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ORIGINAL ARTICLE

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# Formation of a linear [3Fe-4S] cluster in a seven-iron ferredoxin triggered by polypeptide unfolding

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**Abstract** Cubic iron-sulfur ([Fe-S]) clusters are common inorganic cofactors in proteins. The presence of a *linear* [3Fe-4S] cluster in a protein was first observed in beef-heart aconitase at high pH, where the protein structure was perturbed. Not long ago, the same linear cluster was discovered upon unfolding of a thermophilic di-cluster seven-iron ferredoxin, suggesting a more general relevance for this type of linear clusters in Nature. Since structure-induced cluster rearrangements may be important regulatory, on-going processes in living systems, we decided to further characterize the formation of the linear iron-sulfur cluster observed upon ferredoxin unfolding. Here we present a kinetic investigation of parameters that affect the linear-cluster formation and disassembly in the *Sulfolobus acidocaldarius* seven-iron ferredoxin. We find the linear cluster to be an intermediate on the protein-mediated cluster-degradation pathway under a wide range of pH and denaturant conditions. The linear species forms in parallel with secondary-structure disappearance. In contrast, the disassembly rate constant for the linear cluster is independent of denaturant concentration but depends strongly on solution pH. At high pH, the disassembly rate is slower and the linear iron-sulfur species has a longer lifetime, than at low pH.

**Keywords** Iron-sulfur proteins · Protein unfolding · Cluster degradation · Kinetics · Mechanism

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## Introduction

Many proteins in living systems bind small cofactors to attain their specific functions. Among the most common inorganic cofactors that interact with proteins are the iron-sulfur ([2Fe-2S], [3Fe-4S], and 4Fe-4S]) clusters, which upon protein incorporation facilitate electron transfer in various metabolic reaction chains. When and how cofactors incorporate into proteins in vivo is poorly resolved; it has been speculated that cofactor binding may sometimes take place before the corresponding polypeptide has adopted its native structure, thus acting as a folding trigger [1, 2]. Spontaneous assembly of iron-sulfur clusters has been shown in vitro, but it is believed that the clusters are enzymatically inserted (ligated to cysteine residues) into the target proteins in vivo (possibly before polypeptide folding) [3].

The interconversion of iron-sulfur proteins between apo and holo forms, and the interconversion of iron-sulfur clusters of high and low nuclearity (i.e. between [4Fe-4S] and [2Fe-2S] forms), are effective mechanisms for a variety of organisms to deal with oxidative stress and changes in intracellular iron concentrations. Iron-sulfur proteins that are dependent on cluster disassembly and conversion include novel transcriptional and translational regulators [4]. In addition, a growing number of [4Fe-4S] cluster-containing proteins (such as biotin synthase, lipoate synthase, and anaerobic ribonucleotide reductase) have been found to undergo reversible degradation to [2Fe-2S] forms in the presence of dioxygen, possibly a protective mechanism against effects of $O_2$ on the cluster [5, 6]. Iron-sulfur clusters can also undergo acid- and base-catalyzed hydrolysis. The kinetics and mechanisms for hydrolysis of both model [4Fe-4S] clus-

ters and [4Fe-4S]-containing proteins have been characterized. Acid-facilitated degradation was found to proceed via protonation of one or more bridging sulfides. The process was extremely slow in the proteins, unless the polypeptide structure was partially perturbed [7].

A good model system for [3Fe-4S] cluster studies is the family of di-cluster, seven-iron ferredoxins from archaea belonging to the order *Sulfolobales* [8, 9]. These thermostable organisms live optimally at low pH and at temperatures around 70–80 °C. Their ferredoxins are small, monomeric proteins with mostly $\beta$-sheet structure that contain one $[3Fe\text{-}4S]^{+/0}$ center and one $[4Fe\text{-}4S]^{2+/+}$ center. The clusters in the *Sulfolobus* ferredoxins were shown to be extremely resistant to thermally induced degradation [8, 9]. We recently characterized the thermodynamic stability and equilibrium-unfolding reaction in detail for one of these proteins [10, 11]: the *Acidianus ambivalens* ferredoxin for which the complete amino-acid sequence and a structural model are available. This ferredoxin is highly resistant to both temperature ($T_m = 122$ °C; pH 7) and chemical perturbation [addition of 8 M of the chemical denaturant guanidine hydrochloride (GuHCl) did not unfold the *A. ambivalens* ferredoxin at pH 7, 20 °C].

Upon unfolding of the *A. ambivalens* ferredoxin, the 410-nm absorption ultimately disappears, supporting that the iron clusters disassemble in the unfolded state [10]. GuHCl-induced protein unfolding at pH 10, however, promoted conversion of the native brownish protein to a transient intermediate (black/purple) form with absorption features at 520 nm and 610 nm [10], before complete degradation (yielding a clear solution) occurred. Based on comparisons with model complexes and the resemblance to beef-heart aconitase at high pH [12, 13], the *A. ambivalens* unfolding intermediate was assigned to be a linear [3Fe-4S] cluster [10]. Formation of the intermediate occurred in parallel with the disappearance of the polypeptide's secondary structure, but the new cluster was shown by gel filtration to remain bound to the unfolded polypeptide [10]. The intermediate species was also observed at low pH with another *Sulfolobales* seven-iron ferredoxin [11]: the *Sulfolobus acidocaldarius* ferredoxin which shares 89% amino-acid identity with the *A. ambivalens* ferredoxin.

A protein-bound iron-sulfur cluster with absorption peaks at 520 and 610 nm was observed for the first time when beef-heart aconitase was incubated at pH > 9.5 or treated with 4–8 M urea (the so-called purple aconitase) [12]. This partially unfolded form of the protein was shown by absorption, EPR, and Mossbauer spectroscopies (and comparison to model complexes) to contain a linear [3Fe-4S] cluster, which resulted from the rearrangement of the cubic [3Fe-4S] center. Purple aconitase is stable for days at 4 °C in the presence of air [12]; it appears to be the most stable tri-nuclear iron cluster described to date [12]. It has also been shown that the $[4Fe\text{-}4S]^{2+/+}$ cluster in *Escherichia coli* dihydroxy-acid dehydratase converts to a linear [3Fe-4S] form upon exposure to oxygen [14].

The reported observations on three independent groups of iron-sulfur proteins (aconitase, dehydratase, and ferredoxins) suggest that linear [3Fe-4S] clusters may exist, and perhaps have a function, in Nature. To further characterize these unique clusters, poised to form by polypeptide structural changes, we have studied the kinetics of linear-cluster formation and degradation using *S. acidocaldarius* seven-iron ferredoxin (Fd) as our model system. We find that the rate of linear-cluster formation correlates with protein-unfolding speed (secondary structure disappearance), whereas the subsequent degradation rate is independent of denaturant concentration but depends on pH; degradation is much slower at basic pH.

## Materials and methods

### Reagents

The chemical denaturants guanidine hydrochloride (GuHCl) and guanidine thiocyanate (GuSCN) were of the highest purity. All chemicals were from Sigma. *S. acidocaldarius* Fd was purified as detailed in [9]. Protein purity was confirmed by SDS/PAGE. *S. acidocaldarius* Fd contains seven iron atoms and one zinc atom, as determined by atomic absorption.

### Denaturant-induced unfolding

Equilibrium-unfolding data were collected at all pH conditions studied, to determine appropriate denaturant concentrations for kinetic studies. GuHCl was used to promote protein unfolding at pH 10, 8.6, and 2.5; GuSCN was used to promote unfolding at pH 11. Denaturant titrations were performed at 20 °C with 10–20 μM Fd in Tris-HCl (pH 8.5) or glycine (pH 2.5, 10, and 11) buffers. Samples were incubated for 60 min before measurements. Unfolding was monitored by far-UV circular dichroism (CD) (200–300 nm) on an OLIS instrument by visible absorption (300–700 nm) on a Cary-50 spectrophotometer, and by tryptophan emission (300–450 nm, excitation at 280 nm) on a Varian Eclipse. In accord with apparent two-state equilibrium transitions, there was no protein-concentration dependence for the profiles and the three detection probes gave identical results.

### Cluster-rearrangement kinetics

Kinetic measurements (at pH 8.6, 10, and 11) were performed by manual mixing, using either far-UV CD (signal at 220 nm) or visible absorption (signal at 610 nm) detection, at 20 °C. Reactions were triggered by addition of Fd to solutions of desired pH and denaturant concentration. Dead-time (due to mixing) was less than 20 s. All time-resolved experiments were repeated at least twice. The kinetic data were fitted to one-exponential (far-UV CD) and two-exponential (with opposite signs) decay equations (see Table 1) using the fitting module in KaleidaGraph v3.5. The pH 2.5 data were collected on an Applied Photophysics SX.18MV stopped-flow reaction analyzer (absorption at 610 nm). For each time range, eight kinetic traces were averaged and fit to monophasic decays using a non-linear least-squares algorithm supplied by Applied Photophysics.

### Thermally induced unfolding

Samples of 10–20 μM Fd in 2, 3, and 4 M GuHCl (pH 10) and in 2 and 3 M GuSCN (pH 11) were heated in a Cary-100 spectrophotometer with a digitally controlled thermostat. The temperature

was increased in 5 °C increments, from 20 to 90 °C, with 10 min of incubation at each temperature before 300–700 nm spectra were collected.

## Results and discussion

The equilibrium-unfolding process of *S. acidocaldarius* seven-iron Fd can be monitored by three independent spectroscopic probes: far-UV CD (secondary structure), visible absorption (cluster integrity), and tryptophan emission (tertiary structure). The protein is highly stable towards chemical as well as thermal perturbation, especially around neutral pH. Addition of 7.5 M of the chemical denaturant GuHCl does not promote any spectroscopic changes at pH 7 and 20 °C. However, at pH 10 (20 °C), the *S. acidocaldarius* Fd is fully unfolded in 6 M or higher GuHCl concentrations; at pH 2.5 (20 °C) the stability is lower, 4 M GuHCl being enough to promote complete polypeptide unfolding (data not shown).

Upon GuHCl-promoted unfolding at pH 10, the protein solution turns purple within minutes after the denaturant addition, before becoming clear on a 30–40 min time scale (Fig. 1A). The comparison of the visible absorption spectrum (peaks at 510 and 610 nm) for the transiently purple form of *S. acidocaldarius* Fd to spectra of model complexes and beef-heart aconitase at high pH conditions [12, 13] strongly suggests that the purple color originates from a linear three-iron [3Fe-4S] cluster. Further evidence for the presence of a linear iron-sulfur cluster comes from EPR experiments. At pH 6.5 the (oxidized) *S. acidocaldarius* Fd exhibits a typical $[3Fe\text{-}4S]^+$ cluster resonance at $g = 2.02$, and a minor contribution at $g = 4.3$ from adventitious iron in solution (Fig. 1B). Increasing the pH from 6.5 to 10 results in no significant spectral changes on the trinuclear cluster region, indicating that cluster integrity is not affected. However, upon incubation of oxidized *S. acidocaldarius* Fd in 6 M GuSCN for 1 min at the same pH, the signal from the $[3Fe\text{-}4S]^+$ center completely disappears, with the concomitant four-fold increase of the $g = 4.3$ resonance (Fig. 1B). This observation is compatible with the presence of a linear 3Fe cluster, and is reminiscent of the one observed in purple aconitase [12]. Gel filtration (within the first minutes after denaturant addition) shows that the purple cluster is still bound to the unfolded polypeptide. However, the cluster is not very stable in the unfolded protein; further incubation results in complete cluster degradation.

The kinetics of linear-cluster formation and its subsequent degradation in *S. acidocaldarius* Fd was first studied at pH 10 (20 °C) upon addition of sufficient amounts of GuHCl (6.0–7.5 M) to promote complete polypeptide unfolding. We find that the rate of visible absorption increase at 610 nm, correlating with linear-cluster formation, is identical to the rate of secondary-structure disappearance (as monitored by the decrease in CD signal amplitude at 220 nm) at all experimental conditions (see example in Fig. 2). Thus, the polypeptide-unfolding process dictates the speed by which the native cluster is forced to rearrange into the new linear cluster. Upon investigating the formation and decomposition rate constants as functions of denaturant (GuHCl) concentration at pH 10 (Fig. 3, Table 1), a clear denaturant dependence is found for the linear-cluster formation rates, as is expected since they correlate with the protein-unfolding kinetics. In

**Table 1** Kinetic data for *Sulfolobus acidocaldarius* ferredoxin cluster rearrangement, triggered by chemically induced protein unfolding, in solution conditions as indicated (20 °C). Errors in the reported rate constants are (±0.02) for $k_{form}$ and (±0.004) for *k*deg

| Denaturant | pH | $k_{form}$ ($min^{-1}$)[a] | $k_{deg}$ ($min^{-1}$)[b] |
|---|---|---|---|
| 6.0 M GuHCl | 10 | 0.07 | 0.013 |
| 6.5 M GuHCl | 10 | 0.10 | 0.021 |
| 7.0 M GuHCl | 10 | 0.14 | 0.018 |
| 7.5 M GuHCl | 10 | 0.16 | 0.014 |
| 4.0 M GuSCN | 11 | 0.158 | 0.016 |
| 5.0 M GuSCN | 11 | 0.30 | 0.016 |
| 6.0 M GuSCN | 11 | 0.39 | 0.013 |
| 7.0 M GuHCl | 8.6 | 0.32 | 0.062 |
| 4.0 M GuHCl | 2.5 | 35.3 | 0.50 |

[a] $k_{form}$ = linear-cluster formation rate constant. Measured by absorption increase at 610 nm and far-UV CD disappearance at 220 nm
[b] $k_{deg}$ = linear-cluster degradation rate constant. Measured by absorption decrease at 610 nm

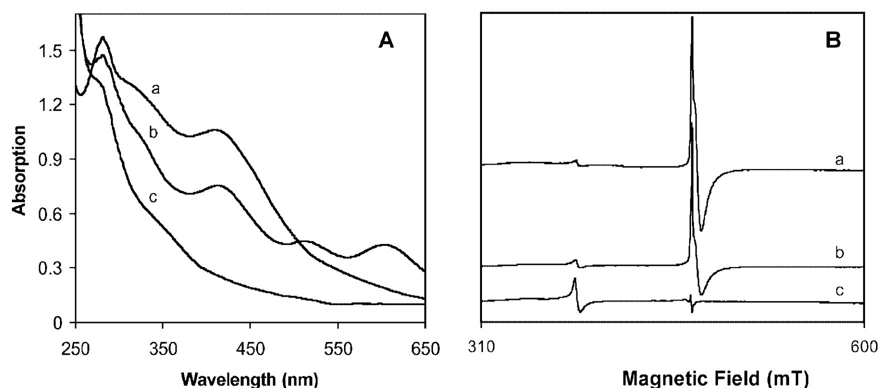


**Fig. 1** **A** Visible absorption of folded *Sulfolobus acidocaldarius* Fd (trace *a*), intermediate Fd, i.e. protein unfolded for a few minutes in 7 M GuHCl, pH 10 (trace *b*), and fully denatured Fd, i.e. unfolded protein incubated for >1 h (trace *c*). **B** EPR spectra of native *S. acidocaldarius* Fd at pH 6.5 (trace *a*, corresponding to trace *a* from **A**), upon pH increase from 6.5 to 10 (trace *b*), and intermediate Fd, i.e. after 1 min in 6 M GuSCN, pH 10 (trace *c*, corresponding to trace *b* from **A**)

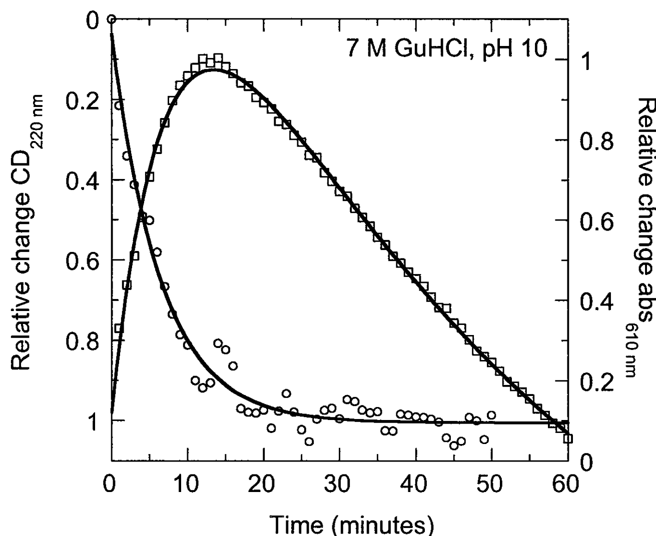


**Fig. 2** Example of kinetic traces, monitored by absorption at 610 nm (*squares*) and far-UV CD at 220 nm (*circles*), upon triggering ferredoxin unfolding by denaturant addition (pH 10, 7 M GuHCl)

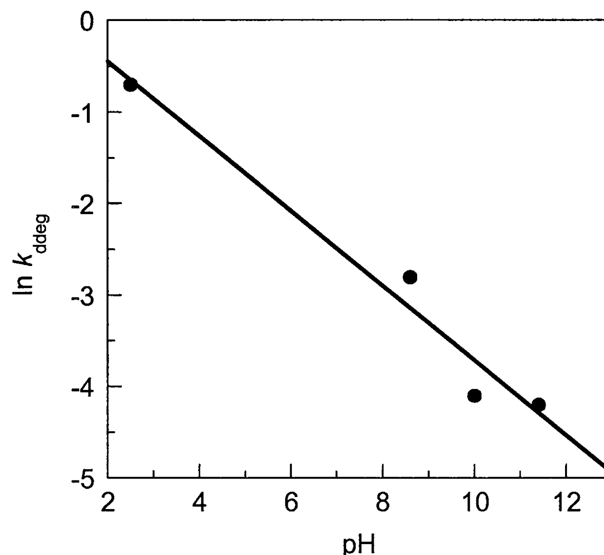


**Fig. 4** Rate constants for linear-cluster degradation (ln $k_{deg}$) as a function of pH. See Table 1 for exact values

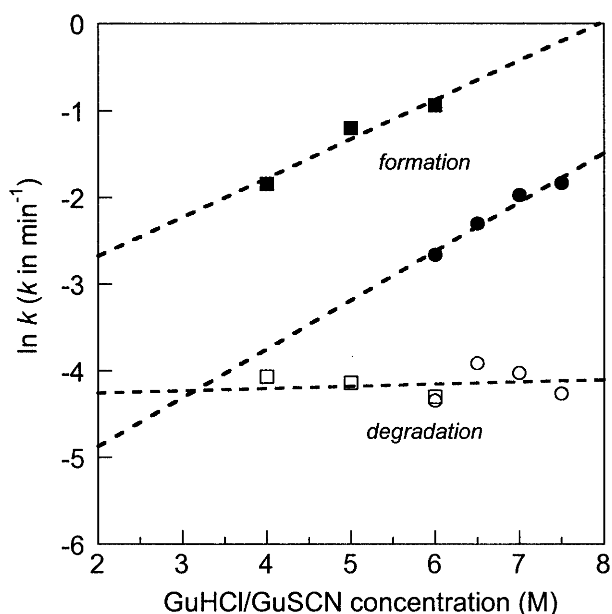


**Fig. 3** Natural logarithm of observed rate constants (in min$^{-1}$) for linear-intermediate formation (ln $k_{form}$, *filled symbols*) and degradation (ln $k_{deg}$, *open symbols*) as a function of GuHCl (at pH 10, *circles*) or GuSCN (at pH 11, *squares*) concentration. See Table 1 for exact values

contrast, the degradation rates do not depend on the denaturant concentration, but appear constant ($\tau_{1/2}$≈42 min, 20 °C, pH 10). This result implies that if the rates are extrapolated towards lower GuHCl concentrations, they will approach each other so that at some point (around 3 M) the linear intermediate will not be observed (cluster formation is slower than decomposition).

To investigate if the linear species in *S. acidocaldarius* Fd can also be observed when using another chemical denaturant, i.e. to reveal if the cluster-dis assembly mechanism is general (and not linked to the presence of GuHCl), we induced Fd unfolding by GuSCN (at pH 11). Equilibrium-unfolding studies of Fd in GuSCN at pH 11 show that 4 M GuSCN is sufficient to promote complete Fd unfolding. Also, in the presence of GuSCN the linear species is detected transiently in the time-resolved protein-unfolding experiments (Fig. 3, Table 1; 4–6 M GuSCN). The cluster-formation rate is faster here than in the same concentration of GuHCl, in accord with the more rapid protein unfolding since GuSCN is a stronger denaturant than GuHCl [15]. The subsequent decomposition rates, however, are very similar to those determined when using GuHCl as the denaturant ($\tau_{1/2}$≈46 min, 20 °C, pH 11). Linear-cluster formation and its subsequent decomposition must therefore be independent processes, such that the kinetics for the latter is not dependent on the nature, or concentration, of the chemical denaturant (it is de-coupled from the protein-unfolding reaction).

Purple aconitase was shown to form when yet another chemical denaturant, urea, was added. The degradation rate for purple aconitase at pH 8, 6 M urea, 4 °C, was reported to be 0.017 min$^{-1}$ ($\tau_{1/2}$ = 41 min) [12]. This value is very similar to the linear-cluster degradation rates reported here for Fd in GuHCl and GuSCN (at 20 °C). Unfortunately, urea is too weak a denaturant to promote unfolding of the highly stable *S. acidocaldarius* Fd. Instead, we tested for the possibility of thermally inducing the linear species, by slowly heating protein samples in presence of 2–4 M GuHCl (pH 10) and 2–3 M GuSCN (pH 11) while monitoring visible absorption changes. In all

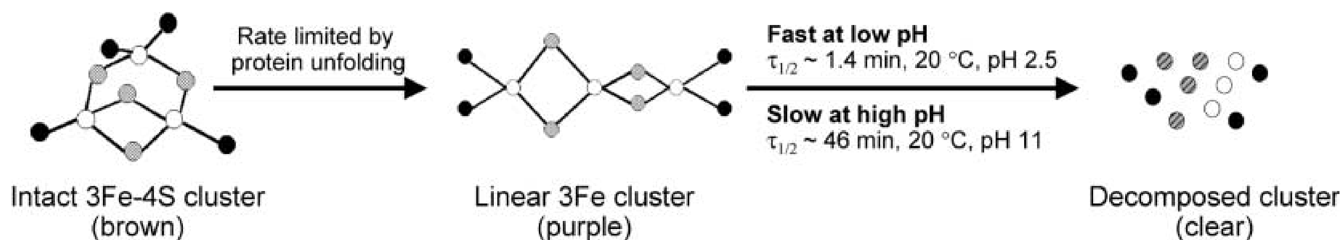


**Fig. 5** Schematic structures of the species involved in the polypeptide-promoted cluster-degradation pathway. A native cubic [3Fe-4S] cluster rearranges to a linear 3Fe species (first step) with a speed that correlates with polypeptide unfolding. The linear species subsequently degrades (second step) by a rate by which is strongly dependent on solution pH

denaturant-protein mixtures, folded Fd was favored at the starting temperature (20 °C). From the midpoints of the cooperative transitions, which were observed as decreases in 410-nm absorption upon heating, at various GuHCl concentrations, we estimated a thermal midpoint ($T_m$) for *S. acidocaldarius* Fd, in the absence of denaturant (at pH 10), of $94 \pm 3$ °C (data not shown). In accord, from observed $T_m$ values in various GuSCN concentrations, a similar $T_m$ ($93 \pm 6$ °C), in the absence of denaturant (at pH 11), could be derived. The linear species was never observed in the GuHCl-containing samples. However, in the presence of 3 M GuSCN the absorption characteristics for the linear species (peaks at 510 nm and 610 nm) were clearly present at temperatures around 45 °C, a temperature within the thermal transition where unfolded molecules have started to accumulate (data not shown).

Having established that the decomposition rate constants for the linear clusters do not depend on the choice of chemical denaturant, we tested the possible importance of solution pH (at 20 °C). The linear species, although still bound to the polypeptide, should be solvent exposed since the polypeptide is in its unfolded state; its stability is therefore likely to be affected by solution pH. In Fig. 4 we present linear-cluster degradation rates in unfolded Fd as a function of solution pH (from 2.5 to 11; 20 °C). At pH 2.5, protein unfolding and cluster decomposition processes are too fast for manual mixing experiments. By the use of stopped-flow mixing, however, accurate rate constants for the two reactions were resolved (Table 1). We detected a strong pH dependence in the cluster decomposition kinetics: the linear clusters disassemble much more rapidly at lower pH values ($\tau_{1/2} \approx 1.4$ min at pH 2.5, 20 °C). This result explains why the linear species were originally observed, in both aconitase and ferredoxin, at high pH conditions.

The equilibrium-unfolding reactions of *S. acidocaldarius* and *A. ambivalens* [10] Fd are not reversible. Denatured, colorless Fd samples cannot retain either native-like secondary structure or native iron-sulfur clusters, by dilution to conditions favoring the native state. To test if refolding and native-cluster formation could perhaps be observed from the intermediate, purple form, we performed double-jump experiments in which only 4–8 min in the presence of high-denaturant concentrations were allowed (resulting in polypeptide unfolding and linear-cluster formation but not degradation), before the protein samples were brought back to conditions favoring the native state. However, no regain of native-like 410-nm absorption, and thus native cubic clusters, as well as no increase in the negative far-UV CD signal at 220 nm, were observed in these experiments. We therefore conclude that formation of linear clusters, when promoted by polypeptide unfolding, is not reversible.

It is possible that the linear clusters, observed upon ferredoxin unfolding, originate from both the [3Fe-4S] and the [4Fe-4S] centers present in the native protein. In the case of aconitase, it is a cubic three-iron cluster that rearranges to a linear cluster [12]; in the case of dihydroxy-acid dehydratase, it is instead a four-iron cluster that converts to a linear three-iron species [14]. We have no evidence yet for which one of ferredoxin's two clusters, or if both, rearrange upon polypeptide unfolding. However, by analogy with the two cases mentioned above, it appears reasonable to assume that both clusters form linear three-iron centers. Nevertheless, further spectroscopic information is needed. In addition to the iron-sulfur centers, this ferredoxin coordinates a zinc ion [9]. The zinc has been suggested to play a role in protein stability and the metal was found to remain associated with the unfolded polypeptide [11].

## Conclusions

Protein-bound linear iron-sulfur clusters are shown to form transiently upon unfolding of *S. acidocaldarius* ferredoxin in a wide range of solution conditions (denaturants, pH, temperature). The cluster formation rate is determined by the protein-unfolding speed; its subsequent degradation is a pH-dependent process (Fig. 5). Although no biological function for linear iron-sulfur clusters is known, they may be common intermediates after unfolding (or prior to folding) of various ferredoxins as well as other iron-sulfur containing proteins. It appears reasonable to postulate that protein-mediated structural perturbations can regulate iron-sulfur cluster rearrangements. These events are likely to be on-going during normal cellular metabolism, perhaps used to modulate biological functions.

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