electrochemical titration cell with a Ag/AgCl reference electrode and a platinum coil working electrode. A high-impedance voltmeter measured the potential across the working and reference electrodes. All data manipulations were performed using the program IgorPro 2.04 (Wavemetrics, Lake Oswego, Oregon). The Nernst equation functions were fit to the data using the Levenberg–Marquardt algorithm to determine coefficients giving a minimum χ^2 value.

EPR spectroscopy

EPR spectroscopy was performed at cryogenic temperatures using a Bruker ECS 106 spectrometer fitted with an ER/4102 ST resonator. Cryogenic temperatures were maintained using an Oxford Instruments ESR 900 liquid helium cryostat controlled by an ITC 4 temperature controller. The sample (250 μL) was reduced by the addition of 50 μL glycine buffer, pH 10.0, to 0.3 M and about 1 mg solid sodium hydrosulfite. Unless otherwise noted, the EPR conditions were field modulation frequency, 100 KHz; field modulation amplitude, 8.83 G; receiver gain, 2×10^4; sweep time, 168 s; time constant, 163 ms.

Acknowledgments

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