Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 104-111

MUTATED FORMS OF A [2Fe-2S] FERREDOXIN WITH SERINE LIGANDS TO THE IRON-SULFUR CLUSTER

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Received May 21, 1993

ABSTRACT: The [2Fe-2S] ferredoxin from *Clostridium pasteurianum* contains five cysteine residues in positions 11, 14, 24, 56 and 60. Residues 24, 56 and 60 have been separately mutated into serine. The modified ferredoxins have been purified and were all found to contain a [2Fe-2S]-type cluster. The electronic absorption and EPR spectra of the C24S protein were only slightly different from those of the native one. In contrast, the C56S and C60S ferredoxins displayed spectroscopic features witnessing the presence of an oxygen ligand to the iron-sulfur cluster: the UV-visible absorption bands were shifted to higher energy by ca. 20 nm, and the high field components of the EPR spectra were shifted from g_x=1.92 and g_y=1.95 to g_x=1.88 and g_y=1.92, respectively.

In an overwhelming majority of iron-sulfur proteins, the coordination sphere of the iron atoms consists of a tetrahedral array of sulfur atoms (1). In few but significant cases, the presence of ligands other than sulfur has been evidenced. Prominent among the latter are aconitase, where one of the iron atoms of the [4Fe-4S] cluster is coordinated to an oxygen atom from hydroxide, water, or substrate (2), and the Rieske-type proteins, in which two among the ligands of the [2Fe-2S] cluster are probably histidyl nitrogens (3). In addition, the occurrence of non-sulfur ligands is likely in some [4Fe-4S] ferredoxins (4), in the P clusters of nitrogenase (5), and in the H clusters of Fe hydrogenases (6,7).

In view of the growing importance of non-cysteinyl ligation in iron-sulfur proteins, it is timely to develop model systems allowing a systematic investigation of this phenomenon. One successful approach, over the past decade, has consisted in studying synthetic iron-sulfur clusters with at least partial non-sulfur

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0006-291X/93 $4.00

ligation (8,9, and references therein). Another opportunity is afforded by site-directed mutagenesis, which allows the replacement of cysteine ligands of iron-sulfur clusters in proteins. Among the latter, ferredoxins are particularly attractive, as their small size and high solubility allows the implementation of a wide range of spectroscopic techniques. Substitutions of cysteine ligands by serine residues have previously been attempted in two [2Fe-2S] ferredoxins, putidaredoxin (10) and adrenodoxin (11). In neither case were any iron-sulfur containing mutated proteins isolated or even detected in cell extracts (10,11).

We have recently overexpressed in *E. coli* the gene encoding the [2Fe-2S] ferredoxin from *Clostridium pasteurianum*, and shown the recombinant protein to be identical to its native counterpart (12). The sequence of this protein contains five cysteine residues (12-14), of which four are presumably ligands of the iron-sulfur cluster. We have now individually converted three of these cysteine residues into serine, and we show here that the UV-visible and EPR spectra of two of these mutated ferredoxins are consistent with serine ligation to their iron-sulfur cluster.

MATERIALS AND METHODS

All common DNA manipulations were as described (7,12,15). Restriction enzymes were purchased from Eurogentec, T4 DNA ligase and DNase I from Boehringer Mannheim, RNase A from Sigma, Taq DNA polymerase from Appligene, bacterial alkaline phosphatase from Amersham, and competent DH5α *E. coli* cells from Gibco-BRL. Oligonucleotides were synthesized by phosphoramidite chemistry on a 381A Applied Biosystems synthesizer.

Site-directed mutagenesis was performed by a modification (16) of a method (17) which uses two successive rounds of polymerase chain reaction (PCR) to create a mutation and amplify a DNA fragment surrounding it. The DNA on which mutations were introduced was the pTCP2F plasmid (12), where a sequence encoding the [2Fe-2S] ferredoxin from *Clostridium pasteurianum* was cloned between the *Nde*I (upstream) and *Hin*dIII (downstream) restriction sites of the pT7-7 expression vector (18). Two heptadecamer oligonucleotides were designed from sequences of the pT7-7 vector flanking the ferredoxin gene. O4 (5' TAATACGACTCACTATA 3') hybridized to the non coding strand and was located ca. 60 bp upstream of the gene. O9 (5' TTGGTAACTGTCAGACC 3') hybridized to the coding strand and was located ca. 70 bp downstream of the gene. The oligonucleotide (O10) for the C24S mutation was a 31-mer hybridizing to the coding strand: 5' GGAATTTTTGGAGTAACTAAAACCTTGCTGC 3' (mutated base underlined). For the C56S and C60S mutations, a single fourfold degenerate 35-mer oligonucleotide (O7) was used, which was coding for cysteine or serine in both positions. O7 hybridized to the non coding strand and had the following sequence: 5' TAATACAGGTT(GC)CTTTGGTATAT(GC)CAGTCAAGGCC 3' (mutated bases underlined).

The C24S mutated gene was obtained from a first round of PCR with O4 and O10, followed by a second round using the product of the first round and O9 as primers. The C56S and C60S mutated genes were obtained from a first round of PCR with O7 and O9, followed by a second round using the product of the first round and O4 as primers. The first rounds of PCR were performed as follows: plasmid pTCP2F (10 ng) was denatured for 5 minutes at 95°C in the presence of

0.3 μM of each primer in a final volume of 100 μL containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3 and 0.2 mM of each dNTP. Taq DNA polymerase (2.5 U) was then added, the reaction volume was covered with mineral oil, and 27 reaction cycles (1 min at 94°C, 2 min at 50°C, 3 min at 72°C) were run, followed by a 7 min elongation at 72°C. The second rounds of PCR were as above, except that 10 μL of the first PCR were used as one of the primers.

The products of the second rounds of PCR were precipitated with ethanol, cut with *Nde*I and *Hin*dIII, and electrophoresed through a 2% low melting agarose gel (Nusieve, FMC). The DNA fragments having the size expected for the ferredoxin encoding sequence were excised and ligated into the pT7-7 vector (18) cleaved with the same enzymes and dephosphorylated. The ligation mixtures were used to transform *E. coli* DH5α cells. Clones were first scanned by restriction analysis and partial sequencing. In case they contained the desired mutations, the ferredoxin gene was completely sequenced (19) on both strands to ensure the absence of additional unwanted mutations.

The mutated plasmids were used to transform *E. coli* K38 cells harboring the pGP1-2 plasmid (18). The expression of the mutated ferredoxins with this double plasmid system, and their purification, were as described (12) for the wild type ferredoxin.

RESULTS AND DISCUSSION

The three mutated ferredoxins displayed the brownish red color expected from [2Fe-2S] chromophores, which provided evidence that iron-sulfur clusters were present in all of them. However, color differences suggestive of variations in chromophore structure, were observed: the C56S and C60S ferredoxins displayed a distinctly more intense brownish cast than the wild type and C24S ones.

The UV-visible absorption spectra of the C24S, C56S, C60S and wild type proteins are shown in Figure 1. All of these spectra display the characteristic pattern of absorption bands arising mainly from ligand to metal charge transfer in [2Fe-2S] clusters. The positions of the UV-visible absorption bands are gathered in Table I. In all cases, Cys ⇒ Ser mutations resulted in hypsochromic shifts of the spectra. However, this effect was much weaker for the C24S protein than for the C56S and C60S ones: for the former, the shifts were no larger than 6 nm, whereas they were in the 10-20 nm range (and even 80 nm for the lowest energy band) for the two latter ones. The blueshifts observed above for the Cys ⇒ Ser mutated ferredoxins are consistent with similar observations reported for synthetic [2Fe-2S] clusters upon replacement of sulfur terminal ligands by oxygen (20-22). Interestingly, the substitution of four thiophenolate ligands by four phenolates resulted in hypsochromic shifts of 70-80 nm in the 400-500 nm range (20,21), *i.e.* four times larger than those observed here for the single replacements in the C56S and C60S proteins (Table I). The possible additivity of these ligand effects will be tested by preparing ferredoxins with multiple Cys ⇒ Ser mutations.

The absorption ratios of the ca. 450 nm maxima to the 280 nm peak have been found to be 0.50, 0.48, 0.40 and 0.33 for the purified wild type, C24S, C56S, and C60S ferredoxins, respectively (Figure 1). The lower ratios for the C56S and

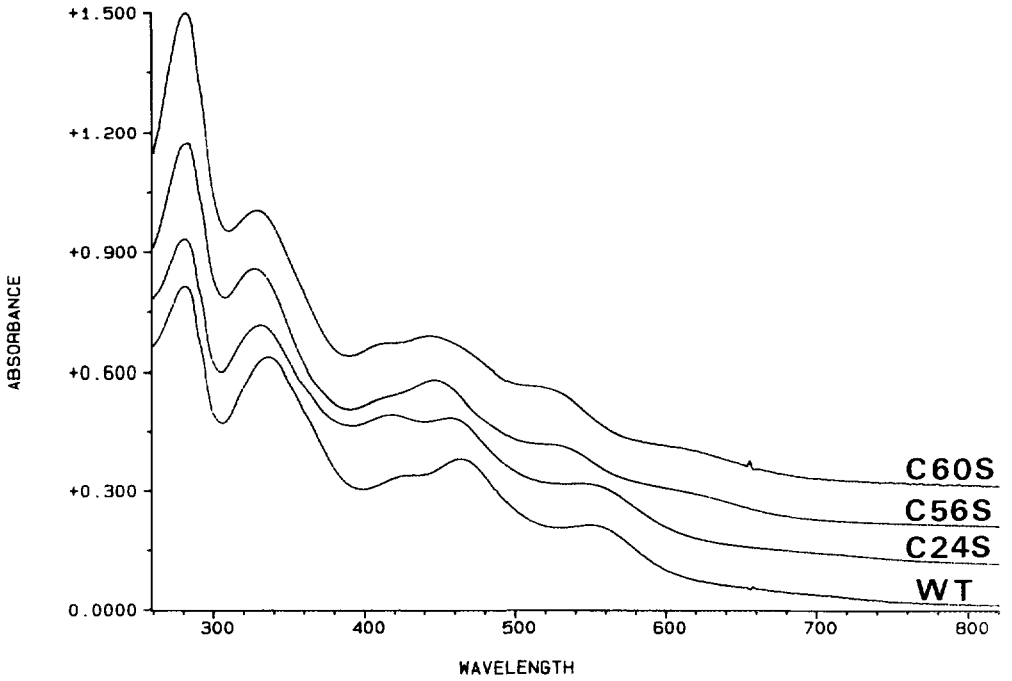

FIGURE 1. UV-visible absorption spectra of wild type and mutated [2Fe-2S] *C.pasteurianum* ferredoxins. The spectra were recorded with a Hewlett-Packard 8452 diode-array spectrophotometer. For the sake of clarity, offsets of +0.1, +0.2 and +0.3 absorbance units have been applied to the spectra of the C24S, C56S and C60S mutants, respectively. The protein concentrations were 5 mg/ml, and the optical pathlength was 1 mm.

C60S proteins probably arise, at least in part, from the lower stability of the iron-sulfur clusters in these proteins.

EPR spectra of the purified reduced ferredoxins are shown in Figure 2. The C24S protein differs marginally from the wild type protein (Table II). The spectra of

Table I: UV-visible absorption bands (nm) of the iron-sulfur chromophores of the wild type and mutated [2Fe-2S] ferredoxins from *C. pasteurianum*

Wild type	C24S	C56S	C60S
336	332	328	328
420 (sh)	418	410 (sh)	410 (sh)
464	458	446	444
550	548	530 (sh)	530 (sh)
700 (sh)	700 (sh)	620 (sh)	620 (sh)

Abbreviation: sh, shoulder.

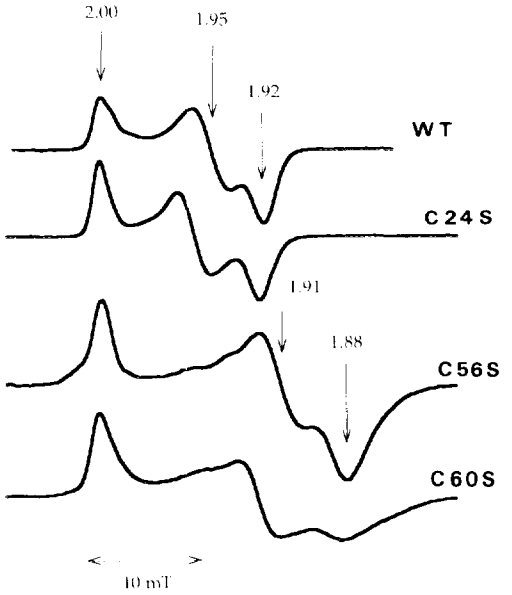

Figure 2. EPR spectra of wild type and mutated [2Fe-2S] *C.pasteurianum* ferredoxins. The spectra were recorded with a X-band Varian E-109 spectrometer equipped with a liquid helium transfer system (Oxford Instruments ESR 900) at a klystron frequency of 9.226 GHz and a temperature of 12K with a microwave power of 0.01 mW. All proteins (ca. 0.3 mM in NaCl 0.1 M, Tris-Cl 10 mM, pH 7.5) were reduced with dithionite (3 mM final concentration).

the C56S and C60S proteins are similar to each other, but differ strongly from those of the wild type and C24S ones by high field shifts of their two lowest g values (Table II). Similar spectral changes, which reveal an increase in rhombicity, have been reported for reduced [2Fe-2S] synthetic analogues upon substitution of sulfur terminal ligation by oxygen (9,21).

One of the purposes of this work was the identification of the cysteine ligands of the [2Fe-2S] cluster. Since cysteines 11 and 14 occur in a CXXC sequence segment which is a nearly ubiquitous bidentate ligand in [2Fe-2S] ferredoxins (1), we have assumed that they were probably ligands of the iron-

Table II: Apparent EPR g-values of the wild type and mutated [2Fe-2S] ferredoxins from *C. pasteurianum*

Wild type	2.004	1.948	1.922
C24S	2.005	1.955	1.921
C56S	2.007	1.916	1.883
C60S	2.005	1.923	1.882

sulfur cluster, and proceeded to mutate the three other cysteines into serines. The spectroscopic properties of the C56S and C60S ferredoxins (Figures 1 and 2) show that cysteines 56 and 60 are most probably ligands of the [2Fe-2S] cluster in the wild type protein. The case of cysteine 24 is less clearcut: the spectroscopic differences between the C24S protein and the wild type one, though small, are nevertheless suggestive of at least an indirect bearing of cysteine 24 on the structure of the [2Fe-2S] cluster. Indeed, preliminary resonance Raman experiments (in collaboration with M. Lutz, unpublished) have indicated that all of the three C24S, C56S and C60S mutations alter the vibrational properties of the iron-sulfur chromophore. The band patterns observed for the C24S protein on one hand, and for the C56S and C60S ones on the other hand, are different, but the frequency shifts are of similar magnitude in the spectra of all three proteins. These data suggest that cysteine 24 might be involved in the iron-sulfur chromophore, although not in the same way as cysteines 56 and 60. Further mutagenesis experiments, including replacements of cysteines 11 and 14 by serine, and of cysteine 24 by alanine, will be necessary to understand the role of cysteine 24.

The C56S and C60S mutated proteins display spectroscopic properties which, by comparison with those of structurally well characterized synthetic analogues (9,20-22), afford strong evidence that serines 56 and 60 are ligands of the iron-sulfur cluster. This is the first report of such an occurrence in a ferredoxin, as similar substitutions in putidaredoxin (10) and adrenodoxin (11) have not yielded proteins containing iron-sulfur chromophores.

Cys $\Rightarrow$ Ser substitutions have been carried out in several other iron-sulfur proteins (23-29). In some cases EPR spectra (23,24,29) and redox potentials (23) of serine-ligated clusters have been reported. In another case a [4Fe-4S] cluster has been converted into a [3Fe-4S] one (26), and in two other occurrences Cys $\Rightarrow$ Ser substitutions impeded the assembly of the iron-sulfur active sites (25,27). In all of these studies, the ligand-substituted iron-sulfur clusters have been difficult to characterize thoroughly for one or several among the following reasons: the large size of the proteins, their association in membrane-bound complexes, the presence of several iron-sulfur clusters, and the uncertainty of the assignments of the cysteine ligands. It was therefore important to obtain serine-ligated iron-sulfur clusters in small, soluble, and well characterized proteins, *e.g.* ferredoxins. Some advantages of the latter approach have already been underscored in this report, which shows for the first time UV-visible absorption spectra of serine-ligated iron-sulfur clusters. The mutated ferredoxins obtained here will also provide a unique opportunity of studying these structures by, for instance, resonance Raman or low temperature magnetic circular dichroism spectroscopy. Further developments will include substitutions of cysteines 11 and 14, and the preparation of multiply mutated ferredoxins which should afford [2Fe-2S] clusters with a variety of ligand combinations difficult to access by chemical modelling.

ACKNOWLEDGMENTS

We thank Dr. J.-P. Issartel for the synthesis of oligonucleotides, Professor S. Tabor for providing the T7 RNA polymerase expression system and information on its use, and Dr. J.-M. Moulis for helpful suggestions and for carefully reading the manuscript.

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