Divalent metal cofactor binding in the kinetic folding trajectory of *Escherichia coli* ribonuclease HI

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Abstract

Proteins often require cofactors to perform their biological functions and must fold in the presence of their cognate ligands. Using circular dichroism spectroscopy, we investigated the effects of divalent metal binding upon the folding pathway of *Escherichia coli* RNase HI. This enzyme binds divalent metal in its active site, which is proximal to the folding core of RNase HI as defined by hydrogen/deuterium exchange studies. Metal binding increases the apparent stability of native RNase HI chiefly by reducing the unfolding rate. As with the apo-form of the protein, refolding from high denaturant concentrations in the presence of Mg^{2+} follows three-state kinetics: formation of a rapid burst phase followed by measurable single exponential kinetics. Therefore, the overall folding pathway of RNase HI is minimally perturbed by the presence of metal ions. Our results indicate that the metal cofactor enters the active site pocket only after the enzyme reaches its native fold, and therefore, divalent metal binding stabilizes the protein by decreasing its unfolding rate. Furthermore, the binding of the cofactor is dependent upon a carboxylate critical for activity (Asp10). A mutation in this residue (D10A) alters the folding kinetics in the absence of metal ions such that they are similar to those observed for the unaltered enzyme in the presence of metal.

Keywords: cofactors; folding kinetics; metal ions; protein folding; ribonuclease H

A major goal of modern biophysics is to understand quantitatively where, when, and why structure accumulates during the folding of a protein and how critical tertiary interactions are maintained within living cells. Many proteins require the binding of cofactors to perform their biochemical activity, and these molecules fold in a cellular environment where their cognate cofactors are present. Previous studies with dihydrofolate reductase (Iwakura et al., 1993; Jennings et al., 1993) and α -lactalbumin (Kuwayama et al., 1989; Forge et al., 1999; Troullier et al., 2000) have shown that the presence of cofactors can perturb the folding behavior of these proteins; however, protein folding studies are frequently conducted in the absence of potentially complicating ligands to simplify biophysical experiments. Hence, despite the functional importance of cofactors, the manner in which they affect the folding pathway of proteins remains poorly understood.

To address these questions in a well-studied model system, we examined the consequences of divalent-metal ion binding to the stability and folding pathway of *Escherichia coli* ribonuclease HI. RNase H catalyzes the hydrolysis of RNA within RNA/DNA hybrids (Crouch, 1990; Hostomsky et al., 1993), and *E. coli* RNase HI binds a single Mg^{2+} ion cofactor in the active site of the enzyme (Katayanagi et al., 1990, 1992, 1993b; Black & Cowan,

1994). The stability and folding pathway of RNase HI in the absence of Mg^{2+} ions have been extensively characterized by circular dichroism (CD) and by hydrogen/deuterium exchange (Dabora & Marqusee, 1994; Yamasaki et al., 1995; Chamberlain et al., 1996; Raschke & Marqusee, 1997; Raschke et al., 1999). These studies indicated that a central core composed of α -helices and a β -strand is the most stable region under native conditions (Fig. 1). This same region (helices A and D and strand IV) folds first in kinetic experiments that are well described by a three-state scheme ($U \leftrightarrow I \leftrightarrow N$) containing a productive (on-pathway) intermediate.

Divalent metal binding is known to stabilize RNase HI (Kanaya et al., 1996). The metal-binding site in RNase HI is formed by carboxylate side chains (Fig. 1), which are interfacial to the putative kinetic intermediate (defined in the absence of metal) and to the more slowly folding remainder of the protein. One might predict that under the influence of metal, this rapidly collapsing intermediate would expand to include the metal-binding site, thereby changing the form and stability of the kinetic intermediate. Conceivably, the metal-free three-state folding pathway could convert to a two-state pathway ($U \leftrightarrow N$) when cofactor is included. An alternate possibility is that a Mg^{2+} ion may bind only the fully folded state of the protein. In this model, stabilization of the protein would then occur exclusively by decreasing the unfolding rate of the protein. Thus, the previously determined folding pathway would be a close approximation of the biologically relevant folding reaction.

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Fig. 1. Structure of RNase H*. Ribbon diagram of the crystal structure of RNase H* (the cysteine-free variant of *E. coli* RNase HI). The three conserved active site residues (D10, E48, D70) that bind a single Mg^{2+} ion are shown in red. The secondary structural elements (Helices A and D and Strand IV) known to be structured early in the folding trajectory of metal-free RNase H* at pH 5.5 (Raschke & Marqusee, 1997) are colored blue.

To uncover the mechanism by which the enzyme is stabilized via metal binding, we examined the folding trajectory of *E. coli* RNase HI in the presence of Mg^{2+} ions using CD spectroscopy. Our results indicate that Mg^{2+} ions do not perturb the overall three-state kinetic folding pathway, that metal binding stabilizes the protein by reducing its unfolding rate, and that formation of the native fold is required for divalent metal binding. These findings bolster the notion that simplified kinetic protein folding studies can provide information about biologically relevant folding pathways of proteins.

Results

Characterization of cysteine-free RNase H by crystallography

As a model system for studies on protein folding, we have utilized RNase H*, a variant of *E. coli* RNase HI lacking cysteines. This eliminates the necessity of using reducing agents in folding and activity experiments, and is more amenable to long-term storage (Kanaya et al., 1990a; Dabora & Marqusee, 1994). Many of the previous folding studies upon *E. coli* RNase HI including residue-specific hydrogen exchange experiments (Chamberlain et al., 1996; Dabora et al., 1996; Raschke & Marqusee, 1997) have focused upon this fully active variant of the enzyme. To obtain high-resolution structural data for this folding model, we solved the structure of *E. coli* RNase H* using X-ray crystallography (Fig. 1). We crystallized RNase H* (a C13A, C63A, C133A mutant of

E. coli RNase HI) based upon conditions used previously to grow crystals of the wild-type protein (Yang et al., 1990a, 1990b) (see Materials and methods). These crystals diffract to 1.4 Å resolution, and the structure was solved via molecular replacement. The structure of RNase H* was refined to an overall *R*-factor of 21.7% and a free-*R* of 25.4% with good geometry (Table 1).

The overall structure of RNase H* (Fig. 1) is highly similar to the two previously determined structures (Protein Data Bank (PDB) accession codes 1rnh, 2rn2) of the wild-type RNase HI protein (Katayanagi et al., 1990; Yang et al., 1990a). Comparison of the C- α coordinates (Holm & Sander, 1993) of RNase H* to the previously determined structures gave a root-mean-square deviation (RMSD) of 0.7 Å to 1rnh and 1.0 Å to 2rn2. The most substantial change in the structure of RNase H* compared to the probe model used for molecular replacement (2rn2) was the position of the flexible histidine containing loop (residues 123–128). In RNase H* this region was observed to adopt an alternate conformation in which the conserved histidine (His124) flips in toward the active site, similar to that for the 1rnh RNase HI structure. This indicates that our solution was not biased by the initial model used in molecular replacement. The sites where cysteines were replaced with alanines show no significant perturbations. Most side chains neighboring these mutations differ little from the wild-type structures, while those that were displaced are also seen to adopt different positions between the 1rnh and 2rn2 structures.

The negatively charged active site residues known to have important roles in metal binding (Kanaya et al., 1990b, 1996; Katayanagi et al., 1990, 1992, 1993b) are shown in Figure 1. One residue (Glu48) is part of the previously defined kinetic folding intermediate of RNase H* (Raschke & Marqusee, 1997). The other carboxylates (Asp10 and Asp70) are at the interface of the previously defined the kinetic intermediate of the apo-protein and the more slowly folding regions of the enzyme.

Table 1. RNase H* crystallographic statistics^a

Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell (Å)	<i>a</i> = 37.27, <i>b</i> = 40.83, <i>c</i> = 85.45
Resolution (Å)	20.0–1.4
<i>R</i> _{sym} (%) ^b	6.3 (13.6)
Completeness (%)	92.8 (59.8)
<i>I</i> / σ	16.7 (9.8)
<i>R</i> _{work} (%) ^c	21.7
<i>R</i> _{free} (%) ^c	25.4
Total atoms included	1,378
H ₂ O included	168
Ramachandran distribution	
Most favored regions (%)	88.5
Additionally allowed regions (%)	11.5
Generously allowed regions (%)	0.0
Disallowed regions (%)	0.0
RMSD bonds (Å)	0.006
RMSD angles (deg)	1.8

^aValues in parentheses are for highest resolution bin (1.45–1.4 Å).

^b $R_{sym} = \sum |I_{hkl} - \langle I_{hkl} \rangle| / \sum I_{hkl}$, where I_{hkl} is the intensity measurement for reflection with indices *hkl* and $\langle I_{hkl} \rangle$ is the mean intensity for multiply recorded reflections.

^c $R_{work, free} = \sum ||F_{obs}| - |F_{calc}|| / \sum |F_{obs}|$, where the *R*-factors are calculated using the working and free reflection sets, respectively. The free reflections comprise a random 10% of the data held aside for unbiased cross-validation throughout refinement.

Metal binding to RNase H is pH-dependent

Because the activity and metal-binding of RNase H* is pH-dependent, we surveyed a variety of conditions for metal-binding prior to beginning detailed kinetic studies. As a probe for ligand binding, we monitored thermal denaturation by CD spectroscopy in the presence and absence of divalent cations (Fig. 2). In these experiments, metal binding will be evident as increases in the melting temperature of RNase H* via preferential stabilization of the native state. We found that below pH 6.0 metal binding is minimal, but significant stabilization against thermal denaturation was observed at higher pHs. We also noted that the intrinsic stability of apo-RNase H* in the absence of metal at lower pHs was greater than it was at higher pHs

Given that metal binding was more pronounced at pH 8.0 than at lower pHs, we selected pH 8.0 as the standard conditions in which to evaluate metal-dependent folding. A comparison of urea denaturant melts on RNase H* in the presence and absence of 50 mM Mg^{2+} ions showed significant stabilization by the addition of divalent metal (Fig. 2C). Fitting these equilibrium melts to a two-state model with linear dependence of denaturant (Santoro & Bolen, 1988) gave apparent free energies of folding of -9.6 and -7.0 kcal/mol in the presence and absence of Mg^{2+} . Assuming that Mg^{2+} ions bind only the folded state, and at only one site in the enzyme, these stabilities indicate a metal-binding constant (K_d) of 0.7 mM (see Materials and methods). This number is roughly similar to that obtained for *E. coli* RNase HI under a variety of different conditions (0.1 – 3 mM) (Oda et al., 1991; Casareno & Cowan, 1996; Kanaya et al., 1996). We also noted that the stability of RNase H* was decreased by ~ 3 kcal/mol relative to its stability at pH 5.5 (Table 2).

RNase H refolds with a burst phase and one observable phase in the presence of metal

We used stopped-flow CD to monitor the refolding of RNase H* in the presence and absence of metal. Within the dead time of the instrument (~ 15 ms), we observed a significant acquisition of signal ($\sim 70\%$ at low denaturant concentrations), or burst phase, that is followed by a slow single kinetic phase easily fit to a first order rate constant (Fig. 3). Qualitatively similar three-state kinetics (unfolded $\rightarrow$ burst $\rightarrow$ native) were observed both in the presence and absence of divalent metal. The addition of Mg^{2+} did not, therefore, substantially alter the folding reaction of RNase H* and must stabilize the protein by a more subtle effect.

As was observed in metal-free studies at pH 5.5 (Raschke & Marqusee, 1997), unfolding reactions at pH 8.0 were well described by a single observable rate constant regardless of the presence of divalent metal. The rates, burst, and final amplitudes of both refolding and unfolding experiments are shown in Figure 4. The ionic strength of the solution is significantly perturbed by the addition of MgCl_2 . Therefore, as a control, we examined the kinetics of RNase H* in the presence of high KCl (200 mM), which mimics the ionic strength used in the presence of divalent cofactor (50 mM MgCl_2 , 50 mM KCl). There was little effect on the rates or amplitudes by the addition high KCl relative to lower ionic strength (Mg^{2+} -free) reactions (Fig. 4).

Mg^{2+} stabilizes RNase H by reducing the unfolding rate

We fit our rates and amplitudes simultaneously using a three-state, on-pathway model ($U \leftrightarrow I \leftrightarrow N$) (Table 2). The existence of burst

Fig. 2. Mg^{2+} ions stabilize RNase H* in a pH-dependent manner. **A:** Thermal melts of RNase H* monitored by CD: open circles (no divalent metal), black circles (10 mM MgCl_2), gray squares (50 mM MgCl_2). Buffer conditions were 16.7 mM HEPES pH 8.0, 0.8 M GdmCl (included to ensure $>90\%$ reversibility). **B:** pH-Dependence of melting temperature (T_m) of RNase H* in the presence of divalent metal. All samples contained 0.8 M GdmCl and 16.7 mM buffering agent where the buffering agent was potassium acetate at pH 5.0, MES at pH 5.7 and 6.2, and HEPES at pH 7.6 and 8.0. No metal (white), 1 mM MgCl_2 (light gray), 10 mM MgCl_2 (dark gray), 50 mM MgCl_2 (black), 50 mM NaCl (stippled). All T_m s are approximately $\pm 1^\circ\text{C}$. **C:** Urea denaturation of RNase H* in the presence (black circles) and absence (white circles) of 50 mM MgCl_2 monitored by CD at 227 nm. Buffer condition were 20 mM Tris pH 8.0, 50 mM KCl at 25°C .

Table 2. Equilibrium denaturant melts of RNase H* in the presence and absence of Mg^{2+} ions^a

Condition	$\Delta G_{F,app}$ (kcal/mol)	m, app (kcal/mol M ⁻¹)	C_m (M urea)	K_D (mM)
No divalent metal	-7.0 ± 0.2	1.82 ± 0.06	3.9	—
50 mM $MgCl_2$	-9.6 ± 0.3	2.03 ± 0.06	4.7	0.66

^aDenaturation was monitored by following the CD signal at 227 nm at 25 °C. Buffer conditions were 25 μ g/mL protein, 20 mM Tris pH 8.0, 50 mM KCl.

phase amplitudes and “rollovers” (deviations from linearity at low denaturant concentrations) suggests a folding intermediate (*I*) in the presence or absence of divalent metal (Fig. 4). The overall stability ($U \leftrightarrow N$) generated from global fits (-9.5 and -7.3 kcal/mol for the presence and absence of $MgCl_2$) agrees well with that obtained by two-state equilibrium melts (-9.6 and -7.0 kcal/mol). The majority of the stabilization obtained from the addition of Mg^{2+} ions stems from the ~ 15 -fold reduction of the unfolding rate constant (k_{ni}). An approximately twofold increase was observed in k_{in} when Mg^{2+} ions were added to the folding reactions and there was no significant difference in the stability of the intermediate.

Stabilization by metal can be mimicked by reducing the pH

The stability of RNase H* at pH 5.5 (~ 10 kcal/mol) is similar to that of RNase H* in the presence of 50 mM $MgCl_2$ at pH 8.0 (Table 3). The kinetic folding behavior of the enzyme in these conditions is also similar (Fig. 4). A reduction in the unfolding rate constant (k_{ni}), therefore, stabilizes RNase H* relative to the metal-free (pH 8.0) form either when the pH is reduced or when Mg^{2+} binds the enzyme. This suggests that the folding pathway of RNase

Fig. 3. Refolding of RNase H* in 50 mM $MgCl_2$. Protein folding was unfolded overnight in 6.4 M urea, rapidly diluted to 1.55 M urea and monitored by stopped-flow CD. Buffer conditions were 20 mM Tris pH 8.0, 50 mM KCl, 50 mM $MgCl_2$ at 25 °C. The gray curve is a single exponential fit of the data.

Fig. 4. RNase H* folding kinetics monitored by CD and fit to a three-state on-pathway model: rate constants (squares), burst phase amplitudes (triangles), and final amplitudes (circles). Gray symbols are 50 mM KCl data (fit is dashed curve), black symbols are 200 mM KCl (fit is solid curve), white symbols are 50 mM $MgCl_2$, 50 mM KCl, pH 8.0 (fit is short dashed curve). All experiments were carried out in 20 mM Tris pH 8.0 at 25 °C. Data in the long dashed curves are that for 50 mM KCl, pH 5.5.

H* at the lower pH is largely equivalent to that under conditions when the enzyme's cofactor binding site is occupied.

Without Asp10, RNase H does not bind metal and the unfolding rate is also slowed

The pK_a of active-site residue Asp10 has been measured to be upshifted to 6.1 in the absence of metal and reduced to 4.2 in the presence of Mg^{2+} ions (Oda et al., 1994). Because Asp10 is crucial for metal affinity in *E. coli* RNase HI (Kanaya et al., 1990b, 1996), we characterized a version of this protein lacking this residue (D10A). [The crystal structure of D10A is virtually identical to the RNase H* structure with minimal changes in the active site (E.R. Goedken & S. Marqusee, unpubl. obs.).] Mg^{2+} does not significantly alter the melting temperature of D10A RNase H* (data not shown). As previously observed (Kanaya et al., 1996; Raschke et al., 1999), this inactive mutant protein is significantly stabilized relative to unaltered RNase H*. We found that this is chiefly due to a reduced unfolding rate, which is not affected by the presence of metal (Fig. 5; Table 3). Therefore, the reduction in the unfolding

Table 3. Kinetic parameters of RNase H* folding^a

Protein	wt*	wt*	wt*	wt* ^b	D10A	D10A	D10A ^b
[KCl] (mM)	50	200	50	50	50	50	50
[MgCl ₂] (mM)	0	0	50	0	0	50	0
pH	8.0	8.0	8.0	5.5	8.0	8.0	5.5
k_{in} (s ⁻¹)	1.4 ± 0.1	1.8 ± 0.2	3.3 ± 0.5	0.7 ± 0.02	4.1 ± 1	2.5 ± 0.4	3.93 ± 0.2
min (kcal/mol M ⁻¹)	0.67 ± 0.02	0.66 ± 0.02	0.67 ± 0.04	0.454 ± 0.005	0.27 ± 0.08	0.26 ± 0.04	0.59 ± 0.01
k_{ni} (s ⁻¹)	6(±1) × 10 ⁻⁴	4(±1) × 10 ⁻⁴	4(±1) × 10 ⁻⁵	1.7(±0.04) × 10 ⁻⁵	4(±2) × 10 ⁻⁷	2(±0.7) × 10 ⁻⁶	9(±0.1) × 10 ⁻⁸
m_{ni} (kcal/mol M ⁻¹)	-0.36 ± 0.03	-0.42 ± 0.02	-0.39 ± 0.03	-0.42 ± 0.03	-0.49 ± 0.06	-0.40 ± 0.05	-0.54 ± 0.01
K_{ui}	100 ± 30	170 ± 50	120 ± 70	400 ± 20	400 ± 170	800 ± 200	1,660 ± 80
m_{ui} (kcal/mol M ⁻¹)	0.80 ± 0.05	1.0 ± 0.05	1.0 ± 0.07	1.24 ± 0.08	1.30 ± 0.09	1.36 ± 0.05	1.45 ± 0.01
ΔG_{un} (kcal/mol)	-7.3 ± 0.4	-8.0 ± 0.4	-9.5 ± 0.7	-9.7 ± 0.4	-13.1 ± 0.8	-12.4 ± 0.6	-15 ± 1
m_{un} (kcal/mol M ⁻¹)	1.9 ± 0.2	2.1 ± 0.1	2.0 ± 0.2	2.1 ± 0.1	2.1 ± 0.2	2.0 ± 0.2	2.4 ± 0.2
ΔG_{ui} (kcal/mol)	-2.7 ± 0.2	-3.0 ± 0.2	-2.8 ± 0.5	-3.6 ± 0.03	-3.5 ± 0.3	-4.0 ± 0.2	-4.4 ± 0.1
ϕ_i^c	—	0.4 ± 0.5	0.05 ± 0.2	0.38 ± 0.1	0.14 ± 0.07	0.25 ± 0.07	0.22 ± 0.04
$\phi_{\ddagger}^c$	—	0.66 ± 0.4	0.27 ± 0.3	0.13 ± 0.2	0.25 ± 0.1	0.34 ± 0.1	0.33 ± 0.01

^aReported values are in absence of denaturant.^bpH 5.5 kinetic data from Raschke et al. (1999).^c ϕ -Value of intermediate relative to pH, 8.0, 50 mM KCl, no MgCl₂^d ϕ -Value of transition state to pH 8.0, 50 mM KCl, no MgCl₂

rate of the unaltered RNase H* in the presence of Mg²⁺ is dependent upon metal binding to the active site. The kinetic behavior of D10A at pH 8.0 is also quite similar to that of D10A at pH 5.5. The stability of D10A does not change nearly as much with pH as does RNase H*. RNase H* can, therefore, be stabilized by the addition to the active site pocket of a positively charged cofactor or the removal of negatively charged side chain, and the effects upon the folding pathway of binding metal, protonating Asp10 or removal of Asp10, are similar in nature.

Discussion

The stability and folding pathway of *E. coli* RNase HI in the absence of divalent metal ions have been studied extensively by spectroscopy and hydrogen/deuterium exchange (Dabora & Marqusee, 1994; Yamasaki et al., 1995; Chamberlain et al., 1996; Raschke & Marqusee, 1997; Raschke et al., 1999). Those experiments have lent credence to a "hierarchical model" of folding in which the most stable portions of the molecule are the first to fold and serve as a scaffold assisting the assembly of less stable, more slowly folding regions (Chamberlain & Marqusee, 1997). A core group of helices (Helices A and D) and a beta strand (Strand IV) in the protein are the most stable under native conditions and comprise an early folding intermediate (Fig. 1). The kinetic folding pathway of RNase HI has been previously described as a three-state process with an on-pathway obligatory intermediate ($U \leftrightarrow I \leftrightarrow N$). The biologically active form of this enzyme, however, contains a divalent metal ion cofactor. In this study, we sought to assess whether the folding trajectory of RNase HI was significantly perturbed by the inclusion of divalent metal or whether minor alterations of the previously determined pathway were sufficient to describe its folding pathway.

RNase HI carries a single Mg²⁺ ion in its active site, which is required for the hydrolytic attack of RNA in RNA/DNA hybrids. A negatively charged metal-binding pocket is formed by the side chains of residues Asp10, Glu48, Asp70 (Fig. 1); this metal-

Fig. 5. D10A RNase H* folding kinetics: rate constants (squares), burst amplitudes (triangles), and final amplitudes (circles). Black and gray symbols are the presence and absence of 50 mM MgCl₂, respectively, in 20 mM Tris pH 8.0 with resulting fits (long dashed and short dashed, respectively). Shown for comparison are the curves determined for D10A at pH 5.5 (dashed curve) and for RNase H* at pH 8.0 (dotted curve) in the absence of divalent metal.

binding site is proximal to the “folding core” of the apo-protein as defined by hydrogen/deuterium exchange studies (Chamberlain et al., 1996; Dabora et al., 1996; Raschke & Marqusee, 1997). We found that in the presence of metal, rather than adopting an alternate folding scheme [such as a two-state process ($U \leftrightarrow N$) or a pathway having multiple channels to the native state], the folding of RNase HI is well described by a three-state folding trajectory ($U \leftrightarrow I \leftrightarrow N$).

The binding of Mg^{2+} ions ($K_D \sim 1$ mM) stabilizes the native fold of the protein and, therefore, must alter one or more of the steps in the on-pathway folding trajectory (see Model 1 in Materials and methods). A priori, there are several ways to increase overall stability of the native state (N) given the model above. For example, metal binding could increase the relative population of the intermediate state (I) by increasing K_{ui} , or the cofactor could increase the rate at which I proceeds to N (i.e., increasing k_{in}). Both of these possibilities accelerate flux through the folding pathway toward N . Alternatively, metal binding could stabilize N via reducing the unfolding rate k_{ni} . In our work with RNase HI, the most dramatic effect we observed was an ~ 15 -fold reduction in the unfolding rate (k_{ni}) in the presence of Mg^{2+} (Table 3). Figure 6 shows a reaction coordinate depiction of the folding trajectory of RNase H* in the presence and absence of divalent metal. Note that the transition-state height estimated using Eyring's equation (see Fig. 6 caption) is unchanged under all conditions examined regardless of the native state stability. Because CD spectroscopy is an inherently insensitive structural probe, we cannot rule out subtle metal-induced differences in the burst phase intermediate; however, neither the burst phase signal nor the stability of the intermediate suggest that the burst phase is influenced by Mg^{2+} ions. Hence, either in the presence or absence of cofactor, the route from the unfolded to the native state is similar, and protein stability is perturbed by the cofactor only after it reaches N .

Fig. 6. Reaction coordinate diagram for RNase H* at pH 8.0 in the presence of 50 mM $MgCl_2$ (gray circles), pH 8.0 in the absence of divalent metal (black squares), pH 5.5 in the absence of divalent metal (white triangles). The stabilities of the native states (N), the intermediates (I) relative to the unfolded state (U) are from Table 3. The activation energies of the transition states (TS) were determined using Eyring's equation and k_{ni} data from Table 3, and are intended to illustrate differences between folding conditions, not to accurately represent the absolute folding barriers.

ϕ -Value analysis of kinetic data can also provide useful interpretations regarding whether a certain interaction is important in either the intermediate (ϕ_I) or transition state ($\phi_{\ddagger}$) (Fersht et al., 1992). A ϕ -value near zero indicates that a certain interaction is not significant for a given state's formation while a ϕ -value near 1 indicates that such interactions are important for a given state. The ϕ -values in Table 3 are given relative to the pH 8.0 metal-free form of RNase HI. The ϕ -values of the metal-bound form both for the intermediate and the transition state are near zero, suggesting that Mg^{2+} makes little contribution to either state. Nonetheless, fractional ϕ -values are difficult to interpret, but in conjunction with other results reported here, our data indicate that the binding of metal to the active site of RNase HI occurs after the native fold has been adopted.

The carboxylate side chains in the active site of RNase HI make this pocket highly negatively charged, and this undoubtedly plays an important role in liganding divalent metal. Interestingly, active-site residue Asp10 has an unusually high pK_a (6.1), while the pK_a s of the other active site carboxylates are near expected ranges (2.6–4.4) (Oda et al., 1994). At pH 5.5, where most of the previous studies on the folding of RNase HI have been conducted, Asp10 is mostly protonated, leading to a reduction in the net charge of the active site. This likely accounts for the increase in stability observed at this pH and the inability to bind metal ions. At higher pHs, metal binding to the active site helps relieve negative charge repulsion and thereby decreases the unfolding rate. The overall stability and denaturant dependence of I is similar both when the pH is decreased to 5.5 or when Mg^{2+} ions are added. This furthers the notion that the kinetic pathway of the protein is not affected by the negative-charge repulsion in the active site until after the native structure is formed. Prior to this event, folding is essentially independent of the metal ions. Once the active site is properly oriented for metal binding, the divalent cation serves to further lock down the native fold and enables RNase HI to function catalytically.

Mutation of active site residue Asp10 to alanine (D10A) results in an enzyme that is inactive (Kanaya et al., 1996; Raschke & Marqusee, 1997) and does not bind metal. D10A is stabilized relative to the wild-type enzyme. This effect is largely due to a reduction in the unfolding rate (k_{ni}). Thus, the stability of the protein is affected by completely removing Asp10 from the active site pocket in a manner that is qualitatively similar to that observed when metal binds. The ϕ -values for D10A are low, suggesting that the active site is not formed in these states. Ideally, we would like to be able to compare the residue-specific stabilities of metal-bound RNase HI with the residue specific stabilities of apo-RNase HI determined via the recently developed native-state exchange experiment (Chamberlain et al., 1996). However, RNase HI does not bind its cofactors in pH ranges where these studies are accessible due to technical considerations. D10A may, therefore, provide a model for studying the structural energetics of the protein when the active site charge density is similar to that in the presence of metal ions.

We have found that the kinetic refolding trajectory of RNase HI in the presence of its metal cofactor remains largely similar to that in the absence of metal ions. Our results indicate that the protein binds metal only once it has achieved its native state, and that the stability of RNase HI is increased upon metal binding by reducing the unfolding rate. Similar results have been reported for staphylococcal nuclease A (Sugawara et al., 1991) where calcium ions stabilize the enzyme by reducing its unfolding rate. It remains to be seen if other enzymes using metal ions or other small cofactors also fold via similar pathways, pre-forming an apo-enzyme that is

then competent to ligand requisite cofactors. One example of a protein whose folding pathway is more substantially perturbed by the introduction of metal ions is α -lactalbumin. This metal-binding protein refolds much more quickly in the presence of calcium ions, which appear to prevent the sampling of nonnative pathways (Forge et al., 1999; Troullier et al., 2000). In contrast to RNase H, the unfolding behavior of α -lactalbumin remains largely unchanged in the presence of metal (Kuwajima et al., 1989). Additional studies will be necessary to determine if the acceleration of refolding or the deceleration of unfolding is a more general property of native state stabilization in metal-binding enzymes.

Materials and methods

Protein purification and crystal growth

RNase H* and D10A proteins were overexpressed in *E. coli* and purified by cation-exchange chromatography as described previously (Raschke et al., 1999). (RNase H* is cysteine-free variant of *E. coli* RNase HI.) Crystals of RNase H* were grown using conditions similar to those used for wild-type *E. coli* RNase HI (Yang et al., 1990b) (20 mM HEPES pH 7–8, 5–15% PEG-3350, 4–10 mg/mL protein) by the hanging-drop vapor diffusion method. Needle-like crystals frequently appeared after several weeks, but did not diffract sufficiently to be useful for X-ray analysis. Large crystals suitable for X-ray diffraction were produced by micro-seeding, growing to detectable sizes within 1 h and to maximum size ($\sim 500 \times \sim 200 \times \sim 70 \mu\text{m}$) within ~ 2 days. Crystals were briefly incubated (~ 1 min) in cryo-protectant (20% MPD, 20 mM HEPES pH 8.0, 10% PEG-3350) and flash frozen in liquid nitrogen.

X-ray diffraction and refinement of model

X-ray diffraction data were collected at the Advanced Light Source (Beamline 5.0.2) at the Lawrence Berkeley National Laboratory. Diffraction data were integrated and scaled using DENZO and SCALEPACK (Otwinowski & Minor, 1997). The RNase H* structure was solved by molecular replacement using the wild-type RNase HI structure (PDB accession number 2rn2) (Katayanagi et al., 1992) and AMoRe (Navaza, 1994). RNase H* models were refined using REFMAC (Murshudov et al., 1997) with automated water-building via ARP (Lamzin & Wilson, 1993). Between refinement cycles, manual rebuilding into $2F_o - F_c$ and $F_o - F_c$ maps was performed using O (Jones et al., 1991). Refinement statistics are summarized in Table 1.

The coordinates of the RNase H* structure have been deposited in the Protein Data Bank (1F21).

Equilibrium CD measurements

Circular dichroism (CD) data were collected using an Aviv 62DS spectropolarimeter with a Peltier temperature-controlled sample holder and 1 cm pathlength cuvette. Protein denaturation studies were conducted by monitoring the ellipticity at 222 or 227 nm as a function of temperature or denaturant concentration. Free energy of folding [assuming a two-state equilibrium transition and linear dependence on denaturant (Santoro & Bolen, 1988)], midpoint denaturant concentrations (C_m s), and midpoint temperatures (T_m s) were calculated as described previously (Dabora & Marqusee, 1994) using KaleidaGraph (Abelbeck Software) or Sigma Plot (Jandel Scientific, Austin, Texas).

Calculation of metal binding constants from apparent folding free energies

Metal was assumed to bind in a single site to the enzyme and not to bind to the unfolded state by the following model:

$$\begin{aligned} M + U &\xrightleftharpoons{K_F} M + N \xrightleftharpoons{K_A} MN \\ K_F &= [N]/[U] \\ K_A &= [MN]/([M][N]) \\ K_{F,app} &= [N]_{tot}/[U] = ([N] + [MN])/[U] \\ &= [N]/[U] (1 + [M]K_A) \\ K_{F,app} &= K_F (1 + [M]K_A) = K_F (1 + [M]/K_D) \end{aligned}$$

where M represents the metal ion, U and N represent the enzyme's unfolded and native states, K_F is the folding equilibrium constant between the unfolded and native states in the absence of metal binding, K_A and K_D are the association and dissociation constants between metal ions and the native state, and $K_{F,app}$ is the observed folding equilibrium constant in the presence of metal. When total metal concentration $[M]_{tot} \gg [N]$, then $[M] \approx [M]_{tot}$, and

$$K_{F,app} = K_F (1 + [M]K_A) \approx K_F (1 + [M]_{tot}K_A).$$

Folding/unfolding kinetics

Refolding/unfolding reactions were initiated by 1:11 dilution from high urea concentrations (6–8 M), either by using an Aviv 202-SF stopped-flow CD spectrometer (1 mm pathlength) or via manual mixing in an Aviv 62DS spectrometer (10 mm pathlength). Reaction conditions were 20 mM Tris pH 8.0, 50–200 mM KCl, 0–50 mM MgCl_2 with protein concentrations of 200 $\mu\text{g/mL}$ protein (stopped-flow) or 20 $\mu\text{g/mL}$ (manual mixing) at 25 °C.

Kinetic traces were fit to the following single exponential equations using Sigma plot (Jandel Scientific):

$$\text{CD signal} = A \exp(-k_{obs}t) + C$$

where A is the amplitude of the observable phase, k_{obs} is the observed rate constant, t is time, and C is the final amplitude (CD signal). The burst phase amplitude A_{bp} was calculated using

$$A_{bp} = C - A.$$

To correct for variations in protein concentration and other experimental errors, final amplitudes were multiplicatively scaled to match those expected from equilibrium values; burst amplitudes were scaled by the same relative factor.

The parameters determined above were fit via least-squares regression using Sigma Plot (Jandel Scientific) to a three-state on-pathway model (reviewed in Baldwin, 1996):

using the following equations:

$$K_{un} = K_{ui} k_{in} / k_{ni}$$

$$\ln k_{obs} = \ln((K_{ui}/(1 + K_{ui}))k_{in} + k_{ni})$$

K_{un} and K_{ui} , describe the equilibrium constant between U and N or U and I , respectively. The values k_{in} and k_{ni} represent the microscopic rate constants between I and N where these parameters have the following dependence upon denaturant concentration $[D]$:

$$k_{xy} = k_{xy(H_2O)} \exp((-m_{xy}[D])/RT).$$

$$K_{xy} = K_{xy(H_2O)} \exp((-m_{xy}[D])/RT).$$

Together with the kinetic model above, the burst and final amplitudes and observed rates of each kinetic experiment were simultaneously fit to two-state unfolding equilibria ($U \leftrightarrow I$ and $U \leftrightarrow N$) with each state modeled having a denaturant-dependent extinction coefficient (Santoro & Bolen, 1988). All of the kinetic parameters reported in Table 3 (K_{ui} , m_{ui} , k_{in} , m_{in} , k_{ni} , and m_{ni}) therefore contain contributions from the rates, burst, and final amplitudes.

ϕ -Values were calculated using the following equations:

$$\phi_I = (\Delta G_{ui,r} - \Delta G_{ui,t}) / (\Delta G_{un,r} - \Delta G_{un,t})$$

$$\phi_{\ddagger} = 1 - ((RT \ln(k_{ni,t}/k_{ni,r})) / (\Delta G_{un,r} - \Delta G_{un,t}))$$

where t is the reference condition and r is the test condition.

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