Slow formation of $[3Fe–4S]^{1+}$ clusters in mutant forms of *Desulfovibrio africanus* ferredoxin III

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Abstract ***Desulfovibrio africanus*** **ferredoxin III (*Da* FdIII) readily interconverts between a 7Fe and an 8Fe form with Asp-14 believed to provide a cluster ligand in the latter form. To investigate the factors important for cluster interconversion in Fe/S cluster-containing proteins we have studied two variants of *Da* FdIII produced by site-directed mutagenesis, Asp14Glu and Asp14His, with cluster incorporation performed in vitro. Characterisation of these proteins by UV/visible, EPR and ^{1}H NMR spectroscopies revealed that the formation of the stable 7Fe form of these proteins takes some time to occur. Evidence is presented which indicates the $[4Fe–4S]^{2+}$ cluster is incorporated prior to the $[3Fe–4S]^{1+}$ cluster.**

Key words: Di-cluster ferredoxin; Cluster formation; ^{1}H NMR spectroscopy

1. Introduction

Ferredoxins, small proteins containing one or more iron-sulphur clusters of the type [2Fe–2S], [3Fe–4S] or [4Fe–4S], are widespread. Such clusters carry out a diverse range of functions including: electron transfer through reversible oxidation reduction, catalysing dehydratase reactions, and acting as structure-forming cross-links [1–3]. Cluster transformation reactions occur in some ferredoxins, such as ferredoxin II from *Desulfovibrio gigas* (*Dg* FdII) [4], *Pyrococcus furiosus* Fd (*Pf* Fd) [5] and FdIII from *Desulfovibrio africanus* (*Da* FdIII) [6–8]. In these ferredoxins the reduced $[3Fe–4S]^0$ cluster can take up Fe^{2+}, to become a $[4Fe–4S]^{2+/1+}$ cluster, or a variety of metals, M, to form $[M:3Fe–4S]^{2+/1+}$ clusters [9–13]. The ease and reversibility of these reactions are, however, ferredoxin dependent, and neither the structural features that allow ready cluster transformation reactions to occur, nor the mechanisms of de novo cluster incorporation are well understood. The presence of a suitable ligand must, however, be a pre-requisite for covalently binding the Fe/S cluster to the polypeptide backbone. For example, *Dg* FdII, which is isolated as a [3Fe–4S] cluster-containing protein, contains a classic [4Fe–4S] binding motif of CysI–$(Xaa)_2$–CysII–$(Xaa)_2$–CysIII–$(Xaa)_n$–CysIV–Pro. Thus, its conversion into a [4Fe–4S] cluster, by coordination of a fourth Fe atom at the previously vacant CysII in the motif, would be expected based on its amino acid sequence. However, although Cys is the most common [xFe–xS] cluster ligand, the presence of four cysteines is not necessary for cluster interconversion and other amino acids have also been shown to ligate Fe/S clusters [14–20]. For example, in the cases of *Da* FdIII and *Pf* Fd, both of which also undergo cluster interconversion, an aspartic acid is located at position II in the binding motif. This aspartic acid has been identified as the ligand in the case of *Pf* Fd [17] and postulated as so in *Da* FdIII [6,13]. Although the suitability of aspartic acid as a ligand to [xFe–xS] clusters has also been demonstrated in model compounds [21], interconversion is not a phenomenon exhibited by all $[3Fe–4S]^{1+/0}$ cluster-containing Fds with an Asp at position II in the binding motif. Factors other than the presence of a suitable ligand must therefore be operative in determining cluster interconversion within Fe/S proteins.

We have selected *Da* FdIII to study cluster formation [6–8,22]. *Da* FdIII consists of 61 amino acids and has a molecular mass of 6585 Da [23]. It is isolated as a 7Fe Fd containing a $[3Fe–4S]^{1+/0}$ and a $[4Fe–4S]^{2+/1+}$ cluster. Isolation and purification of the recombinant protein in its holo form has not been reported and cluster incorporation has been achieved through in vitro reconstitution procedures [7,24]. Mutations of Asp-14 have been made to introduce amino acids that could provide a ligand to an Fe atom of the cluster. Aspartic acid was mutated to glutamic acid (D14E) and to histidine (D14H). In the former case, the carboxylate group is retained whilst increasing the size of the side chain, whereas mutation of Asp to His (D14H), in the latter case, was undertaken as His is a known ligand in other Fe/S cluster-containing proteins [14–16].

Here we report the characterisation of his_6-tagged D14E and D14H variants of *Da* FdIII using UV/visible, EPR and ^{1}H NMR studies. This is the first reported case of in vitro cluster incorporation of a di-cluster 7Fe Fd monitored by ^{1}H NMR. These studies demonstrate that the formation of the stable 7Fe forms of the D14E and D14H variants of *Da* FdIII takes some time to occur, and that the lag phase is associated with the formation of the $[3Fe–4S]^{1+}$ cluster.

2. Materials and methods

2.1. Sample preparation

The expression and purification of apo D14E and D14H mutants of *Da* FdIII was achieved using the protocol developed for wild-type *Da* FdIII [24]. Both mutants, with a his_6-tag C-terminus extension (ht), were overexpressed in *Escherichia coli* and purified in accordance with procedures described by Busch et al. [24]. Site-directed mutagenesis enabled the replacement of Asp-14 with a glutamic acid or histidine

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residue [25]. Reconstitution of the apo protein into the holo form was achieved in the presence of NifS by a procedure modified from that of Zheng and Dean [26]. The reconstitution, carried out with DTT, L-cysteine and ferrous ammonium sulfate under anaerobic conditions, is described in detail by Busch et al. [24].

Samples for the various spectroscopic analyses were exchanged into the appropriate buffer using an Amicon 8 MC unit equipped with a YM3 membrane (m. wt. cut-off 10 000 Da). All data reported have been recorded on the ht forms of the proteins.

2.2. Spectroscopy

UV/visible absorption spectra were recorded with a Hitachi U-4001 spectrophotometer or a HP845x diode array spectrometer (Hewlett Packard). Sample conditions were typically 120–149 μM protein concentration in 20 mM HEPES, pH 7.4, 0.1 M NaCl, 100 μM EGTA. Protein concentrations were determined using the molar extinction coefficient $\varepsilon = 28.6\ mM^{-1}$ at 395 nm and 397 nm for D14E and D14H FdIII, respectively [25]. Incorporation of Fe/S clusters was monitored by the purity index of each sample based on the ratio of the absorption maxima for each (i.e. 395 or 397 nm) and the protein absorption at 280 nm, e.g. A_{395}/A_{280} for D14E FdIII.

^{1}H NMR spectra were recorded on a Varian Unity Inova spectrometer operating at a frequency of 599.167 MHz. NMR samples contained 1.3–2.5 mM protein concentration in 50 mM deuterated Tris buffer (95% H_2O/5% D_2O) at pH 7.4 or 100 mM phosphate buffer, pH 7.6 (99.9% D_2O). The samples were stored under these solution conditions for a period of 3 months at 277 K.

One-dimensional (1D) spectra were recorded with the superWEFT sequence [27] as described previously [22]. For temperature dependence studies 1D spectra were recorded over the temperature range of 278–203 K for all samples, except the D14E *Da* FdIII sample prior to storage, where data were only collected at 288 K and 298 K. T_1 values were determined from 1D inversion recovery data using the pulse sequence of Vold et al. [28]. Linewidths at half-height were measured from superWEFT 1D spectra acquired at 298 K. Data were processed using VNMR software on the spectrometer or using Felix 95 (Biosym Technologies, San Diego, CA, USA) running on a Silicon Graphics Indigo workstation. Some data sets were processed with the application of an exponential line-broadening window function prior to Fourier transformation. All spectra were referenced to the residual HDO peak at 4.75 ppm from the methyl resonance of 2,2-dimethyl-2-silapropanesulphonic acid at 0 ppm (298 K).

EPR spectra were recorded on an X-band Bruker ER-200D SRC spectrometer (perpendicular mode, 9.64 GHz) equipped with an Oxford Instruments ESR-900 helium-flow cryostat and a TE-102 microwave cavity. Field intensity was measured using a microwave counter (Marconi Instruments, model 2440). Data were collected using a dedicated Bruker ESP-1600 computer. Spin densities of paramagnetic samples were estimated from integrations of EPR absorption spectra using a 1 mM Cu(II)/10 mM EDTA as a standard solution [29]. EPR data were recorded on the initial NMR samples (×10-fold dilution) and again after a 3 month storage period at 277 K.

3. Results and discussion

3.1. D14E Da FdIII

The UV/visible spectrum of freshly prepared D14E *Da* FdIII had an A_{395}/A_{280} ratio of 0.55. Taken together with the relative absorbencies of the native 7Fe *Da* FdIII [23], this indicates that the sample was ~70% loaded with iron. The X-band EPR spectrum of D14E FdIII contained a slightly anisotropic signal with a g value of 2.01, comprising a sharp positive peak with a shoulder and a broad negative tail at higher field. A small signal at $g = 4.3$ was also present, originating from adventitiously coordinated Fe(III). The signal at $g = 2.01$ is typical of an oxidised [3Fe–4S]$^{1+}$ cluster with an electronic ground state of $S = 1/2$, as observed in a range of ferredoxins [30]. However, the EPR spectrum of freshly prepared D14E FdIII indicated that only ~5% of the sample contained a [3Fe–4S]$^{1+}$ cluster (data not shown). After storage at 277 K for 3 months the EPR spectrum of the mature D14E FdIII showed that there was 0.7 ± 0.1 [3Fe–4S]$^{1+}$ cluster spins/mol.

In the ^{1}H NMR spectrum of freshly prepared D14E *Da* FdIII, eight broad peaks occur downfield of the diamagnetic region (at 10.23 ppm, 10.63 ppm, 11.53 ppm, 11.97 ppm, 13.31 ppm, 13.64 ppm, 17.62 ppm and 18.34 ppm) with linewidths at half-height ranging from 120 to 222 Hz (Fig. 1, upper panel). Fast relaxing peaks, dispersed downfield of the diamagnetic region, are characteristics exhibited by αCH and βCH protons of cysteinyl residues which form covalent bonds to paramagnetic [xFe–xS] cluster(s) (e.g. [31–38]). Peak 1, and one of the degenerate peaks designated as 4a/b, exhibited Curie-type temperature dependent behaviour over the range examined (288–298 K), atypical of that commonly observed for oxidised 4Fe or 8Fe ferredoxins [33–37], but characteristic of a [3Fe–4S]$^{1+}$ cluster-containing ferredoxin [31,32,35,38]. However, with the EPR data for the freshly prepared NMR sample indicating the presence of only 5% [3Fe–4S]$^{1+}$ cluster, these Curie temperature dependent peaks cannot arise from a 'classic' [3Fe–4S]$^{1+}$. These various spectroscopic measurements suggest that the freshly prepared protein contains a novel iron-sulphur centre (see later).

After storage at 277 K for 3 months, the ^{1}H NMR spectrum was found to be significantly different from that of the freshly prepared sample (Fig. 1, middle panel). It contained 16 resolved hyperfine shifted peaks downfield of the diamagnetic envelope ranging from 9.91 ppm to 23.03 ppm, and with linewidths within the range 94–430 Hz. A further three peaks, degenerate at 298 K, were resolved using data recorded over the temperature range of 278–303 K (peaks 14b/c and peak 16b, Fig. 1, middle panel). All the resonances shifted downfield of the diamagnetic region displayed temperature dependence to their observed shifts: Curie-type behaviour was exhibited by four of the peaks, anti-Curie by the remainder (Fig. 1, middle panel). Nine of these peaks had T_1 values ≤10 ms, with the T_1 values of the remaining three measured being below 21 ms. These values are characteristic of βCH or αCH cysteinyl protons of cluster ligands, respectively. Signals dispersed to >20.00 ppm is a characteristic associated with [3Fe–4S]$^{1+}$ clusters, as is the Curie temperature dependence of

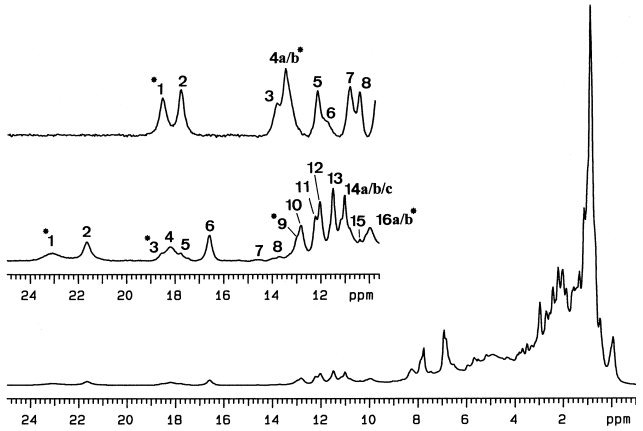

Fig. 1. 600 MHz ^{1}H NMR spectra of oxidised ht D14E *Da* FdIII at 298 K (100 mM phosphate, 99% D_2O, pH 7.6). (upper) Downfield region of freshly prepared protein. (middle) Downfield region of sample 'a' after storage at 277 K for 3 months. In (lower) the full spectrum of the sample after storage is given. * denotes peaks exhibiting a Curie-type temperature dependence.

at least one of the two furthest downfield peaks [31,32,35,38]. Both these characteristics are observed in the ^{1}H NMR data of mature D14E *Da* FdIII. The chemical shift dispersion pattern of the downfield hyperfine shifted peaks of this mature form of D14E *Da* FdIII is similar to that observed for wild-type ht *Da* FdIII, previously identified as a 7Fe ferredoxin [22,24]. The ^{1}H NMR data, therefore, support the other spectroscopic data in identifying mature D14E *Da* FdIII as an Fe/S protein containing both a $[3Fe–4S]^{1+/0}$ and a $[4Fe–4S]^{2+/1+}$ cluster. No experimental evidence for two $[4Fe–4S]^{2+/1+}$ clusters, or ligation by Glu-14, has been found. The differences observed between the chemical shift pattern of D14E *Da* FdIII and other 7Fe Fds, including wild-type *Da* FdIII [22], probably reflect small changes in the $[3Fe–4S]^{1+/0}$ cluster environment. Such subtle changes are not detectable by other methods and may arise as a result of the replacement of Asp-14 by the bulkier glutamic acid residue [22].

3.2. D14H Da FdIII

The UV/visible spectrum of freshly prepared D14H *Da* FdIII had an A_{397}/A_{280} ratio of 0.47. Taken together with the relative absorbencies of the native 7Fe *Da* FdIII [23], this indicates that the sample was ~60% loaded with iron. The EPR spectrum of D14H FdIII contained a $g = 2.01$-type signal and a small signal at $g = 4.3$, similar to the spectrum reported for D14E FdIII. For the freshly prepared protein double integration of the $g = 2.01$ signal gave 0.1 ± 0.15 spin/mol, indicating that only ~10% of the protein contained a typical $[3Fe–4S]^{1+}$ cluster. However, after storage for 3 months at 277 K, the mature form of the protein had a more intense signal at $g = 2.01$ which integrated to 1.0 ± 0.15 spin/mol, consistent with the presence of one $[3Fe–4S]^{1+}$ cluster per monomer. These data indicate that D14H *Da* FdIII behaved in a similar fashion to D14E *Da* FdIII. In both of these, the species observed in the freshly prepared sample only contained a small proportion of a detectable 3Fe cluster, which, with time, gradually changed to yield a stable species with spectral properties indicative of a 7Fe ferredoxin.

The ^{1}H NMR spectrum of the freshly prepared oxidised D14H *Da* FdIII contained seven peaks downfield of the diamagnetic envelope (at 10.16 ppm, 10.47 ppm, 10.78 ppm, 11.68 ppm, 13.37 ppm, 17.49 ppm and 18.27 ppm), with two of the peaks arising from overlapping resonances (peaks 3 and 4, Fig. 2, upper panel). This chemical shift dispersion resembles that observed for the freshly prepared D14E FdIII species: two distinctive downfield shifted signals are observed between 17 ppm and 19 ppm, but no peaks are shifted downfield of 20 ppm (cf. Fig. 1, upper panel and Fig. 2, upper panel). Linewidths at half-height ranged from 98 to 300 Hz. With the exception of peak 7, at 10.16 ppm, all of the hyperfine signals for which T_1 values could be measured had T_1 values of less than 10 ms. With linewidths >100 Hz, these protons exhibited characteristics of βCH protons of cysteine residues involved in the coordination of iron-sulphur clusters. Peak 7, with its longer T_1 value of 19 ms, and its comparatively narrow linewidth (~98 Hz), is likely to originate from the αCH proton of a cluster coordinating cysteine residue. Two peaks, peaks 1 and 3a, show Curie-type temperature dependencies, the same number as observed for the freshly prepared sample of D14E FdIII. As stated previously, this behaviour is typically observed for $[3Fe–4S]^{1+}$ clusters and not for $[4Fe–4S]^{2+}$ clusters. This observation cannot readily be reconciled with the EPR data which indicate that only ~10% of the sample contains a normal $[3Fe–4S]^{1+}$ cluster.

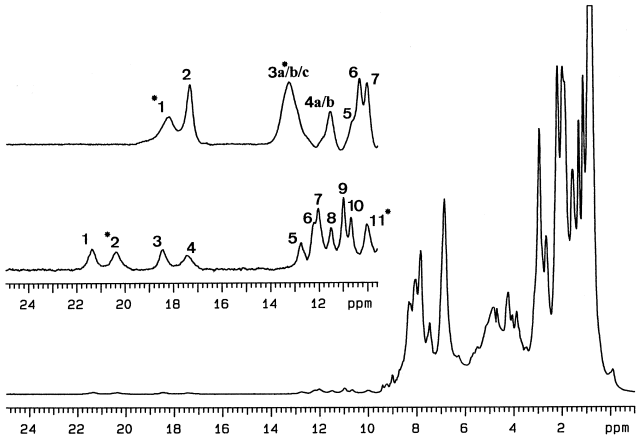

Fig. 2. 600 MHz ^{1}H NMR spectra of oxidised ht D14H *Da* FdIII (50 mM deuterated Tris, 95% H_2O/5% D_2O, pH 7.4). (upper) Downfield region of freshly prepared protein. (middle) Downfield region of sample 'a' after storage at 277 K for 3 months. In (lower) the full spectrum of the sample after storage is given. (a) was recorded at 288 K and (b/c) at 298 K. * denotes peaks exhibiting a Curie-type temperature dependence.

The ^{1}H NMR spectrum of D14H *Da* FdIII after storage contains 11 hyperfine shifted signals between 10 ppm and 22 ppm (Fig. 2, upper/lower panels). The increased chemical shift dispersion observed for the mature sample of D14H FdIII, compared to the freshly prepared sample, indicates a change in the cluster species present (Fig. 2). The enhanced relaxation exhibited by these peaks; T_1 values ranging between 2.6 ms to 16.7 ms, and half-height linewidths between ~86 and 223 Hz, again permitted the assignment of these peaks to βCH and αCH protons of cysteinyl residues ligating an Fe/S cluster(s). Although the number of resolved peaks shifted downfield of the diamagnetic region is smaller than that observed for wild-type FdIII [22] and D14E FdIII (this study), two of the main characteristics which enable the designation of a $[3Fe–4S]^{1+}$ cluster are observed; peaks shifted to >20.00 ppm and the Curie temperature dependence of one of the peaks shifted furthest downfield [31,32,35,38]. The identification of the presence of a $[3Fe–4S]^{1+}$ in the NMR data is consistent with the other spectroscopic data reported above.

Thus, in summary, no evidence for histidine coordination to a $[4Fe–4S]^{2+}$ cluster in D14H *Da* FdIII was found in this study, and the NMR characteristics of the clusters of the mature form of this protein were found to be similar to those of wild-type *Da* FdIII [22]. The mature form of D14H *Da* FdIII has thus been identified as a 7Fe ferredoxin. Differences observed in the ^{1}H NMR data between D14H and wild-type *Da* FdIII may be attributed to a combination of a change in the electrostatic environment of the $[3Fe–4S]^{1+/0}$ cluster and/or a change in the packing of the protein around the cluster. Comparison of the ^{1}H NMR data of wild-type FdIII [22] and the two mutants (this study) indicates that the Asp-14 to His-14 mutation has a greater effect on the immediate cluster environment in *Da* FdIII than the Asp-14 to Glu-14 mutation.

3.3. Cluster formation in Da FdIII

The role that iron-sulphur clusters play in determining the overall fold of the polypeptide chain has been recognised for

many years [39]. However, less progress has been made in understanding the processes by which peptide folding, accompanied by the insertion or assembly of the metal cofactor, actually occurs. NMR has been shown to be a technique suited to studying this relationship through both reconstitution of apo Fe/S proteins [40] and the denaturation of holo Fe/S proteins [41–43]. Intermediate species have been observed in the $[4Fe–4S]^{2+}$ HiPIPs of *Chromatium vinosum* (*Cv* HiPIP) and *Ectothiorhodospira halophilia*, and also the 8Fe Fd of *Clostridium pasteurianum* (*Cp* Fd) [40–43]. However, no intermediate was identified for the [7Fe–8S] *Bacillus schlegelii* Fd, or the [2Fe–2S] Fd from *Porphyra umbilicalis* [42]. The nature of the intermediate species, with respect to the state of the cluster and the polypeptide fold, has been shown to be dependent on the direction of the study, i.e. reconstitution or denaturation. The reconstitution study suggests that the iron-sulphur cluster assembles within a polypeptide framework that already possesses to a large extent, the secondary and tertiary structural elements associated with the native protein [40]. In this case, the cluster motif of the intermediate species is a [4Fe] species that lacks the disulphide bridges. Upon denaturation, however, it is the secondary and tertiary structure of the protein which is lost, with the cluster stabilising the intermediate species and structuring the protein only in the vicinity of the cluster ligands [43].

For *Da* FdIII, the ^1H NMR spectra of its apo form indicated that the polypeptide chain is unstructured in the absence of the clusters [24]. A similar lack of structure has also been observed for the apo forms of a [2Fe–2S] Fd and a [4Fe–4S] HiPIP [40,44]. After in vitro reconstitution holo ht wild-type *Da* FdIII is a structured protein containing both a $[3Fe–4S]^{1+/0}$ and $[4Fe–4S]^{2+/1+}$ cluster [24]. These observations suggest that protein folding is initiated by incorporation of the Fe/S cluster(s). However, with cluster incorporation complete for wild-type *Da* FdIII within the time course of the reconstitution reaction, and with no intermediate state observed, it is not possible to determine whether the Fe and S^{2-} atoms are incorporated as an [xFe–xS] unit or as an Fe_x unit, similar to those observed in the denaturation and reconstitution experiments described above. The data presented in this paper however, clearly identified an intermediate for the D14E and D14H mutants of *Da* FdIII.

Both the freshly prepared and mature forms of D14E and D14H FdIII contained a $[4Fe–4S]^{2+}$ cluster, but a normal $[3Fe–4S]^{1+}$ cluster was only present in the mature protein. However, since NMR signals with a Curie-type temperature dependence were present for the freshly prepared D14E and D14H FdIII mutants, a paramagnetic species other than the $[4Fe–4S]^{2+}$ cluster was also present. Though this was not a normal $[3Fe–4S]^{1+}$ cluster it had to be a poly-iron species so that magnetic coupling would render it EPR silent. Also as the mutants contained sufficient iron and S^{2-} for formation of two clusters in the mature protein, and as the UV/visible spectra monitor charge transfer transitions of the type Cys-S^- or S^{2-} to Fe^{3+}, a substantial proportion of the iron ions that eventually formed the $[3Fe–4S]^{1+}$ cluster must have been bound to sulphur ligands. Interestingly, the *Cv* HiPIP intermediate was postulated to contain an Fe cluster that lacked sulphide bridges but was stabilised by ligation to cysteine thiolates from the protein and molecules of DTT [40]. In this case, the addition of S^{2-} was needed for completion of the $[4Fe–4S]^{2+}$ cluster. This was not necessary for the proteins studied in this work, since all the components for the assembly and incorporation of the $[3Fe–4S]^{1+}$ and $[4Fe–4S]^{2+}$ clusters were present; time was the determining factor as to the cluster types observed.

Our results indicate that the $[4Fe–4S]^{2+}$ cluster is incorporated prior to the $[3Fe–4S]^{1+}$ cluster. This preferential incorporation may be connected with the positions of the ligands within the primary sequence. The covalent ligands of the $[3Fe–4S]^{1+}$ cluster are Cys11, Cys17 and Cys51, located near the termini of the polypeptide chain. Therefore, if this cluster was incorporated first the terminal regions would have to be anchored to provide the binding site, leaving a large loop region for insertion of the second cluster. In contrast, the initial incorporation of the $[4Fe–4S]^{2+}$ cluster, ligated by Cys41, Cys44, Cys47 and Cys21, would have a stabilising effect on the flexible polypeptide, both structuring the core of the protein and helping to anchor the tail regions. These tail regions would then be structured by the subsequent incorporation of the $[3Fe–4S]^{1+}$ cluster. Stabilisation of the polypeptide fold by the $[4Fe–4S]^{2+}$ cluster in a 7Fe ferredoxin is supported by a study on 7Fe *Sulfolobus* sp. strain 7Fd, where the likely function of this cluster was suggested to be structural rather than redox [45]. In addition, in the case of the 8Fe *Cp* Fd, the cluster structurally equivalent to the $[3Fe–4S]^{1+}$ cluster of *Da* FdIII was shown to be more susceptible to geometric changes on denaturation than the other, more buried, cluster [42].

Since an intermediate was not seen during the reconstitution of apo wild-type *Da* FdIII to the holo 7Fe form, but was for the D14E and D14H FdIII mutants, the mutations within the binding domain must be affecting the time course of the cluster incorporation. Differences in the local environment of the cluster imposed by the mutations of Asp to Glu or His are reflected in the chemical shift dispersions of the mature proteins (this study, [22]), and may reflect a conformational rearrangement of the polypeptide chain around the cluster binding motif. Bertini et al. [42] have proposed a correlation between the absence or presence of a structural intermediate containing a cluster with factors important for stabilising the actual protein structure. In cases where a cluster-containing intermediate is observed 'the prosthetic group is an essential structural element', but in the absence of an observable intermediate 'structural elements other than the cluster are determining folding and stability properties'. Whether the $[3Fe–4S]^{1+}$ cluster of *Da* FdIII is an essential structural element by this definition cannot be determined from the data available but the processes of cluster incorporation and protein folding are clearly linked.

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