Investigating the Role of Metal Ions in the Catalytic Mechanism of the Yeast RNA Triphosphatase*

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The Saccharomyces cerevisiae RNA triphosphatase (Cet1) requires the presence of metal ion cofactors to catalyze its phosphohydrolase activity, the first step in the formation of the 5′-terminal cap structure of mRNAs. We have used endogenous tryptophan fluorescence studies to elucidate both the nature and the role(s) of the metal ions in the Cet1-mediated phosphohydrolase reaction. The association of Mg2+, Mn2+, and Co2+ ions with the enzyme resulted in a decrease in the intensity of the tryptophan emission spectrum. This decrease was then used to determine the apparent dissociation constants for these ions. Subsequent dual ligand titration experiments demonstrated that the metal ions bind to a common site, for which they compete. The kinetics of real-time metal ion binding to the Cet1 protein were also investigated, and the effects on RNA and nucleotide binding were evaluated. To provide additional insight into the relationship between Cet1 structure and metal ion binding, we correlated the effect of ion binding on protein structure using both circular dichroism and guanidium hydrochloride-induced denaturation as structural indicators. Our data indicate that binding of RNA, nucleotides, and metal ion cofactors does not lead to significant structural modifications of the Cet1 architecture. This suggests a model in which Cet1 possesses a preformed active site, and where major domain rearrangements are not required to form an active catalytic site. Finally, denaturation studies demonstrate that the metal ion cofactors can act by stabilizing the ground state binding of the phosphohydrolase substrate.

Eukaryotic mRNAs possess a 5′-terminal cap structure that plays a critical role in the translation, stability, splicing, and transport of these mRNAs from the nucleus to the cytoplasm (1). The capping of mRNAs occurs immediately following the synthesis of pre-mRNAs, and involves three distinct enzymatic activities. First, the 5′-end of the pre-mRNA is hydroxylated to a diphosphate by an RNA triphosphatase. This diphosphate end is then capped with GMP by an RNA guanyltransferase, and finally methylated by an RNA (guanine-7) methyltransferase (2).

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The Saccharomyces cerevisiae RNA triphosphatase elegantly demonstrated that each of these activities is essential for yeast cell growth (3–5).

The S. cerevisiae RNA triphosphatase (Cet1)† is a member of a family of metal-dependent phosphohydrolases that all possess the ability to hydrolyze the triphosphate end of mRNAs to a diphosphate in the presence of magnesium while also being able to hydrolyze NTPs to NDPs in the presence of either manganese or cobalt (9–13). The protein has been extensively studied by a wide range of techniques, including crystallography, kinetics, and site-directed mutagenesis (14–17). Additional studies showed that Cet1 shares many structural and mechanistic similarities with the RNA triphosphatases of various fungi, protozoan parasites, and DNA viruses (8, 11, 13, 14, 19–23).

Analysis of the Cet1 crystal structure reveals that the active site of the protein is located in a tunnel-like structure composed of eight antiparallel β strands (14). Mutational studies have demonstrated that amino acid residues located in each of the β strands contribute to the activity of the protein (8, 11, 15, 16). Furthermore, analysis of the crystal structure of Cet1 revealed the presence of a single manganese ion, coordinated by three glutamate residues (Glu-305, Glu-307, and Glu-496), located on the floor of the tunnel (14). No information is currently available on the precise affinity of Cet1 for the metal ion cofactors nor on their possible roles in catalysis. However, metal ions have the potential of fulfilling multiple functional roles in phosphohydrolase catalysis, including: (i) increasing the affinity of the enzyme for the RNA/NTP substrate; (ii) increasing the stability of the protein; (iii) being directly involved in catalysis by promoting the activation of nucleophiles; and (iv) stabilizing the transition state.

As a first step toward elucidating the nature and the role(s) of the metal ion cofactors in the Cet1-mediated phosphohydrolase reaction, we have utilized endogenous tryptophan fluorescence to evaluate the interactions of metal ions with Cet1. Quenching of the fluorescence signals by titration of the protein with metal ions provides a straightforward and powerful technique for evaluating the binding of metal ions to proteins. To provide additional insight into the relationship between Cet1 structure and metal ion binding, we correlated the effect of ion binding on protein structure using circular dichroism and guanidium hydrochloride-induced denaturation as structural indicators. Additionally, we investigated the effect of RNA and nucleotide binding on the structure and stability of the Cet1 protein. Our data provide insights into the precise role of metal ions in the Cet1-mediated RNA triphosphatase reaction, as well as on the effect of ligand binding on Cet1 structure.

† The abbreviations used are: Cet1, S. cerevisiae RNA triphosphatase; ATP-S, adenosine 5′-O-thiotriphosphate; Gdm-HCl, guanidium hydrochloride; CD, circular dichroism.
**Experimental Procedures**

**Cet1 Expression and Purification**—A plasmid for the expression of a full-length Cet1 protein (549 amino acids) was generated by inserting the *S. cerevisiae* Cet1 gene between the NheI and BamHI cloning sites of the pET28a expression plasmid (Novagen). In this context, the Cet1 protein is fused in-frame with a C-terminal peptide containing 6 tandem histidine residues, and expression of the His-tagged protein is driven by a T7 RNA polymerase promoter. The resulting recombinant plasmid (pET-Cet1) was transformed into *Escherichia coli* BL21(DE3) and a 100-ml culture of *E. coli* BL21(DE3)pET-Cet1 was grown at 37 °C in Luria-Bertani medium containing 0.1-mg/ml ampicillin until the A₆₀₀ reached 0.5. The culture was adjusted to 0.4 mM isopropyl-β-D-thiogalactopyranoside, and the incubation continued at 18 °C for 20 h. The cells were then harvested by centrifugation, and the pellet stored at −80 °C. All subsequent procedures were performed at 4 °C. Thawed bacteria pellets were resuspended in 5 ml of lysis buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10% sucrose), and cell lysis was achieved by adding lysozyme and Triton X-100 to final concentrations of 50 μg/ml and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and any insoluble material was removed by centrifugation at 13,000 rpm for 45 min. The soluble extract was applied to a 2-ml column of nickel-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer A containing 0.1% Triton X-100. The column was washed with the same buffer, and then eluted stepwise with buffer B (50 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, and 10% glycerol) containing 50, 100, 200, 500, and 1000 mM imidazole. The polypeptide composition of the column fractions was monitored by SDS-PAGE. The recombinant Cet1 protein was retained on the column and was recovered predominantly in the 0.2 M NaCl fraction. Following dialysis against buffer C, the phosphocellulose preparation was stored at −80 °C. Protein concentration was determined by the Bio-Rad dye binding method using bovine serum albumin as the standard.

**Fluorescence Measurements**—Fluorescence was measured using an Hitachi F-2500 fluorescence spectrophotometer. Background emission was eliminated by subtracting the signal from either buffer alone or buffer containing the appropriate quantity of substrate.

The extent to which ligands bind to the Cet1 protein was determined by monitoring the fluorescence emission of a fixed concentration of proteins and titrating with a given ligand. The binding can be described by Equation 1,

\[ K_d = \frac{[\text{Cet1}][\text{ligand}]}{[\text{Cet1}][\text{ligand}]} \]  
(Eq. 1)

where \( K_d \) is the apparent dissociation constant, [Cet1] is the concentration of the protein, [Cet1-ligand] is the concentration of complexed protein, and [ligand] is the concentration of unbound ligand. The proportion of ligand-bound protein as described by Equation 1 is related to measured fluorescence intensity by Equation 2,

\[ \Delta F/\Delta F_{\text{max}} = \frac{[\text{Cet1}][\text{ligand}]}{[\text{Cet1}][\text{ligand}]} \]  
(Eq. 2)

where \( \Delta F \) is the magnitude of the difference between the observed fluorescence intensity at a given concentration of ligand and the fluorescence intensity in the absence of ligand, \( \Delta F_{\text{max}} \) is the difference at infinite [ligand], and [Cet1] is the total protein concentration.

If the total ligand concentration, [ligand]ₜₐₜ, is in large molar excess relative to [Cet1]ₜₐₜ, then it can be assumed that [ligand] is approximatively equal to [ligand]ₜₐₜ. Equations 1 and 2 can then be combined to give Equation 3.

\[ \Delta F/\Delta F_{\text{max}} = \frac{[\text{ligand}]_\text{eq} / (K_d + [\text{ligand}]_\text{eq})}{[\text{Cet1}][\text{ligand}]} \]  
(Eq. 3)

The \( K_d \) values were determined from a nonlinear least square regression analysis of titration data by using Equation 3, and the stoichiometry of binding was established from a linear version of the Hill equation (Equation 4),

\[ \log(\Delta F/\Delta F_{\text{max}} - \Delta F) = n \log[\text{ion}] - \log K' \]  
(Ex. 4)

where \( n \) is the order of the binding reaction with respect to ligand concentration and \( K' \) is the concentration of ions that yields 50% of \( \Delta F_{\text{max}} \).

**Fig. 1.** Expression, purification, and fluorescent properties of Cet1. A, an aliquot (2 μg) of the purified preparation of Cet1 was analyzed by electrophoresis through a 12.5% polyacrylamide gel containing 0.1% SDS and visualized by staining with Coomassie Blue dye. The positions and sizes (in kDa) of the size markers are indicated on the left. B, background-corrected fluorescence emission spectra of Cet1. 1, purified protein in 50 mM Tris-HCl, and 50 mM KOAc, pH 7.5; 2, purified protein after a 2-h exposure to an 8 M solution of urea at 25 °C. Fluorescence spectra were recorded at an excitation wavelength of 290 nm. C, molar fluorescence of Cet1. Various concentrations of the purified Cet1 protein were assayed in 50 mM Tris-HCl, and 50 mM KOAc, pH 7.5. Emission was monitored at 339 nm, and excitation was performed at 290 nm.

**Circular Dichroism Spectroscopy Measurements**—Circular dichroism measurements were performed with a Jasco J-810 spectropolarimeter. The samples were analyzed in quartz cells with path lengths of 1 mm. Far-UV and near-UV wavelength scans were recorded from 290 to 250 nm and from 250 to 340 nm, respectively. All the CD spectra were corrected by subtraction of the background for the spectrum obtained with either buffer alone or buffer containing the ligand. The average of six wavelength scans is presented. The ellipticity results were expressed as mean residue ellipticity, [θ]d, in degrees-cm²-dmol⁻¹. Analysis of Competitive Metal Ion Binding—Analysis of the effect of a fixed concentration of one metal ion ligand (ion₀) on the binding of a second ion ligand (ionₐ) was performed in a manner analogous to that previously reported for analyzing the kinetics of a system in which two alternative substrates compete for the same enzyme binding site (24).
where $f$ probe was determined according to Equation 6, infinite concentrations of ion a and ion b, respectively. The concentrations of divalent ions were modified in some experiments using supplemental concentrations of KCl to generate the desired ionic strength. The concentrations of Co $^{2+}$ ions used in these experiments were 0 mM ( ), 20 mM (C), and 50 mM (D).

**Nucleotide Binding Assay**—Nucleotide binding was assessed by analyzing the interaction of $[^{35}S]ATP$ with the Cet1 protein. Protein-nucleotide complexes were precipitated by addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.2 volumes of ethanol, and quantified by spectrophotometry at 260 nm after dissolving in water.

**Chemical RNA Synthesis**—An RNA molecule of 50 nucleotides was synthesized using 2'-aceate ester chemistry (Invitrogen Research Inc., Lafayette, CO) and deprotected according to the manufacturer's recommended protocol. The 5'-hydroxyl-terminated RNA was then purified on a 20% acrylamide-8 M urea gel. The product was visualized by UV-shadowing, and the band was cut out. The RNA molecule was then eluted by incubating overnight at room temperature in a solution containing 0.1% SDS and 0.5 M ammonium acetate. The RNA was then precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.2 volumes of ethanol, and quantified by spectrophotometry at 260 nm after dissolving in water.

**Equilibrium Unfolding Experiments**—For all unfolding experiments, a 50 mM solution of purified Cet1 was adjusted to the desired final concentration of guanidium hydrochloride (Gdm-HCl) and incubated for 60 min at 25 °C. The intrinsic fluorescence of the protein was then monitored as a function of the Gdm-HCl concentration. The parameters $\Delta G_0$ (Gibbs free energy of unfolding), $\Delta G_0^m$ (Gibbs free energy of unfolding in the absence of denaturant), $m$ (cooperativity of unfolding), and $C_m$ (midpoint concentration of denaturant required to unfold 50% of the protein) were obtained as previously outlined (25) using Equations 7 and 8.

\[
\Delta G_0 = -RT \ln K_u \quad (Eq. 7)
\]
\[
\Delta G_0^m = \Delta G_0 - m[Gdm-HCl] \quad (Eq. 8)
\]

**RESULTS**

**Expression, Purification, and Intrinsic Fluorescence Properties of Cet1**—The S. cerevisiae Cet1 protein has the ability to hydrolyze the 5'-triphosphate end of RNAs to a diphosphate in the presence of magnesium. In addition, it is also able to hydrolyze NTPs to NDPs in the presence of either manganese or cobalt (8, 9). To investigate the metal ion binding activity of the enzyme, the Cet1 protein was expressed in Escherichia coli as described under "Experimental Procedures." The protein was sequentially purified from soluble bacterial extracts by affinity chromatography. SDS-PAGE analysis showed that the protein concentration, and $K_u$ the dissociation constant for the binding reaction.

**TABLE I**

| Ligand | $K_u$ (mM) | $\Delta F/F_0$ (max) | $n$ | Association rate ($\mu M^{-1} s^{-1}$) |
|--------|------------|----------------------|-----|-------------------------------------|
| $\text{Mg}^{2+}$ | 7 | 0.16 | 1.1 | 1.0 |
| $\text{Mn}^{2+}$ | 11 | 0.20 | 1.4 | 0.1 |
| $\text{Co}^{2+}$ | 26 | 0.19 | 1.1 | 0.1 |

The change in fluorescence ($\Delta F$) observed upon titration of Cet1 with ion a in the presence of a fixed concentration of competing substrate (ion b) can be described by Equation 5.

\[
\Delta F = \Delta F_{\text{march}} ([\text{ion}_a]/K_a) + \Delta F_{\text{march}} ([\text{ion}_b]/K_b) + ([\text{ion}_a]/K_a + [\text{ion}_b]/K_b) \quad (Eq. 5)
\]

where $\Delta F_{\text{march}}$ and $\Delta F_{\text{march}}$ are the changes in fluorescence produced at infinite concentrations of ion a and ion b, respectively. $K_a$ and $K_b$ are the apparent dissociation constants for ion a and ion b, respectively. Equation 5 was fit to the simple sigmoid saturation isotherms for both ion a and ion b.

**Equilibrium Binding Equations**—The interactions of Cet1 with various metal ions were described by Equation 6, for each probe was determined according to Equation 6.

\[
f_0 = \frac{[\text{Cet1}]}{K_u + [\text{Cet1}]} \quad (Eq. 6)
\]

where $f_0$ represents the fraction of bound nucleotides, [Cet1] the total concentration of the enzyme, and $K_u$ the dissociation constant for the specific binding reaction.

**Kinetic Analysis**—The kinetic parameters of Cet1 MgCl 2 were determined by fitting the change in fluorescence ($\Delta F$) at 339 nm as a function of added MgCl 2 to the simple ligand saturation isotherms for both ion a and ion b. A double-reciprocal plot is shown in the inset. C, the effect of increasing ionic strength on the apparent dissociation constant of Cet1 for MgCl 2 was investigated. Increasing concentrations of MgCl 2 were added to the reactions to generate the desired ionic strength. Kinetic analysis of real-time binding of MgCl 2 to the Cet1 protein. A 50 mM solution of the enzyme was incubated with 100 mM MgCl 2. Emission was monitored for 30 s at 339 nm, and excitation was performed at 290 nm. E, dual ligand titration using Mn $^{2+}$ as ion a, and Co $^{2+}$ as ion b. Standard titration assays were performed using Mn $^{2+}$ ions in the presence of increasing amounts of Co $^{2+}$ ions.

**FIG. 2** Titrations of Cet1 with Mg$^{2+}$ ions. A, increasing amounts of MgCl 2 were added to a 50 mM Tris-HCl and 50 mM KOAc, pH 7.5, and the emission spectrum was scanned from 310 to 440 nm. B, a saturation isotherm can be generated from these data by plotting the change in fluorescence intensity at 339 nm as a function of added MgCl 2. A double-reciprocal plot is shown in the inset. C, the effect of increasing ionic strength on the apparent dissociation constant of Cet1 for MgCl 2 was investigated. Increasing concentrations of MgCl 2 were added to the reactions to generate the desired ionic strength. D, kinetic analysis of real-time binding of MgCl 2 to the Cet1 protein. A 50 mM solution of the enzyme was incubated with 100 mM MgCl 2. Emission was monitored for 30 s at 339 nm, and excitation was performed at 290 nm. E, dual ligand titration using Mn $^{2+}$ as ion a, and Co $^{2+}$ as ion b. Standard titration assays were performed using Mn $^{2+}$ ions in the presence of increasing amounts of Co $^{2+}$ ions. The changes in fluorescence ($\Delta F$) observed upon titration of Cet1 with ion a in the presence of a fixed concentration of competing substrate (ion b) can be described by Equation 5.

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\]

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\]

where $f_0$ represents the fraction of bound nucleotides, [Cet1] the total concentration of the enzyme, and $K_u$ the dissociation constant for the specific binding reaction.
64-kDa Cet1 protein was the predominant polypeptide in the purified fraction (Fig. 1A). The amount of Cet1 protein in this fraction was estimated to be 150 ng/ml.

The fluorescence emission spectrum of the purified Cet1 protein in standard buffer at 22°C is shown in Fig. 1B. To obtain the maximal emission peak at the low concentrations of protein required to accurately determine $K_d$ values, excitation was carried out at 290 nm. Both tyrosine and tryptophan absorb at this wavelength (24). However, varying the excitation wavelength from 254 nm, where the contribution of tryptophane to the emission spectrum would be the greatest, to 295 nm, where the emission spectrum would arise almost exclusively from tryptophan, produced no change in the position of $\lambda_{\text{max}}$ (339 nm) or in the spectral bandwidth (65 nm at half-height) (data not shown). Thus, despite the fact that Cet1 contains 14 tyrosines in addition to the 3 tryptophans, the emission spectrum is dominated by the indole fluorophores. This dominance is due, in part, to the higher extinction coefficient of tryptophan, and to resonance energy transfer from tyrosine to tryptophan. The emission maximum of the enzyme (339 nm) is blue-shifted relative to the free L-tryptophan, which under the same conditions is observed to be at 350 nm. The $\lambda_{\text{max}}$ of tryptophan is highly sensitive to the polarity of the microenvironment in which its indole side chain is located. Blue shifts of protein emission spectra have been ascribed to the shielding of the tryptophan residues from the aqueous phase (26). This shielding is the result of the protein’s three-dimensional structure. Accordingly, denaturation of Cet1 with 8 M urea results in a red shift of $\lambda_{\text{max}}$ toward 350 nm (Fig. 1B).

The molar intensity of the fluorescence emission spectrum of Cet1 was also evaluated. This spectrum was determined to see if significant protein aggregation, or if the loss of protein from solution through adhesion, could influence the data. As can be seen in Fig. 1C, a decrease in fluorescence is observed with increasing concentrations of Cet1. A linear change of 0.28 fluorescence intensity units/nM protein was observed over the range examined. This relatively small change can be attributed to minor aggregation occurring at higher protein concentrations. Therefore, all the binding experiments were subsequently performed at a protein concentration of 50 nM, with the assumption that the binding equilibrium was not complicated by the presence of an aggregation equilibrium.

**Binding of Metal Ions to the Cet1 Protein**—The binding of metal ions to free enzymes has been shown to result in a significant decrease in emission fluorescence intensities (27–29). We initially sought to investigate the binding of the metal ion cofactors necessary for the Cet1-mediated phosphohydro-lase activity by titrating the binding of metal ions to a fixed concentration of the Cet1 protein. Typical emission spectra obtained from the titration of MgCl$_2$ are shown in Fig. 2A. We observed that the binding of Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ ions to the Cet1 protein resulted in a modification of the intensity of the intrinsic fluorescence of the protein. The addition of increasing amounts of Mg$^{2+}$ produced a decrease in the fluorescence intensity; however, both the emission maximum (339 nm) and spectral bandwidth were unaffected. The corresponding saturation isotherm, generated by plotting the change in fluorescence intensity at 339 nm as a function of added MgCl$_2$, is shown in Fig. 2B. Quenching saturated at millimolar Mg$^{2+}$ concentrations, and a 7 mM $K_d$ value could be estimated for Mg$^{2+}$ from a fit of Equation 3 to the generated saturation isotherm. About 16% of the intrinsic protein fluorescence was accessible to the quencher Mg$^{2+}$ ion (Fig. 2B). Analysis of a Hill plot, generated from the Mg$^{2+}$ ion binding data, yielded a Hill coefficient of 1.1, indicating a lack of cooperativity (Table 1). Furthermore, a Scatchard plot of Mg$^{2+}$ binding data is linear, providing no evidence for multiple classes of independent magnesium ion binding sites, nor of cooperative binding sites (data not shown). Note that the binding of the Mg$^{2+}$ ions could not be detected after heating the enzyme at 60°C in the presence of 0.1% SDS prior to the titration (data not shown). Furthermore, the addition of EDTA to the reaction reversed the effects on fluorescence and showed that the change in fluorescence observed by the addition of the metal ion is not solely due to a change in the ionic strength of the solution (data not shown). Accordingly, electrostatic interactions appear to make only minor contributions to the overall binding energy, as illustrated by the minimal effect of ionic strength on the apparent $K_d$ value of Cet1 for Mg$^{2+}$ ions (Fig. 2C).

The kinetics of real-time Mg$^{2+}$ ion binding to the Cet1 protein were investigated by monitoring the intrinsic protein fluorescence of Cet1 following the addition of Mg$^{2+}$ ions (Fig. 2D). The progress of the binding reaction was followed and showed that there was a rapid exponential decrease in fluorescence following the addition of the Mg$^{2+}$ ions. An apparent association rate of 0.1 μM⁻¹ s⁻¹ was estimated from the data. Half-maximal quenching was observed at −2 s, whereas maximal quenching was achieved after 6 s of incubation with Mg$^{2+}$ ions and remained constant thereafter. The exponential decrease in fluorescence observed following the addition of metal ions was not due to photobleaching, because similar results were obtained when the Cet1 protein was incubated away from the light source (data not shown).

The binding of Mn$^{2+}$ and Co$^{2+}$ ions to the Cet1 protein was
investigated in an analogous manner by monitoring the decrease in the intrinsic protein fluorescence following binding of the respective metal ion. The kinetics parameters were determined and are shown in Table I. Again quenching saturated at millimolar concentrations, and $K_d$ values of 11 mM and 26 mM were evaluated for manganese and cobalt ions, respectively. About 20% of the intrinsic protein fluorescence was accessible to the quencher Mn$^{2+}$ and Co$^{2+}$ ions.

We next sought to investigate whether or not magnesium, manganese, and cobalt ions bind to the same site of Cet1. Competitive alternative ligand binding experiments were carried out to determine if the different metal ions compete for a common binding site. In the first experiment, the combined $\Delta F$ produced at 339 nm by the addition of various Co$^{2+}$ concentrations was plotted against the concentration of Mn$^{2+}$ ions. Three concentrations of Mn$^{2+}$ were used in this competition experiment. The saturation isotherm, resulting from the competitive ligand experiment conducted with Mn$^{2+}$ as iona and Co$^{2+}$ as ionb, is shown in Fig. 2E. In accordance with a previously established model (24), the pattern of lines indicates that the Co$^{2+}$ and Mn$^{2+}$ ions are competitive ligands, because the binding is mutually exclusive (24). A quantitative analysis of the compliance of the experiment with the competitive binding model was made by comparing the $K_d$ and $F_{\text{max}}$ values derived from the fit of Equation 5 to the dual titration data with the $K_d$ and $F_{\text{max}}$ values determined in single ligand titrations. In each case, the values were statistically indistinguishable. Similar competitive experiments also demonstrated that Mg$^{2+}$ ions are competing for the same site as Mn$^{2+}$ and Co$^{2+}$ ions (data not shown).

importance of the metal ions in the nucleic acid binding activity of Cet1—Because metal ions have previously been shown to be required for the Cet1-mediated phosphohydrolase activity (8, 9), we were interested in determining if the metal ion binding activity could stimulate the binding of Cet1 to the RNA substrate. Fluorescence spectroscopy was used to monitor the RNA binding activity of Cet1 and revealed that the binding of RNA to the Cet1 protein results in a significant decrease in emission fluorescence intensities (Fig. 3A). The binding of Cet1 to a 5'-hydroxyl-terminated RNA transcript of 50 nucleotides was initially evaluated in the presence of 10 mM MgCl$_2$. Quenching saturated around 3 $\mu$M, and higher concentrations of RNA did not cause a further decrease in emission fluorescence intensity, suggesting that the reaction had come to an equilibrium. An apparent $K_d$ of 0.7 $\mu$M could be estimated for the RNA substrate (Fig. 3A).

The divalent ion requirements for the binding of the Cet1 protein to nucleic acids were then investigated. The results clearly showed that RNA binding can occur in the absence of
ions and that the addition of metal ions only modestly affects the binding of the RNA substrate (Fig. 3B). Similar conclusions were also drawn from electrophoretic mobility shift assays performed in both the presence and the absence of metal ions (data not shown).

**Importance of the Metal Ions in the Nucleotide Binding Activity of Cet1**—The effect of metal ions on the binding of free nucleotides to Cet1 was then investigated. A nonhydrolyzable ATP analog, ATPγS, was used in this assay to monitor the effect of metal ions on nucleotide binding, because the addition of metal ions to the classic ATP substrate would result in hydrolysis of the substrate and its release from the active site of the protein. Control experiments showed that ATPγS is not hydrolyzed by Cet1 either in the absence or the presence of metal ions (data not shown). Radiolabeled ATPγS was used as a probe in the binding assay, and nucleotide binding was analyzed by quantitation of bound substrate following trichloroacetic acid precipitation. The extent of binding was proportional to the amount of input Cet1 protein as seen in Fig. 4A. A typical titration experiment, using ATPγS as the substrate, is shown in Fig. 4B. An apparent $K_d$ value of 0.5 μM could be estimated from the generated saturation isotherm.

To determine the role of metal ions in NTP binding, the enzyme was titrated with ATPγS in the presence of increasing concentrations of Mg2+, Mn2+, or Co2+ ions. The addition of increasing concentrations of metal ions, up to a concentration of 50 mM, did not significantly modify the apparent $K_d$ value for ATPγS (Fig. 4C). These results suggest that metal ions do not significantly influence the NTP substrate binding activity of the Cet1 protein.

**Effect of Metal Ion Binding on the Structure and Stability of Cet1**—Far-UV circular dichroism (CD) spectra can provide useful information on the secondary structural features of a protein, whereas the CD spectra in the near-UV region reflect the environments of the aromatic amino acid side chains, giving information about the tertiary structure of a protein. To determine if the binding of metal ions results in the modification of the Cet1 structure, far- and near-UV CD spectra were recorded both in the presence and the absence of metal ions. Analysis of the far-UV CD spectra (Fig. 5A) revealed that the binding of Mg2+ ions to the Cet1 protein does not induce a significant modification of the secondary structure of the protein. Similar results were obtained when saturating concentrations of Mn2+ and Co2+ ions were added to the binding reactions (data not shown). Thus, the far-UV CD spectra suggest that the Cet1 protein maintains a comparably ordered secondary structure following the binding of the metal ions. Although the far-UV CD data indicate that no significant changes in secondary structure are occurring, analysis of the near-UV CD spectra was also performed to verify that the decrease in fluorescence intensity observed upon binding of metal ions indeed reflects a conformational change. Analysis of the near-UV CD spectra of the Cet1 protein in both the absence and presence of Mg2+ ions was performed from 250 to 340 nm. As can be seen in Fig. 5B, only a very minor reduction of the amplitude of the signal is observed over the 280- to 300-nm region when the protein is incubated with Mg2+ ions. Similar results were obtained when the protein was incubated with either Mn2+ or Co2+ ions (data not shown). Additionally, no meaningful changes in the patterns of proteolytic digestion were observed upon the binding of metal ions to Cet1 (data not shown). Overall, the CD spectra suggest that the protein does not undergo significant conformational change upon the binding of metal ions and that no radical modifications of the overall protein architecture are occurring.

To investigate whether or not the binding of metal ions could increase the stability of Cet1, guanidinium hydrochloride-induced denaturation and from the concentration of denaturant, $m$ (cooperativity of unfolding), and $C_m$ (midpoint concentration of denaturant required to unfold half of the protein) were determined by guanidinium hydrochloride denaturation and from the integration of the fluorescence intensity. The differences in $C_m$ and $\Delta G_m^\circ$ values in comparison to the free Cet1 protein are also shown ($\Delta C_m$ and $\Delta \Delta G_m^\circ$, respectively).

![Graph](image)

**Table II: Thermodynamic unfolding parameters measured by equilibrium guanidinium chloride denaturation**

| Protein | $C_m$ | $\Delta C_m$ | $m$ | $\Delta G_m^\circ$ | $\Delta \Delta G_m^\circ$ |
|---------|------|-------------|------|-------------------|------------------|
| Cet1    | 1.60 | 0.00        | 2.76 | 6.30              | 0.00             |
| Cet1-Mg | 1.52 | -0.08       | 3.03 | 6.06              | -0.24            |
| Cet1-Mn | 1.60 | 0.00        | 2.89 | 6.44              | 0.14             |
| Cet1-Co | 1.65 | 0.05        | 2.67 | 6.37              | 0.07             |
| Cet1-ATP| 0.78 | -0.82       | 1.93 | 4.44              | -1.86            |
| Cet1-ATPγS | 0.78 | -0.82 | 2.02 | 4.63              | -1.67            |
| Cet1-ATPγS-Mn | 1.10 | -0.50 | 2.45 | 5.71              | -0.59            |
| Cet1-RNA | 0.75 | -0.85       | 2.74 | 4.38              | -1.92            |
| Cet1-RNA-Mg | 1.20 | -0.40 | 2.86 | 5.44              | -0.86            |

**Structural and Mechanistic Implications**—Because our circular dichroism and unfolding analyses indicated no structural differences before and after metal ion binding to Cet1, our data
suggest that the binding of metal ions results in a minimal perturbation of the secondary and tertiary structures of the protein. This raises the possibility that the active site of Cet1 is preformed and that its formation does not involve significant conformational changes. Therefore, spectroscopic analyses were performed to determine if binding of RNA or nucleotides can result in a modification of the Cet1 structure.

Circular dichroism analyses were carried out to monitor potential structural modifications that could occur in Cet1 upon substrate binding. As can be seen in Fig. 7, analysis of the CD spectra revealed that the binding of RNA or ATP to Cet1 does not significantly alter either the secondary or the tertiary structure of the protein. Furthermore, no changes were detected when RNA or the nucleotide substrate was added to Cet1 that had been preincubated with metal ions (data not shown). The addition of other nucleotides (GTP, CTP, or UTP) and the nonhydrolyzable ATP analog ATP\(_{\text{S}}\) yielded similar results (data not shown). These data clearly demonstrate that Cet1 does not undergo significant conformational changes upon binding of RNA, nucleotides, or metal ion cofactors.

The effect of RNA and ATP binding on the stability of Cet1 was investigated by guanidium hydrochloride-induced denaturation studies. The change of the integrated fluorescence intensity as a function of the Gdm-HCl concentration for the Cet1 protein is shown in Fig. 7. The Gibbs free energy of unfolding of Cet1 bound to different ligands was thus determined (Table II). As can be seen from our denaturation studies, binding of RNA or ATP induces a modification of the protein stability. Our data indicate that the Gibbs free energy of unfolding of the Cet1 protein bound to ATP\(_{\text{S}}\) is increased by 1.06 kJ-mol\(^{-1}\) in the presence of Mg\(^{2+}\) ions. Clearly, metal ion cofactors are involved in the stabilization of the ground state binding of the RNA substrate and involve interactions unrelated to those at the scissile phosphate or specific to the transition state.

**DISCUSSION**

The RNA triphosphatase activity of the *S. cerevisiae* Cet1 protein has been extensively characterized during the past few years (8, 9, 11, 15–17). Elucidation of the Cet1 crystallographic structure provided numerous insights into the reaction chemistry (14). The amino acids involved in the binding of both the substrate and the metal ion cofactor have been identified, and mutational studies have confirmed their importance in catalysis (8, 11, 15, 16). However, a number of questions about the reaction chemistry remain to be addressed. One of these is the understanding of the precise role of metal ions in the phosphohydrolase reaction. As a first step toward elucidating the nature and the role(s) of the metal ion binding to the Cet1 protein, we have utilized the endogenous tryptophan fluorescence of the protein to precisely quantitate the interactions of metal ions with the enzyme.

Quenching of the fluorescence signals by titration of the protein with metal ions provides a straightforward technique for determining apparent \(K_d\) values (27–29). The high intrinsic
fluorescence signal of the Cet1 protein allowed binding assays to be carried out with a high degree of sensitivity. The decrease in fluorescence intensity observed upon saturation of the enzyme with metal ions can be produced by contact of the quenching agent with the indole side chain of a tryptophan and/or by alterations in the microenvironments of tryptophan residues distal to the ion binding site. The fact that no tryptophan residues appear to be located in the active site of the Cet1 crystals makes it unlikely that a tryptophan residue is involved in the binding of the metal ions. We hypothesize that the observed decrease in tryptophan fluorescence arises from a subtle change in the environment of Trp-288, which is located immediately under the tunnel floor in close proximity to the β1 and β11 strands. This tryptophan residue may sense the movement of the β1 and β11 strands that contain the glutamate residues that coordinate the metal ion, thereby providing a valuable spectroscopic signal for the induced formation of the enzyme-ion complex. The only other two tryptophan residues of Cet1 (Trp-247 and Trp-251) are quite far removed from the active site tunnel of the enzyme. Analysis of the Cet1 crystal structure revealed that these two residues are exposed on the protein surface, and studies indicated that they are part of a domain involved in binding to the S. cerevisiae RNA guanylyltransferase (17). The environment and location of these two tryptophan residues make them less likely to be responsible for the observed fluorescence changes, although they do contribute to the total observed fluorescence intensity.

Conformational changes in proteins are frequently critical for their function and/or regulation. We have looked for conformational changes in Cet1 upon the binding of metal ions, RNA, and nucleotides using spectroscopic approaches. Our data indicate that binding of the metal ion cofactors, RNA, and nucleotides does not lead to significant structural modifications of the Cet1 architecture. This suggests a model in which Cet1 possesses a preformed active site and where major domain rearrangements are not needed to form a catalytically active site. In fact, the binding of the nucleotide substrate to the Cet1 protein could not even be detected by fluorescence spectroscopy, indicating that no significant modification of the solvent accessibility of the Cet1 tryptophan residues occurs following binding of the substrate. Analysis of the structure of Cet1, crystallized in the presence of manganese and an analog of phosphate, revealed the presence of a tunnel-like catalytic active site (14). Our data suggest that the eight β strands that constitute the catalytic active site of the protein are already positioned prior to the binding of the substrate and metal ion cofactor. This is in sharp contrast to the RNA guanylyltransferase component of the capping reaction where significant domain rearrangements are required to form the catalytically competent active site of the enzyme (30, 31).

Based on the results of our fluorescence and binding studies, we demonstrated that the binding of metal ions to Cet1 does not significantly stimulate binding to RNA or nucleotide substrates. Equilibrium binding studies showed that enzyme-ATP and enzyme-RNA complexes had very similar dissociation constants with or without metal ions. We also demonstrated that binding of the substrate can occur in the absence of metal ion cofactors, indicating that substrate binding and catalytic activity are separate functions. Although metal ions are required for cleavage, the fact that the enzyme-substrate complex can form in the absence of divalent cations further suggests that the essential role of divalent cations in the reaction is in catalysis. The stoichiometry of the metal ion cofactor that is required to mediate the activity of Cet1 is an important variable for the mechanistic understanding of phosphohydrolase reaction. An important caveat to consider is that our binding experiments do not necessarily indicate the number of metal ions required for catalytic activity. It is possible that an additional metal binding site is present in the enzyme-substrate complex, a site created by the association of metal ions to the nucleotides or RNA substrates. Our data clearly indicate that Cet1 possesses a metal-binding site in the absence of nucleotide substrate, but the binding of an additional metal ion upon substrate binding cannot be excluded. In fact, a two-metal mechanism has recently been proposed for the baculovirus LEP4 RNA triphosphatase domain based on synergistic activation of the enzyme by manganese and magnesium (18). Based on crystallographic analysis of Cet1, a one-step-in-line catalytic mechanism has previously been proposed in which the single metal ion, found in the crystal and coordinated by residues emanating from the floor of the tunnel (Glu-305, Glu-307, and Glu-496), promotes catalysis by stabilizing a pentacoordinate phosphorane transition state (14). Analysis of our denaturant-induced unfolding experiments revealed that the metal ion cofactors are involved in the stabilization of the ground state binding of the phosphohydrolase substrate and involve interactions unrelated to those at the scissile phosphate or specific to the transition state. However, additional roles in the reaction chemistry, such as substrate coordination, stabilization of the intermediate transition state, or activation of nucleophiles, cannot be excluded. It is clear that the Cet1-mediated phosphohydrolase reaction requires a very precise alignment between the residues in the active site of the protein, the substrate, and the metal ion cofactor. The fact that the hydrolysis of RNA triphosphate ends is activated by magnesium, but not by manganese or cobalt, whereas the NTPase activity is supported by manganese and cobalt, highlights the importance of the metal ion cofactors in catalysis. The metal ions probably modify the coordination geometry or induce local conformational perturbations in the active site residues that ultimately influence substrate specificity. Although the complete understanding of the mechanisms underlying the Cet1-mediated phosphohydrolase is still incomplete, characterization of the biochemical properties of the protein should provide the basis for further studies in this direction.

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REFERENCES
1. Furuschi, Y., and Shatkis, S. (2000) Adv. Virology 55, 135–148
2. Shuman, S. (2000) Prog. Nucleic Acids Res. Mol. Biol. 66, 1–49
3. Shihabagaki, Y., Itoh, N., Yamada, H., Nagata, S., and Mizumoto, K. (1992) J. Biol. Chem. 267, 9521–9528
4. Schwer, B., and Shuman, S. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 4328–4332
5. Mao, X., Schwer, B., and Shuman, S. (1996) Mol. Cell. Biol. 16, 475–480
6. Wang, S. P., and Shuman, S. (1997) J. Biol. Chem. 272, 14685–14688
7. Tsukamoto, T., Shihabagaki, Y., Imajoh-Ohmi, S., Murakoshi, T., Suzuki, M., Nakamura, A., Gotoh, H., and Mizumoto, K. (1997) Biochem. Biophys. Res. Commun. 239, 116–122
8. Ho, C. K., Pei, Y., and Shuman, S. (1998) J. Biol. Chem. 273, 34151–34156
9. Ho, C. K., Schwer, B., and Shuman, S. (1998) Mol. Cell. Biol. 18, 5189–5198
10. Pei, Y., Ho, C. K., Schwer, B., and Shuman, S. (1999) J. Biol. Chem. 274, 28865–28874
11. Rodriguez, C. R., Takagi, T., and Buratowski, S. (1999) Nucleic Acids Res. 27, 2182–2188
12. Pei, Y., Lehman, K., Tian, L., and Shuman, S. (2000) Nucleic Acids Res. 28, 1885–1892
13. Pei, Y., Schwer, B., Hausmann, S., and Shuman, S. (2001) Nucleic Acids Res. 29, 387–396
14. Lima, C. D., Wang, L. K., and Shuman, S. (1999) Cell 99, 533–543
15. Bisaillon, M., and Shuman, S. (2001) J. Biol. Chem. 276, 17261–17266
16. Bisaloun, M., and Shuman, S. (2001) J. Biol. Chem. 276, 30514–30520
17. Ho, C. K., Lehman, K., and Shuman, S. (1999) Nucleic Acids Res. 27, 4671–4678
18. Martins, A., and Shuman, S. (2003) Nucleic Acids Res. 31, 1455–1463
19. Jin, J., Dong, W., and Guarino, L. A. (1998) J. Virol. 72, 10011–10019
20. Gross, C. H., and Shuman, S. (1998) J. Virol. 72, 10020–10028
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21. Ho, C. K., Martins, A., and Shuman, S. (2000) J. Virol. 74, 5486–5494
22. Ho, C. K., Gong, C., and Shuman, S. (2001) J. Virol. 75, 1744–1750
23. Ho, C. K., and Shuman, S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3050–3055
24. Painter, G. R., Wright, L. L., Hopkins, S., and Furman, P. A. (1991) J. Biol. Chem. 266, 19362–19368
25. Pace, C. N. (1995) Methods Enzymol. 259, 538–554
26. Eftink, M. R., and Ghiron, C. A. (1976) Biochemistry 15, 672–680
27. van der Wolk, J. P. W., Klose, M., de Wit, J. G., den Blaauwen, T., Freudl, R., and Driessen, A. J. M. (1995) J. Biol. Chem. 270, 18975–18982
28. Zhu, C. X., Roche, C. J., and Tse-Dinh, Y. C. (1997) J. Biol. Chem. 272, 16206–16210
29. de Seny, D., Heinz, U., Wommer, S., Kiefer, M., Meyer-Klaucke, W., Galleni, M., Freire, J. M., Bauer, R., and Adolph, H. W. (2001) J. Biol. Chem. 276, 45065–45078
30. Hakansson, K., and Wigley, D. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1505–1510
31. Hakansson, K., Doherty, A. J., Shuman, S., and Wigley, D. B. (1997) Cell 89, 545–583