Functional Groups Required for the Stability of Yeast RNA Triphosphatase in Vitro and in Vivo*

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Cet1, the RNA triphosphatase component of the yeast mRNA capping apparatus, catalyzes metal-dependent γ-phosphate hydrolysis within the hydrophilic interior of an eight-strand β barrel (the “triphosphate tunnel”), which rests upon a globular protein core (the “pedestal”). We performed a structure-guided alanine scan of 17 residues located in the tunnel (Ser177, Thr177, Gln305, His311, Ser324, Glu348, Thr349, His1391), at the tunnel-pedestal interface (Ile364, Met368) and in the pedestal (Asp315, Lys317, Arg321, Asp325). Alanine mutations at 14 positions had no significant effect on Cet1 phosphohydrolase activity in vitro and had no effect on Cet1 function in vivo. Two of the mutations (R321A and D425A) elicited a thermosensitive (ts) yeast growth phenotype. The R321A and D425A proteins had full phosphohydrolase activity in vitro, but were profoundly thermolabile. Arg321 and Asp425 interact to form a salt bridge within the pedestal that tethers two of the strands of the tunnel. Mutations R321Q and D411N resulted in ts defects in vivo and in vitro, as did the double-mutant R321A-D435A, whereas the R321K protein was fully stable in vivo and in vitro. These results highlight the critical role of the buried salt bridge in Cet1 stability. Replacement of Ser324 by alanine or valine elicited a cold-sensitive (cs) yeast growth phenotype. The S429A and S429V proteins were fully active when produced in bacteria at 37 °C, but were inactive when produced at 17 °C. Replacement of Ser324 by threonine partially suppressed the cold sensitivity of the Cet1 phosphohydrolase, but did not suppress the cs growth defect in yeast.

Saccharomyces cerevisiae Cet1 is an essential enzyme that performs the first step of mRNA cap formation: the hydrolysis of the γ-phosphate of nascent pre-mRNA to form a 5′-diphosphate end (1, 2). Cet1 exemplifies a distinctive family of metal-dependent phosphohydrolases that includes other fungal RNA triphosphatases (e.g. Candida albicans CaCet1, S. cerevisiae Ch1, and Schizosaccharomyces pombe Pct1) and the RNA triphosphatase components of the poxvirus, baculovirus, Chlorella virus, and Plasmodium falciparum RNA capping enzymes (3–12). The signature feature of this enzyme family is the ability to hydrolyze nucleoside triphosphates to nucleoside diphosphates and inorganic phosphate in the presence of either manganese or cobalt. The defining structural elements of the family are two glutamate-rich motifs (strands β1 and β11 in Fig. 1) that are required for catalysis.

The crystal structure of Cet1 illuminates surprising structural complexity for an enzyme that catalyzes a mundane phosphohydrolase reaction (13). Cet1 adopts a novel active site fold whereby an antiparallel eight-strand β barrel forms a topologically closed “triphosphate tunnel” (Fig. 2). The hydrophilic tunnel contained a single sulfate coordinated by multiple basic side chains projecting into the cavity. It was proposed that the side chain interactions of the sulfate reflect contacts made by the enzyme with the γ-phosphate of the triphosphate-terminated RNA or nucleoside triphosphate substrates (13). A manganese ion within the tunnel cavity is coordinated with octahedral geometry to the sulfate, to the side chain carboxylates of the two glutamates in β1, and to a glutamate in β11.

The interior of the tunnel has a distinctively baroque architecture supported by an intricate network of hydrogen bonds and electrostatic interactions, of which a surprisingly high proportion are required for enzyme activity (3, 4, 14). Alanine scanning mutagenesis has identified 15 individual side chains within the tunnel that are important for Cet1 function in vitro and in vivo (Fig. 1). Moreover, each of the eight strands of the β barrel contributes at least one functional constituent of the active site. The relevant structural features of the 15 key amino acids have been determined through the analysis of conservative mutational effects (4, 14). We have grouped the active site residues into three functional classes. Class I residues participate directly in catalysis via coordination of the γ-phosphate (Arg305, Lys306, Arg358) or the essential metal (Glu305, Glu307, Glu308). Class II residues make water-mediated contacts with the γ-phosphate (Asp377, Glu433) or the metal (Asp471, Glu494). Class III residues function indirectly in catalysis via their interactions with other essential side chains and/or their stabilization of the tunnel architecture (Lys409, Arg454, Arg469, Thr473, Glu492).

Based on the structure of the putative product complex and the mutational results, we have proposed a one-step in-line mechanism whereby the metal ion (coordinated by acidic residues on the tunnel floor) plus the Arg305, Arg358, and Lys456 side chains (emanating from the walls and roof) activate the γ-phosphate for attack by water and stabilize a pentacoordinate phosphorane transition state in which the attacking water is apical to the β-phosphate leaving group (14). We further speculated that the Glu433 side chain coordinates the nucleophilic water molecule (Fig. 2) and serves as general base catalyst.

Mutational studies have also identified several functionally important hydrophobic residues located on the “outward” face of the β strands of the tunnel (4, 15). For example, alanine substitutions for Leu306 (in β1) and Val498 or Leu499 (in β11) result in temperature-sensitive yeast growth and thermolabil-
ity of catalytic activity in vitro (4). These hydrophobic residues are in no position to participate directly in catalysis (Fig. 2). It is therefore thought that the deleterious effects of mutating these residues reflects the importance of their hydrophobic interactions with the globular protein core that serves as a pedestal upon which the tunnel floor rests (4, 15).

To embellish the picture of the enzyme mechanism and the interactions supporting the tunnel structure, we have extended the mutational analysis to 17 new amino acids denoted by dots in Fig. 1. We thereby identified two important side chains (Arg321 and Asp425) that stabilize Cet1 via their interaction to form a buried salt bridge within the pedestal. A third residue

Fig. 1. Conservation of primary structure within the catalytic domains of the metal-dependent RNA triphosphatases. The amino acid sequence of S. cerevisiae Cet1 from residue 304 to 498 is aligned to the homologous segments of C. albicans CaCet1, S. cerevisiae Cth1, S. pombe Pct1, P. falciparum Pnt1, and Chlorella virus (cv) Rtp1. Gaps in the alignment are indicated by dashes. The β strands that comprise the triphosphate tunnel of Cet1 are shown above the amino acid sequence. Conserved segments are highlighted in shaded boxes. Previous studies (3, 4, 14, 15) had identified Cet1 residues at which alanine substitution resulted either in loss of function (!), thermosensitive function (!H), or no significant effect on function (+). The 17 amino acids of Cet1 that were targeted for mutation in the present study are indicated by dots.

Fig. 2. The triphosphate tunnel and the hydrophobic back surface of the tunnel floor. Stereo view of a cross-section of the tunnel of S. cerevisiae Cet1. The figure highlights the elaborate network of bonding interactions, especially those that coordinate sulfate (γ-phosphate) and manganese. The manganese (blue sphere) interacts with octahedral geometry with the sulfate, three glutamates, and two waters (red spheres). The putative nucleophilic water is coordinated by Glu433, which is postulated to act as a general base catalyst. The tunnel rests on a globular pedestal domain (not shown). The hydrophobic side chains (Ile304, Leu306, Met308, Phe310, Val493, Leu495) on the back side of the strands of the tunnel floor that comprise the tunnel-pedestal interface are illustrated. The image was prepared using SETOR (17).
Ser^{229} stabilizes Cet1 when the protein is synthesized at reduced temperature.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Mutated Versions of Yeast RNA Triphosphatase**—Missense mutations were introduced into the CET1(201–549) gene by polymerase chain reaction, and the mutated genes were inserted into the bacterial expression vector pET16b as described previously (14). The presence of the desired mutations was confirmed in every case by DNA sequencing; the inserted fragments were sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning. The pET plasmids were confirmed in every case by DNA sequencing; the inserted fragments were sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning. The pET plasmids were confirmed in every case by DNA sequencing; the inserted fragments were sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning. The pET plasmids were confirmed in every case by DNA sequencing; the inserted fragments were sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning.

**Fig. 3. Mutational effects on Cet1 function in vivo and in vitro.** The in vivo phenotypes of the mutants were assessed by plasmid shuffle in a yeast cet1Δ strain as described under “Experimental Procedures.” Triphosphatase specific activity of the purified mutant Cet1(201–549) proteins is normalized to that of the wild-type protein (defined as 100%). The atomic contacts made by the wild-type side chains in the Cet1 crystal structure (13) are indicated in the column on the right.

| Mutant | Triphosphatase Activity (% of wild-type) | In Vivo Phenotype | Contacts |
|--------|-----------------------------------------|-------------------|---------|
| I304A  | 82                                      | ++                | Ile296 Leu430 Leu432 |
| M308A  | 100                                     | ++                | Val426 Phe523 |
| D315A  | 100                                     | ++                | Lys317(NH) Glu334(Oe) |
| K317A  | 95                                      | ++                | none |
| R321A  | 95                                      | ++                | none |
| R321K  | 91                                      | ++                | Ser419(CO) Asp422(CO) |
| R321Q  | 86                                      | ++                | Ser419(CO) |
| R321A-D425A | 73                            | ++                | none |
| S373A  | 110                                     | ++                | none |
| T375A  | 110                                     | ++                | none |
| S378A  | 100                                     | ++                | none |
| Q405A  | 86                                      | ++                | Arg393(NC) |
| H411A  | 91                                      | ++                | Asn431(OE) |
| D425A  | 96                                      | ++                | Arg321(Nc, NC) |
| D425N  | 88                                      | ++                | Ser419(NH) |
| S487A  | 91                                      | ++                | none |
| E488A  | 39                                      | ++                | none |
| T489A  | 95                                      | ++                | Glu476(OE) |
| T490A  | 66                                      | ++                | Glu492(OE) |
| H491A  | 91                                      | ++                | none |

The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; cs, cold-sensitive; ts, thermosensitive; WT, wild-type; 5-POA, 5-fluoro-orotic acid.

**RESULTS**

**In Vivo Mutational Analysis of Yeast RNA Triphosphatase**—We tested the in vivo effects of single alanine mutations at 16 positions: Ile^{304} and Met^{308} in β1; Asp^{312}, Lys^{317}, and Arg^{321} in the β1-β2 loop, Ser^{373}, Thr^{375}, and Ser^{378} in β5; Gln^{405} and His^{411} in β7; Asp^{425} and Ser^{429} in β8; and Ser^{487}, Glu^{488}, Thr^{489}, Thr^{490}, and His^{491} in β11 (the mutated residues are denoted by dots in Fig. 1). The alanine mutations were introduced into the biologically active domain Cet1(201–549), and the CET1(201–549)-Ala genes were cloned into a CEN TRP1 vector so as to place them under the transcriptional
control of the natural CET1 promoter. The plasmids were transformed into a cet1Δ strain in which the chromosomal CET1 locus was deleted and replaced by LEU2. Growth of cet1Δ is contingent on a wild-type CET1 allele on a CEN URA3 plasmid. Therefore, cet1Δ is unable to grow on agar medium containing 5-FOA, a drug that selects against the URA3 plasmid, unless it is transformed with a biologically active CET1 allele.

All 16 of the CET1(201–549)-Ala mutants supported the growth of cet1Δ cells on 5-FOA during selection at 30 °C. The viable CET1(201–549)-Ala strains were then tested for growth on rich medium (YPD) at 16, 22, 30, and 37 °C. I304A, M308A, D315A, K317A, S373A, T375A, S378A, Q405A, H411A, S467A, E488A, T489A, T490A, and H491A cells grew at all temperatures, and their colony sizes were similar to that of “wild-type” CET1(201–549) cells (scored as ++ growth in Fig. 3). R321A and D425A cells displayed a temperature-sensitive (ts) phenotype (Fig. 3); they grew well at 16, 22, and 30 °C, but failed to grow at 37 °C (Fig. 4).

Effects of Alanine Mutations on Triphosphatase Activity—The Cet1(201–549)-Ala polypeptides were expressed as N-terminal His-tagged derivatives in E. coli at 18 °C in parallel with the wild-type Cet1(201–549) protein. The recombinant proteins were purified from soluble bacterial extracts by nickel-agarose chromatography. SDS-polyacrylamide gel electrophoresis analysis showed that the 44-kDa Cet1(201–549) protein was the predominant polypeptide in every case (Fig. 5).

The phosphohydrolase activities of the wild-type and mutant proteins were assayed by the release of 32Pγ from 1 mM [γ-32P]ATP during a 15-min reaction in the presence of 2 mM manganese. Two titration experiments were performed for each protein, and the specific activities were calculated from the average of the slopes of the titration curves in the linear range of enzyme dependence. The wild-type Cet1(201–549) preparation released 210 pmol of 32Pγ/μg of protein. The specific activities of the 16 Ala mutants, normalized to the wild-type value (defined as 100%), are listed in Fig. 3. Most of the mutants displayed full activity, and 15/16 had a specific activity within a factor of two of the wild-type enzyme. E488A was 39% as active as the wild-type; this effect does not meet the criterion of a 4-fold decrement in specific activity that we adopted previously as the threshold for a significant mutational effect (14).

Mutants R321A and D425A Are Thermolabile in Vitro—R321A and D425A were fully active in vitro, yet both mutations elicited a ts growth defect in vivo. To evaluate the basis for the ts phenotype, we compared the thermal stability of wild-type Cet1(201–549) to that of the R321A and D425A mutants. This was done by preincubating the purified enzymes for 10 min at 30, 35, 40, 45, or 50 °C, followed by quenching on ice. The protein samples were then assayed for ATPase activity at 22 °C. The data were expressed as the ratio of ATP hydrolysis by enzyme preincubated at a given test temperature to the activity of the respective unheated control. The thermal inactivation curves are plotted in Fig. 6. The activity of wild-type Cet1(201–549) was stable to preincubation at 30 °C and reduced only modestly by treatment at 40 °C. The activity fell off more sharply at 45 °C (to 40% of the unheated control value) and 50 °C (to 18% of the control value). The R321A and D425A proteins, which were temperature-sensitive in vivo, were clearly thermolabile in vitro (Fig. 6). The inactivation curves for both proteins were shifted 15 °C to the left relative to the wild-type enzyme.

Structure-Activity Relationships at Arg231 and Asp425—Arg231 and Asp425 form a salt bridge within the globular pedestal of Cet1. The salt bridge tethers the inferior portions of the β7 and β8 strands and the intervening loop, which project deep into the pedestal, to the distal end of β1 and the following loop, which comprise the platform-like structure at the entrance of the triphosphate tunnel (13) (Fig. 7). The replacement of only one member of an ion pair by alanine eventuates an unopposed charged residue within the protein core, which may by itself destabilize the structure of the mutant enzyme (16). Thus, the effects of the single R321A and D425A mutations do not indicate whether the salt bridge is inherently stabilizing. To address this issue, we tested the effects of a double-alanine mutation R321A-D425A. The rationale was that if an unopposed buried charge was responsible for destabilization of Cet1, then the double-Ala mutation should restore thermal stability. This is not what was observed. R321A-D425A yeast cells grew at 30 °C, but failed to grow at 37 °C (Fig. 3 and data not shown), just like the R321A and D425A single mutants. The R321A-D425A protein produced in bacteria at 18 °C (Fig. 5) retained phosphohydrolase activity (73% of wild-type specific activity; Fig. 3). Moreover, R321A-D425A displayed the same hypersensitivity to heat inactivation as the singly mutated R321A and D425A proteins (Fig. 6). We conclude that the interaction between Arg231 and Asp425 contributes significantly to the stability of Cet1.

To better understand the stabilizing forces, we introduced conservative changes at Arg231 and Asp425. Arg231 was replaced by lysine and glutamine, and Asp425 was substituted by asparagine. R321K cells grew at 37 °C, whereas R321Q and D425N cells displayed the same ts growth defect as the alanine mutants (Fig. 4). Thus, the ionic interaction between the two residues is essential for Cet1 stability in vivo. The R321K, R321Q, and D425N proteins were produced in E. coli at 18 °C and purified from soluble bacterial extracts by nickel-agarose chromatography (Fig. 5). The phosphohydrolase specific activities of the conservative mutants were 86–91% of the wild-type activity (Fig. 3). R321Q and D425N evinced the same hypersensitivity to heat inactivation as R321A and D425A (Fig. 6). The instructive finding was that replacement of Arg231 by lysine completely restored the thermal stability of R321K to the level of the wild-type enzyme (Fig. 6). Thus, the conservative
Mutational effects on Ceg1 stability in vivo and in vitro were in accord.

Mutations of Ser429 Elicit Cold-sensitive Phenotypes—The present alanine scan also targeted Ser429 in strand β8 (Fig. 1). The effects of the S429A mutation were initially puzzling, insofar as: (i) yeast cet1Δ cells transformed with S429A readily formed 5-FOA-resistant colonies, and the resulting S429A cells grew as well as WT cells on YPD agar at 30 °C, yet (ii) recombinant S429A protein produced in bacteria at 18 °C displayed ≤1% of the phosphohydrolase activity of the wild-type enzyme (data not shown). We considered the possibility that the S429A mutation might have resulted in a cold-sensitive folding defect; thus we examined the growth of yeast cells on YPD agar at 30 °C, but not at 16 °C (not shown). The S429A, S429T, and S429V mutants were catalytically active when the proteins were produced at 37 °C, with specific activities 75–90% of the wild-type enzyme expressed in parallel at 37 °C (Fig. 8B). However, S429A and S429V were grossly defective in γ-phosphate hydrolysis when the proteins were produced at 18 °C (1% and <0.5% of wild-type activity, respectively), consistent with the cs growth defect in yeast. The introduction of threonine partially restored triphosphatase activity, to one-fifth of the wild-type level, when the S429T protein was produced at 18 °C (Fig. 8B). We conclude that the hydroxyl moiety at position 429 is somehow critical for proper folding of the Cet1 when it is synthesized at low temperatures.

**DISCUSSION**

The unique fold of Cet1 and the complex, delicate architecture of its active site provide the impetus for a comprehensive mutational analysis of both the catalytic mechanism and the interactions that stabilize the fold. Here we have probed the function of 17 individual side chains by alanine scanning. The mutated residues were either located in the triphosphate tunnel (Ser373, Thr375, Glu455, His411, Ser429, Glu488, Thr490), on the tunnel’s outer surface (Ser378, Ser487, Thr490, His491), at the tunnel-pedestal interface (Ile304, Met308), or in the pedestal itself (Arg315, Lys317, Arg321, Arg425). Alanine mutations at 14/17 positions had no significant effect on Cet1 phosphohydrolase activity *in vitro* and had no effect on Cet1 function in *vivo*. These negative results are instructive when taken together with prior mutational analyses and the crystal structure of Cet1.

We have now mutated all of the hydrophilic amino acids that project into the triphosphate tunnel. Three of the six tunnel residues found here to be nonessential for Cet1 function (Ser373 and Thr375 in β5 and Glu488 in β11) make no contacts in the crystal structure with the metal cofactor, the γ-phosphate, or other amino acid side chains in the tunnel. Thus, it is sensible that these three residues are unimportant for Cet1 function; indeed they are not conserved in other family members (Fig. 1). However, the four other tunnel residues analyzed presently do participate in the elaborate network of side chain interactions in the tunnel cavity (Fig. 2). Glu455(Oe) engages in a hydrogen bond with Arg393(Nε). Arg393 is an essential catalytic residue...
(14) that makes a bidentate interaction with the γ-phosphate (Fig. 2). The hydrogen bond with Gln405 is apparently not required to correctly orient Arg393 for catalysis by Cet1, which is consistent with the lack of conservation of the Gln405 position in other family members (Fig. 1). His411(N) forms a hydrogen bond with Asn431(O), while Asn431(N) interacts Glu305(O) and Glu307(O) (Fig. 2). Glu305 and Glu307 directly coordinate the metal and are essential for catalysis (3, 4). We showed previously that Asn431 is not important for Cet1 function in vivo or in vitro (14); thus it is sensible that His411, which contacts only Asn431, is also nonessential. Thr490(O) forms a hydrogen bond to Glu492(O). Glu492 is an essential side chain that forms a salt bridge with Arg454 in βγ (Fig. 2). Arg454 is itself essential for Cet1 function, and it has been suggested that Arg454 contacts the α- or β-phosphate of the substrate (4, 14). Apparently, the contact of Thr490 with Glu492 is not required for the essential interaction of Glu492 with Arg454.

Ser378 in β5 and Ser487, Thr489, and His491 in β11 are located on the outer solvent-exposed surface of the tunnel and are noncontributory to Cet1 function. Ser378, Ser487, and His491 make no contacts with other residues in the crystal structure (Fig. 3). Thr489(O) engages in a hydrogen bond with Glu176(O), but this interaction is apparently not important.

Studies performed prior to solving the Cet1 structure showed that hydrophobic residues Leu306, Phe310, Val493, and Leu495 play important roles in Cet1 function and stability (4). It is now apparent from the crystal structure that these side chains are part of a rich network of hydrophobic interactions between residues on the “back” sides of the β strands of the tunnel floor (Fig. 2) and amino acids in the globular pedestal that supports the tunnel. For example, Phe310, which is essential for Cet1 function in vivo and in vitro, makes extensive van der Waals interactions with Val289. Here we found that two other hydrophobic residues in β11, Ile304 and Met308, that comprise part of the tunnel-pedestal interface, are not important for Cet1 stability in vivo or activity in vitro. Ile304 projects into the hydrophobic core of the pedestal and makes van der Waals interactions with Val296, Leu430, and Leu423. Met308 interacts with Val426 and Phe523.
The present study highlights the importance of a buried salt bridge between Arg\textsuperscript{321} and Asp\textsuperscript{425} for the stability of Cet1 \textit{in vivo} and \textit{in vitro}. The Arg and Asp of this ion pair are strictly conserved in other fungal RNA triphosphatases (CaCet1, Cth1, Pct1) and in \textit{P. falciparum} Prt1 (Fig. 1), yet neither the Arg nor the Asp are found in \textit{Chlorella} virus RNA triphosphatase cvRtp1. cvRtp1 has an asparagine in lieu of the Arg\textsuperscript{321} side chain and a glycine instead of the Asp (Fig. 1). We surmise that: (i) there is tight co-evolution of both members of the ion pair, and (ii) the \textit{Chlorella} virus enzyme, which is the smallest member of the metal-dependent RNA triphosphatase family (193-amino acids), has developed alternative strategies to stabilize an active conformation.

The Arg\textsuperscript{321}-Asp\textsuperscript{425} ion pair is part of a wider local network of interactions within the pedestal (Fig. 7). These include hydrogen bonds between Arg\textsuperscript{321}(N\textsubscript{7}) and the main chain carbonyl oxygens of Ser\textsuperscript{419} and Asp\textsuperscript{422}, which are located in the loop that connects strands \(\beta\)7 and \(\beta\)8. Also, Asp\textsuperscript{425}(O\textsubscript{6}) engages in a hydrogen bond to the backbone amide of Ser\textsuperscript{419}. We infer that the salt bridge and associated backbone contacts stabilize the inferior portions of the \(\beta\)7 and \(\beta\)8 strands and the intervening loop as they project down from the wall of the triphosphatase tunnel deep into the pedestal.

The crystal structure provides no immediate explanation for the cold-sensitive folding defects elicited by mutations of Ser\textsuperscript{429} to Ala and Val and the partial defect of the S429T mutant. Ser\textsuperscript{429} projects upward from the tunnel floor into the tunnel cavity, yet it makes no direct contact with other amino acids in the crystal. Ser\textsuperscript{429}(O\gamma) is pointing toward N\textsubscript{7} of Lys\textsuperscript{309} (a non-essential side chain), but the interatomic distance of 3.9 Å is too long for a standard hydrogen bond. It is conceivable that the distance to Lys\textsuperscript{309} is closer in solution. Ser\textsuperscript{429} might also make water-mediated contacts, either with other amino acids or with the 5'-triphosphate substrate, that are not apparent from the crystal structure of the product complex and that assist in the folding of the protein when it is synthesized at low temperatures \textit{in vivo}. Ser\textsuperscript{429} is conserved in all of the other metal-dependent RNA triphosphatases except cvRtp1, which has an alanine instead (Fig. 1).

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