Published in final edited form as:

J Biol Inorg Chem. 2008 August ; 13(6): 981–991. doi:10.1007/s00775-008-0385-4.

“Iron priming” guides folding of denatured aporubredoxins

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Abstract

The relationship between iron uptake by aporubredoxins (apoRds) and formation of native holo-rubredoxins (holoRd), including their Fe(SCys)₄ sites, was studied. In the absence of denaturants, apoRds exhibited spectroscopic features consistent with structures very similar to those of the folded holoRds. However, additions of either ferric or ferrous salts to the apoRds in the absence of denaturants gave less than 40% recovery of the native holoRd circular dichroism and UV-vis spectroscopic features. In the presence of either 6 M urea or 6 M guanidine hydrochloride, the natively-like structural features of the apoRds were absent. Nevertheless, nearly quantitative recoveries of the native holoRd spectroscopic features were achieved by addition of either ferric or ferrous salts to the denatured apoRds without diluting the denaturant. Consistent with this observation, the native spectroscopic features were unaffected by addition of the same denaturant concentrations to the as-isolated holoRds. Denaturing concentrations of urea or guanidine hydrochloride also increased the rates of holoRd recoveries from apoRds and ferrous salts. Mass spectrometry confirmed that ferric iron binding to the denatured apoRds precedes the recoveries of protein secondary structures and Fe(SCys)₄ sites. Thus, iron binding to the apoRds guides, both kinetically and thermodynamically, refolding to the native holoRd structures. Our results imply that the ferrous oxidation state would more efficiently drive formation of the native holoRd structure from the nascent apoprotein in vivo, but that the Fe(SCys)₄ site must attain the ferric state in order to achieve its native structure.

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Electronic supplementary material The online version of this article (doi:10.1007/s00775-008-0385-4) contains supplementary material, which is available to authorized users.

Keywords

Rubredoxin; Metalloproteins folding; Iron uptake; Chaotropes; Hyperthermophilic

Introduction

Thousands of proteins are known to contain active or structural sites in which metal ions are directly coordinated to protein side chains. In a few cases metallochaperones have been shown to deliver metal ions or metal clusters to these proteins [1–4], but for the vast majority the biological pathways and mechanisms of metal insertion and their relationships to formation of the natively folded holoprotein structures remain largely unknown.

In vitro studies have shown that small calcium [5, 6], copper [7–9] and zinc [10–14] proteins in their largely unstructured apo (metal-free) forms can achieve their natively folded structures upon binding of the metal ions delivered as simple metal salts. Relatively little is known about the relationship between metal uptake and folding of small non-heme iron proteins.

Rubredoxins (Rds), which are found in a wide range of mesophilic and thermophilic bacteria and archaea, are small (approximately 54 amino acid residues), monomeric proteins containing a single tetrahedral $[\text{Fe}^{\text{II,III}}(\text{SCys})_4]^{2-,-}$ site within a pair of CXXC loops and a three-stranded β -sheet (Fig. 1). More than 20 X-ray crystal structures of Rds have been deposited in the Protein Data Bank [15], and NMR-derived solution structures of holoRds are also available [16]. Since they contain no inorganic sulfide or other cofactors, Rds may not require a specialized machinery for iron incorporation in vivo [17, 18], and, in fact, no Rd metallochaperones have been reported. Rds, therefore, constitute a seemingly ideal prototype for investigating the relationships between non-heme iron binding and folding into the native protein structure.

The most thoroughly studied Rds are those from the mesophile, *Clostridium pasteurianum* (Cp), and the hyperthermophile, *Pyrococcus furiosus* (Pf). Investigations of thermally induced unfolding of CpRd showed that loss of the metal ion immediately precedes formation of the (apparently) unstructured apoRd [19], but that $\text{Fe}(\text{SCys})_4$ site stability does not control the thermostability of the native folded structure [20]. Engineered V $\leftrightarrow$ A exchanges of residue 44, which forms an N–H...S hydrogen bond to one of the iron-coordinating Cys residues, significantly raised and lowered the respective stabilities of the $\text{Fe}(\text{SCys})_4$ sites in CpRd and PfRd [21–24] but had only minor effects on the thermal stabilities of the folded holoRds. Thiophilic metal ions (Cd^{II} , Zn^{II}) can directly and isomorphically displace iron from the native $\text{Fe}^{\text{II}}(\text{SCys})_4$ site of CpRd under non-denaturing conditions and without unzipping of the β -sheet [25].

ApoCpRds and apoPfRds have both been reported to retain significant secondary and tertiary structure [19, 26] at room temperature and neutral pH, although the apoPfRd structure was, as expected, more thermally stable. An engineered PfRd variant in which all the Cys residues were replaced, although unable to bind iron, still folded into a near-native

tertiary structure at room temperature [27], whereas an analogous mesophilic Cys-free apoRd variant was unfolded [28].

The studies cited above addressed either the structure of the apoRd or irreversible iron loss starting from the native holoRd structures. A more physiologically relevant process may be iron uptake by apoRd and formation of the native holoRd. The studies reported here are to our knowledge the first to examine the sequence of events by which iron binding to apoRd leads to formation of the native holoRd structure. The results point to a central role for iron binding in driving the folding process even in the presence of denaturants.

Materials and methods

Proteins, chemicals and general procedures

Chemicals were reagent grade or better. Ultrapure urea and guanidine hydrochloride (GuHCl) were from Sigma. CpRds and PfRds were expressed and purified as the iron-containing holoRds according to published procedures [29]. Unless otherwise indicated, all the experiments were carried out aerobically at room temperature in 50 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.4 (hereafter referred to as buffer). Stock solutions of 6–8 M urea or 6 M GuHCl were prepared in buffer. The concentrations of holoRds were determined using $\epsilon_{492} = 8,850 \text{ M}^{-1} \text{ cm}^{-1}$ [23, 29]. ApoCpRd and apoPfRd were prepared by treating the holoRds with 10 or 25% trichloroacetic acid, respectively, in the presence of 5 mM 2-mercaptoethanol at 0 °C. The resulting apoRd precipitates were washed twice with water, and redissolved in buffer either with or without denaturants, as described later. ApoRd concentrations were determined using published extinction coefficients [29, 30].

Denaturation/renaturation of apoRds

For denaturation titrations, the washed apoRd precipitates were dissolved in a minimum volume of buffer, then diluted into various mixtures of buffer and stock 8 M urea or 6 M GuHCl solutions to achieve the desired chaotrope concentrations. Renaturation titrations were carried out analogously by dissolving apoRd precipitates in a minimum volume of stock 8 M urea or 6 M GuHCl, then diluting the solutions with various mixtures of buffer and chaotrope solutions. The fraction of folded protein was determined from far-UV circular dichroism (CD) spectra (200–250 nm), using buffer-dissolved apoRd as a reference.

Iron uptake by apoRds and refolding of holoRds

ApoRd precipitates were dissolved in buffer and, when appropriate, denaturants (either 6 M urea or 6 M GuHCl). For experiments in the absence of denaturants, increasing volumes of a freshly prepared 5 mM aqueous solution of ferric ammonium citrate, ferric ammonium sulfate or ferrous ammonium sulfate were progressively added to a buffered apoRd solution (typically, 0.05–0.1 mM protein). Visible-range CD and absorption spectra were recorded 30 min after each addition of the iron stock solution. When iron addition resulted in no further spectral changes, the mixture was left overnight, and a final spectrum was recorded 16–18 h later.

For experiments in the presence of denaturants, unless otherwise specified, 1 equiv of iron (from a freshly prepared 50 mM aqueous solutions of the ferric or ferrous salts) was added to the apoRd solution (typically, 0.05–0.1 mM protein in buffer, containing either 6 M urea or 6 M GuHCl). Changes in far-UV CD (at 225 nm), in visible CD (at 416 or 495 nm) and in absorbance (at 492 nm) were monitored either continuously at the given wavelengths in separate mixtures of identical composition, or by obtaining spectra of the same individual mixture at appropriate time intervals. For experiments involving denaturant dilution after iron addition, unless otherwise specified, the apoRd/denaturant/iron mixture was diluted with buffer within 10–15 s after addition of the iron salt. The UV–vis CD and absorption spectral time courses were then monitored for separate mixtures of identical composition, as described above.

Metal and protein analyses were performed after removal of excess reagents by exhaustive buffer exchange using Centricon® devices. Metal contents of the reconstituted Rds were determined by inductively coupled plasma mass spectrometry (MS) at the Chemical Analysis Laboratory, University of Georgia, Athens, GA, USA. Accessible and total thiols were determined using dithiobisnitrobenzoate in the absence or in the presence of 6 M guanidine, respectively [19].

Spectroscopy

UV–vis absorption spectra were recorded using a PerkinElmer Lambda 2 spectrophotometer. CD spectra were recorded with a JASCO J-810 spectropolarimeter. Both instruments were equipped with computer-controlled, Peltier-driven cell holders for temperature control.

¹H NMR experiments were performed as reported previously [19, 20, 23] using a Bruker AMX600 spectrometer operating at 600 MHz, in anaerobic 90:10 v/v H₂O/D₂O solutions containing 0.6–0.8 mM apoRd in approximately 5 mM buffer.

Mass spectrometry

Electrospray ionization (ESI) MS analysis of holoRds and iron-reconstituted apoRds was performed with a quadrupole time-of-flight hybrid mass spectrometer (Waters) operating in positive ion mode. Samples (10 µL, 25–50 pmol protein) were injected into the ion source at a flow rate of 2 µL/min using a syringe pump driving a gastight syringe connected to the instrument capillary. Spectra were scanned from *m/z* 2,200 to 800 at 10 s per scan. Mass-scale calibration was carried out by using the multiple charged ions of a separate introduction of myoglobin. Quantitative analysis of components was performed by integration of the multiple-charge ions relative to each species. H/D exchange experiments were performed at room temperature by diluting a 0.01-mL aliquot of the appropriate apoRd solution (approximately 0.1 mM protein in 50 mM buffer) into 1 mL of 99.95% D₂O. The resulting mixture was immediately withdrawn with a syringe that was placed in the syringe pump drive mentioned above. Spectra were taken at various times after dilution, during continuous feeding of the D₂O-diluted protein through the ESI capillary. For iron-uptake experiments, 1 molar equiv of ferric ammonium citrate (or ferrous ammonium sulfate, both from aqueous 50 mM solutions) was added to the appropriate apoRd (approximately 1 mM protein in buffer containing 6 M urea), followed by dilution at preset times (minimum 2–3 s

from iron addition) with 100 vol of water. The diluted mixture was immediately withdrawn with a gastight syringe that was placed in the instrument's sample drive system. Spectra were recorded at appropriate times (minimum 20 s) following dilution of the urea/aprotein/iron mixture.

Results

HoloRd but not apoRd structures are stable towards high denaturant concentrations

The UV-vis absorption and CD spectroscopic features of holoCpRd and holoPfRd were completely insensitive to denaturant concentrations of up to 8 M urea and 6 M GuHCl at pH 7.4, even upon overnight incubation at 20 °C (Fig. 2).

On the other hand, the far-UV CD spectral features of the apoRds, which at pH 7.4 are very similar to those of the holoRds (Fig. 2) [19, 23], were differentially affected by denaturants. Six molar urea led to a nearly complete loss of the far-UV CD features of apoCpRd, whereas this concentration of urea had no effect on the far-UV CD features of apoPfRd. Residual far-UV CD features for apoPfRd were lost only upon treatment with at least 6 M GuHCl (Fig. S1).

Midpoint concentration (C_m) values for urea or GuHCl-induced denaturation of apoRds at 25 °C were calculated from semilog plots of fraction unfolded protein versus denaturant concentration from experiments similar to those reported in Figs. 2 and S1. The C_m for urea-induced denaturation of apoCpRd was 1.9 M, whereas C_m values of 1.4 and 2.9 M were obtained for GuHCl-induced denaturation of apoCpRd and apoPfRd, respectively. The fact that GuHCl, but not urea, can denature apoPfRd provides additional evidence that the stability of holoPfRd is "intrinsic" to the folded polypeptide and is not localized at the Fe(SCys)₄ site. We also found that apoPfRd, once denatured by 6 M GuHCl, remained unfolded when exchanged into 6 M urea.

The denaturant-induced changes in the various apoRds were fully reversible (Fig. S1). The far-UV CD spectra at the end of the denaturant addition/dilution cycles were identical to those of the starting apoRds (not shown). All the observed spectral changes were complete within the time (about 10 min) required for recording CD spectra. Rates could not be measured for either the unfolding or the refolding processes by manual mixing. Thus, under these conditions, equilibration between the various folded and unfolded states of the apoRds must occur on the seconds or faster timescale.

Recoveries of holoRds from apoRds are incomplete in the absence of denaturants

Recoveries of the native holoRd structures in buffered, denaturant-free solution of apoRds plus added iron salts were monitored by the appearance of the signature visible CD features of the [Fe^{III}(SCys)₄] site (Fig. 3) [19, 23]. The visible CD spectral changes following addition of iron salts required 30–60 min to reach their end points (i.e., no further spectral changes) at room temperature. However, the yields of holoRds at these end points (with respect to the starting apoRd) were all less than 40% and usually much lower (Fig. 3, Table 1), even in the presence of a fivefold molar excess of iron over apoRds. Under the aerobic conditions used here, these time courses and yields were insensitive to the presence or

absence of 2-mercaptoethanol (up to 70 mM). Ferric ammonium citrate, ferric ammonium sulfate and ferrous ammonium sulfate all gave similarly low yields.

Metal analyses showed that iron was the only metal incorporated under these conditions, and that the extent of iron incorporation was proportional to the observed intensity of the visible-region CD spectra prior to removal of excess reagent. ESI-MS experiments (Fig. S2) also indicated that apoRds and holoRds were the only species present in these mixtures, and that their molar ratios were consistent with both the visible CD spectral intensities and the metal analyses. These results indicate that the low yields of holoRd recovery are not due to nonspecific or spectroscopically undetectable iron binding or to binding of competing metals. The insensitivity of the reaction outcome to the presence or absence of 2-mercaptoethanol also rules out the possibility that cystine formation was responsible for the low yields of holoRd recovery.

¹H-NMR (Fig. 4) of the as-prepared apoCpRd resolved two structurally similar but distinct species. Several of the resonances in Fig. 4 can be assigned on the basis of previous NMR studies [19, 26]. The two distinct species are observed as splitting of these resonances into pairs, as exemplified by that centered at approximately -1.3 ppm assigned to I33 δ-CH₃, and that centered at approximately 11.5 ppm assigned to W37 indole ring NH. Figure 4 also shows that, over the time course of a few days at 25 °C under anaerobic conditions, one of these species converts into the other, and only one species remains after 2 weeks. Concomitantly with these spectroscopic changes, the “aged” apoCpRd lost its ability to take up iron, giving less than 8% holoRd recovery under conditions where the as-prepared apoRd gave approximately 40% holoRd (Table 1). Cystine formation during the aging process was negligible (3.94 and 3.88 thiols equiv/mol apoCpRd before and after the aging, respectively). The as-prepared apoCpRd, thus, contains two discernible structural isoforms, one of which is largely unable to recover the native holoRd structure.

HoloRds were also prepared by progressive dilutions of denatured apoRd solutions initially in 8 M urea (Cp) or 6 M GuHCl (Pf) to below the *C_m* values listed earlier with small volumes of buffered solutions of ferric or ferrous salts (either with or without 2-mercaptoethanol). HoloRd recoveries using this method (data not shown) were no higher than those obtained in the absence of denaturants (Table 1). The similar holoRd recoveries for these two methods indicate that, upon denaturant dilution, the apoRds fold into their nondenatured forms more rapidly than iron incorporation.

Iron uptake and holoRd recovery is associated with a flexible apoRd structure

The rigidity of the apoRd structural elements was assessed by ESI-MS H/D exchange experiments on the as-prepared apoCpRd and apoPfRd. A clear difference was observed between the two apoRds when monitoring the time courses of changes in mass distribution after the as-prepared apoRds in aqueous buffer had been diluted 100-fold into unbuffered D₂O (Fig. S3). H/D exchange was complete in apoCpRd 72 s after dilution into D₂O, whereas it was largely incomplete 144 s after dilution of apoPfRd into D₂O. This comparison indicates a much “stiffer” structure in apoPfRd.

Table 1 shows that the yields of holoRds from apoRds were significantly higher when 2 M of the chaotropic salt, NaClO₄, was present, whereas 2 M of the nonchaotropic salt, NaCl, had no significant effects on holoRd recoveries. This perchlorate effect and the lower holoRd yields from the “stiffer” apoPfRd (Table 1) suggest that “loosening” of structural elements of the apoRds leads to increased yields of holoRds.

Recovery of holoRds from apoRds is complete at denaturant concentrations above the C_m values

A completely different pattern emerged when iron salts were added to the urea- or GuHCl-denatured apoRd solutions prior to dilution with buffer and the resulting mixture was incubated at room temperature. As shown in Fig. 5, approximately 90% yields of holoRds were consistently obtained (Table 1) even when using stoichiometric amounts of ferric or ferrous iron salts and with or without 2-mercaptoethanol. Yields were assessed by UV/vis absorption and CD spectroscopies, and by metal analyses, all of which gave concordant results. Metal analyses and ESI-MS showed that iron at approximately 1 mol/mol apoRd was the only metal incorporated under these conditions. These nearly quantitative yields occurred only when the denaturant concentrations were initially higher than the C_m values for apoRd denaturation (listed earlier) prior to addition of the iron salts.

Denaturation accelerates holoRd recoveries from apoRds and ferrous salts

The yields of holoRds starting from denatured apoRds plus iron were unaffected by the oxidation state of added iron or by dilution of the denaturant after addition of iron salts. However, the rates of holoRd re-formation were considerably affected by both the redox state of the metal and the denaturant concentrations (Table 2). When starting with ferrous salts, recoveries of the native secondary structural elements, as monitored by the far-UV CD spectral features near 225 nm, were complete within the time required for manual mixing (5–10 s) even at denaturant concentrations above the C_m values. These secondary structural recoveries preceded those of the Fe(SCys)₄ sites.

Ellipticity changes at 495 nm and absorbance increases at 492 nm were used to monitor native Fe^{III}(SCys)₄ site recovery. No significant differences between the rates of ellipticity and absorbance recoveries were observed. The absorption feature at 492 nm is characteristic of Fe^{III}(SR)₄ complexes [31] but, unlike CD, does not require the chiral environment provided by the surrounding protein. The parallel absorption and CD spectral recoveries characteristic of the native Fe^{III}(SCys)₄ sites in a 54-residue protein where the two iron-ligating CXXC loops are separated by 29 residues indicates acquisition of the natively folded holoRd structure. As shown in Fig. 6, recoveries of the spectral features of the native Fe^{III}(SCys)₄ sites following addition of ferrous salts to denatured apoCpRds and apo-PfRds followed pseudo-first-order kinetics, were complete within a few minutes, and were faster when the denaturants were maintained above C_m after the addition of ferrous salts. Only minor differences were observed between the recovery kinetics of CpRd and PfRd under these conditions (Table 2). The rates reported in Fig. 6 and Table 2 were unaffected by the addition of 1 mM potassium ferricyanide within 1 min after the addition of ferrous salts, indicating that oxidation of ferrous iron to ferric iron is not the rate-limiting step in the formation of Fe^{III}(SCys)₄ sites. In all our experiments (including those with ferric iron

discussed below) we did not observe at any reaction stage the intense negative CD band at 315 nm characteristic of the native ferrous $\text{Fe}(\text{SCys})_4$ site in Rds [32].

Spectroscopic changes ensuing from the addition of ferric salts (either ferric ammonium citrate or ferric ammonium sulfate) to the apoRds in the presence of denaturants were much slower than those observed with ferrous salts. As shown in Fig. 7 and in Table 2, recoveries of the native $\text{Fe}^{\text{III}}(\text{SCys})_4$ sites starting with ferric salts occurred on the hours time scale, and, as with ferrous salts, were always slower than recovery of secondary structure elements.

Iron binding to denatured apoRds is the earliest detectable event in holoRd recovery

Iron-loaded Rds were the only species detected by MS after 1-min incubations of either apoCpRd or apoPfRd with equimolar ferric salts in buffered 6 M urea (Fig. 8). Urea was used for these experiments because GuHCl was found to be incompatible with MS. Prior to MS, the apoPfRd was buffer-exchanged from 6 M GuHCl into 6 M urea, under which conditions apoPfRd was found to remain unfolded.

The data in Fig. 8 and Table 2 show that binding of ferric iron was much faster than both the secondary structural (monitored by far-UV CD) and native $\text{Fe}^{\text{III}}(\text{SCys})_4$ site (monitored by 495-nm CD) recoveries whether or not the denaturant was diluted to below the C_m values following addition of ferric salts. The relatively rapid iron binding to the unfolded apoRds relative to the subsequent folding and $\text{Fe}^{\text{III}}(\text{SCys})_4$ site recovery is supported by the independence of the rates or yields of the recoveries of holoRds upon variation of the time interval between the addition of ferric salts to the denaturant-unfolded apoRds and the subsequent dilutions to 0.75 M denaturant from 5 s to 10 min.

Thus, regardless of the iron source, the sequence of events following addition of iron salts to chaotrope-denatured apoRds must minimally consist of, first, binding of iron to the apoproteins, second, formation of secondary structural elements and, finally, recoveries of the native $\text{Fe}^{\text{III}}(\text{SCys})_4$ site and holoRd structures.

Discussion

Two significant new results emerge from our studies. First, nearly quantitative recoveries of natively folded holoRds were obtained from apoRds and iron salts only at denaturing concentrations of urea or GuHCl. Much lower recovery yields of natively folded holoRds were obtained upon addition of iron salts to structured apoRds in the absence of denaturant or when denaturant was diluted by addition of iron salt solutions. Consistent with these results, exposure of the as-isolated holoRds to 8 M urea (CpRd) or 6 M GuHCl (PfRd) at neutral pH and room temperature did not alter the spectroscopic properties of the native $\text{Fe}^{\text{III}}(\text{SCys})_4$ sites, including the UV and visible-region CD spectra, indicating preservation of the iron site's chiral protein environment.

The second significant result can be interpreted as “ironpriming” of the denatured apoRds being the key step leading to recoveries of the holoRd structures. As summarized in Scheme 1, a sequence of events consistent with our results is as follows: (1) rapid (seconds or less) iron binding to the denatured apoRd leading to the iron-loaded unfolded apoRd; (2) collapse,

alignment and “zipping” of the three-stranded β -sheet at the protein structural core (Fig. 1), thereby generating the iron-primed, secondary structured Rd (seconds or less for ferrous); (3) slower rearrangements of the iron-primed, secondary-structured Rd that restore the native $\text{Fe}^{\text{III}}(\text{SCys})_4$ site and holoRd structures.

A conceivable alternative sequence of iron priming events to steps 1 and 2 could consist of a facile equilibrium between the denatured apoRd and an undetectably small portion of secondary structured apoprotein to which iron then binds, thereby shifting the equilibrium entirely to the iron-primed folded form. However, this alternative possibility is not consistent with the delay between iron binding and secondary structural recoveries for ferric priming of the denatured apoRds.

Our results indicate that the effect of iron priming on the protein folding is both kinetic and thermodynamic in nature. Since much lower yields and slower rates of recoveries were obtained starting from structured apoRds in the absence of denaturant, these structured apoRds must be largely incompetent for iron uptake, i.e., these apoRds must consist of largely nonnative (albeit folded) structures which cannot easily rearrange to native, iron-uptake competent structures. Thus, iron priming of the denatured apoRds must divert refolding away from nonnative structures onto a pathway leading to the native holoRd structures.

The iron priming events and holoRd formation were several-fold faster when using ferrous rather than ferric salts. This iron oxidation state dependence further supports iron priming as the key step leading to recovery of the native holoRd structure. The inherently faster ligand exchange rates of the ferrous oxidation state would allow the iron to more rapidly find its priming site(s) and more rapidly dissociate from off-pathway sites. The binding site(s) for the spectroscopically silent (even when using ferric) priming iron have not been identified. The native iron ligand Cys residues are obvious candidates, and this possibility is under investigation using site-directed Cys substituted variants.

The CD spectral signature of the native ferrous $\text{Fe}(\text{SCys})_4$ site [32] was not observed during holoRd recovery even when priming with ferrous salts. Our data also indicate that the ferric oxidation level of the $\text{Fe}(\text{SCys})_4$ must be achieved in order to recover the native holoRd structure. Therefore, if this recovery involves formation of a native ferrous $\text{Fe}(\text{SCys})_4$ site as an intermediate, it would have to be extremely rapidly oxidized to a ferric form. However, the only exogenous oxidant present, namely, atmospheric dioxygen, oxidizes native ferrous holoRd on the minutes time scale [33], suggesting that native ferrous $\text{Fe}(\text{SCys})_4$ sites are not part of the holoRd recovery pathway. The on-pathway folding events also must not involve oxidation of proximal Cys residues or intermolecular interactions. Disulfide reducing agents did not affect the sequence of events or yields of holoRd recovery, and our ESI-MS data did not indicate intermolecular iron- or disulfide-bridged protein species.

Our current results do not require different holoprotein recovery pathways for the Rd from the hyperthermophile versus the mesophile. However, a role for protein structural flexibility in promoting folding to the appropriate iron-ligating structure is indicated by the faster holoRd recoveries when maintained at the apo-denaturing concentrations, at least with

ferrous salts (Table 2). The role of protein structural flexibility is also supported by the higher holoRd recovery yields in the presence of the chaotropic salt perchlorate and by lower recovery yields for the “stiffer” apoPfRd in the absence of denaturants.

To our knowledge there are no published data directly addressing the relative time scales of folding and native iron site formation of Rds in vivo. Factors such as the nature of the intracellular iron donor, intracellular metal complexing agents and macromolecular crowding could conceivably affect these time scales. The fact that holoCpRds and holoPfRds can be recombinantly expressed in high yields in *Escherichia coli* [17, 30], which contains no native Rd, indicates that a Rd-specific metallochaperone is not required (although a mixture of ZnRds and FeRds is typically obtained in this heterologous expression system [18]).

In any case, the results reported here demonstrate that iron priming of loosely structured or unstructured apoRds from simple iron salts triggers and guides a viable folding pathway leading to the native holoRd structure. Our results suggest that metal delivery to the nascent apoRd poly-peptide on the ribosome could facilitate folding into the native holoRd structure and that achievement of this native structure from the de novo apoRd would be faster for ferrous than ferric donation. This latter suggestion is consistent with the typically reducing environment within bacterial cells. However, our results also show no evidence for a native ferrous Fe(SCys)₄ site along the refolding pathway and that the ferric Fe(SCys)₄ oxidation level must be achieved for full recovery of the native holoRd structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by funds from the University of Milan (FIRST, to F.B.) and US National Institutes of Health grant GM040388 (to D.M.K.). A.M. is the grateful recipient of a postdoctoral fellowship from the University of Milan.

Abbreviations

CD	Circular dichroism
Cp	<i>Clostridium pasteurianum</i>
ESI	Electrospray ionization
GuHCl	Guanidine hydrochloride
MS	Mass spectrometry
Pf	<i>Pyrococcus furiosus</i>
Rd	Rubredoxin

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Fig. 1.
The holo *Clostridium pasteurianum* (Cp) rubredoxin (Rd) structure. Residues relevant to this study are indicated. The drawing was generated using Rasmol, from coordinates in file 1IRO in the Protein Data Bank [34]

Fig. 2.

Urea-dependent changes in the far-UV circular dichroism (CD) spectrum of as-prepared apoCpRd. Shown are the CD spectra of 0.04 mM apoCpRd in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, containing 5 mM 2-mercaptoethanol and various urea concentrations (from 0 to 6 M, from *bottom* to *top* at 225 nm). The pathlength was 1 mm. The spectra of 0.04 mM holoCpRd in the presence (*dotted line*) and in the absence of 6 M urea are shown as *thin lines*

Fig. 3.

Appearance of the spectral features of holoRds upon addition of a fivefold molar excess of iron to as-prepared apoRds. Shown are the visible-region CD spectra of as-prepared apoRds dissolved in buffer before (*dotted line*) and 15 h after (*dashed line*) the addition of a fivefold molar excess of ferric ammonium citrate. The spectrum of the untreated holoRds at the same concentration is given as a reference (*solid line*). *Pf*, *Pyrococcus furiosus*

Fig. 4. ^1H NMR spectra of apoCpRd at various times after preparation by precipitation with trichloroacetic acid (TCA). Spectra were obtained for anaerobic solutions of 0.8 mM Rd in 5 mM Tris-HCl, pH 7.4, containing 10% D_2O (v/v) at 25 °C

Fig. 5.

Appearance of the spectral features of holoRds upon addition of iron salts to chaotrope-denatured apoRds. Shown are the visible-region CD spectra of apoRds in 6 M of the given chaotrope before (*dotted line*) and 15 h after (*dashed line*) the addition of an equimolar amount of iron as ferric ammonium citrate. Spectra of the untreated holoRds are shown for comparison (*solid line*). All proteins were 0.06 mM

Fig. 6.

Time courses of holoRd formation in mixtures of ferrous salts and chaotrope-denatured apoRds. Ferrous ammonium sulfate was added to a final concentration of 0.2 mM to a 0.2 mM solution of each apoRd in buffered 6 M urea or 6 M guanidine hydrochloride (*GuHCl*), as indicated. The resulting solution was immediately diluted either with 7 vol of plain buffer to give a final chaotrope concentration of 0.75 M, or with 7 vol of buffered 6 M chaotrope. Fractional structural recoveries were monitored by following increases in CD signal

intensities at 495 nm, and scaling to the corresponding spectral intensities of the appropriate holoRd

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Fig. 7.

Time courses of structural recovery in mixtures of ferric salts and chaotrope-denatured apoRds. Ferric ammonium citrate was added to a final concentration of 0.2 mM to a 0.2 mM solution of each apoRd in buffered 6 M urea or 6 M GuHCl, as indicated. The resulting solution was immediately diluted with either with 7 vol of buffer only to give a final chaotrope concentration of 0.75 M (*open symbols*), or 7 vol of buffered 6 M chaotrope (*filled symbols*). Fractional structural recoveries were monitored by following increases in the CD signal intensities at 225 nm (secondary structure, *circles*) or at 495 nm [Fe(SCys)₄]

site, *triangles*], and scaling to the corresponding spectral intensities of the appropriate
holoRd

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Fig. 8.

Electrospray ionization mass spectrometry (ESI-MS) spectra of apoRds, holoRds or a mixture containing apoRd, 6 M chaotrope and stoichiometric iron. *Right:* CpRd (6 M urea as the chaotrope). *Left:* PfRd (buffer-exchanged in 6 M urea after denaturation in 6 M GuHCl). For iron-uptake experiments solutions of each apoRd in the buffered denaturant indicated were diluted 100-fold in water 20 s after addition of an equimolar amount of iron as ferric ammonium citrate, and the ESI-MS spectrum was recorded within 20 s after dilution

Scheme 1.

Table 1

Recovery yields of holorubredoxins (holoRds) starting from aporubredoxins (apoRds)

Rubredoxin	Denaturant	HoloRd recovery (%)		
		In buffer	+2 M NaCl	+2 M NaClO ₄
<i>Clostridium pasteurianum</i>	None	42	39	57
	6 M urea	87	–	–
	6–0.75 M urea	89	–	–
<i>Pyrococcus furiosus</i>	None	14	14	23
	6 M GuHCl	88	–	–
	6–0.75 M GuHCl	86	–	–

ApoRds were prepared as described in “Materials and methods.” Experiments in the absence of denaturants were conducted at 0.04–0.05 mM apoRd in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), pH 7.4, containing a fivefold molar excess of ferric ammonium citrate over apoRd and the denaturants or sodium salts indicated. For experiments in the presence of denaturants, 1 equiv of ferric ammonium citrate was added to the apoRd in either 6 M urea or 6 M guanidine hydrochloride (*GuHCl*) (both in 0.1 M Tris–HCl, pH 7.4), and the resulting mixture was divided into two aliquots, one of which was diluted with 7 vol of 0.1 M Tris–HCl, pH 7.4 to give a final denaturant concentration of 0.75 M, and the other was diluted with buffered 6 M denaturant. Yields of holoRd recoveries were determined after overnight incubation at 20 °C by scaling the visible circular dichroism spectral intensities to those of the corresponding native holoRds at the same concentrations

Rate constants for secondary structural and the Fe^{III}(Cys)₄ site recoveries following additions of either ferrous or ferric salts to denatured apoRds

Table 2

Rubredoxin	Denaturant	k'_{app} (min ⁻¹)			
		Ferrous		Ferric	
		225 nm	495 nm	225 nm	495 nm
<i>Clostridium pasteurianum</i>	6 M urea	>100 ^a	2.9	0.008	0.003
	6–0.75 M urea	>100 ^a	0.46	0.27	0.045
<i>Pyrococcus furiosus</i>	6 M GuHCl	>100 ^a	0.96	0.14	0.067
	6–0.75 M GuHCl	>100 ^a	0.31	>100 ^a	<0.001 ^b

Data were obtained at 25 °C from experiments similar to those reported in Figs. 5, 6 and 7. Rate constants were calculated from semilog plots of fractional signal recovery versus time that were linear up to at least twice the reaction half-times, unless otherwise indicated

^aEstimated lower limit. Spectral changes were complete within approximately 5 s after mixing

^bNon-pseudo-first-order kinetics. The upper limit was estimated from the total recovery time