Structure-Function Analysis of the Active Site Tunnel of Yeast RNA Triphosphatase*

Received for publication, February 1, 2001
Published, JBC Papers in Press, February 13, 2000, DOI 10.1074/jbc.M100980200

Martin Bisaillon and Stewart Shuman‡
From the Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10021

The RNA triphosphatase component of the yeast mRNA capping apparatus, catalyzes metal-dependent γ phosphate hydrolysis within the hydrophilic interior of a topologically closed 8-strand β barrel (the "triphosphatase tunnel"). We used structure-guided alanine scanning to identify 6 side chains within the triphosphatase tunnel that are essential for phosphohydrolase activity in vitro and in vivo: Arg393, Glu433, Arg458, Arg469, Asp471 and Thr473. Alanine substitutions at two positions, Asp377 and Lys409, resulted in partial catalytic defects and a thermosensitive growth phenotype. Structure-function relationships were clarified by introducing conservative substitutions. Five residues were found to be nonessential: Lys309, Ser395, Asp397, Lys397, Asn413, and Lys474. The present findings, together with earlier mutational analyses, reveal an unusually complex active site in which 15 individual side chains in the tunnel cavity are important for catalysis, and each of the 8 strands of the β barrel contributes at least one functional constituent. The active site residues fall into three classes: (i) those that participate directly in catalysis via coordination of the γ phosphate or the metal; (ii) those that make critical water-mediated contacts with the γ phosphate or the metal; and (iii) those that function indirectly via interactions with other essential side chains or by stabilization of the tunnel structure.

RNA 5′ triphosphatase executes the first step of mRNA cap formation, the hydrolysis of the γ phosphate of nascent pre-mRNA to form a 5′ diphosphate end. Two classes of eukaryotic RNA triphosphatases can be distinguished on the basis of their cofactor requirements, structures, and catalytic mechanisms (1). The RNA triphosphatases of metazoans and higher plants belong to the cysteine phosphatase enzyme superfamily (2). Metazoan RNA triphosphatases cleave the β-γ phosphoanhydride bond via the formation and hydrolysis of a covalent enzyme-(cysteinyl-S)-phosphate intermediate (3). The active site cysteine resides within the signature phosphate-loop motif HCXXX(S/T). The cysteine phosphate enzymes do not require a metal cofactor and are characteristically inhibited by divalent cations. In contrast, the RNA triphosphatases of fungal species such as Saccharomyces cerevisiae, Candida albicans, and Schizosaccharomyces pombe are strictly dependent on a divalent cation (5–10). The fungal enzymes belong to a new family of metal-dependent phosphohydrolases that embraces the poxvirus, baculovirus, phycodnavirus, and Plasmodium falciparum RNA capping enzymes (6, 11–15). The signature biochemical property 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 features of the metal-dependent RNA triphosphatases are two glutamate-rich motifs (motifs A and C; see Fig. 1) that are strictly dependent on a divalent cation.

The S. cerevisiae RNA triphosphatase (Cet1) is essential for yeast cell growth (5, 16). The crystal structure of Cet1 illuminates a surprising structural complexity for an enzyme that catalyzes a seemingly mundane phosphohydrolase reaction (17). Cet1 adopts a novel enzyme fold whereby an antiparallel 8-strand β barrel forms a hydrophilic "triphosphate tunnel" (see Fig. 2). Multiple acidic side chains point into the tunnel cavity, including the essential glutamates of motifs A and C. The interior of the tunnel contains a single sulfate ion coordinated by basic side chains projecting into the tunnel. Insofar as sulfate is a structural analog of phosphate, it is proposed that the side chain interactions of the sulfate reflect contacts made by the enzyme with the γ phosphate of the triphosphate-terminated RNA and nucleoside triphosphate substrates (17). 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 motif A, and to a glutamate in motif C.

Initial efforts to identify the Cet1 active site were made without the benefit of an atomic structure and entailed alanine scanning of strands β1 and β11, strand β9, and the connecting loop between strands β10 and β11 (see Fig. 1 and Refs. 6 and 7). Eight of the side chains analyzed, Glu309, Glu307, and Phe310 in β1, Arg494 and Lys496 in β9, and Glu492, Glu494, and Glu496 in β11, were deemed to be important for Cet1 function in vitro and in vivo (indicated by ! in Fig. 1). Alanine substitutions for these residues resulted in a significant decrement in the hydrolysis of RNA or NTP substrates by purified recombinant mutant proteins, and the mutant alleles were unable to complement the growth of a yeast cet1Δ strain. Alanine mutations of aliphatic residues Leu406 in β1 and Val493 and Leu495 in β11 resulted in temperature-sensitive (ts)2 growth and thermolability of enzyme activity in vitro (indicated by A in Fig. 1). Mutations of Thr455, Ser463, His463, Asn481, Lys483, Ser484, and Arg485 (denoted by + in Fig. 1) had no apparent effect on cell growth and either little effect or only a modest effect (2- to 5-fold) on triphosphatase activity in vitro.

* This research was supported in part by National Institutes of Health Grant GM52470 (to S. S.) and a postdoctoral fellowship from the Natural Sciences and Engineering Research Council of Canada (to M. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Fax: 212-717-3623; E-mail: s-shuman@ski.mskcc.org.

1 Changela, A., Ho, C. K., Martins, A., Shuman, S., and Mondragon, A. (2001) Embo J., in press.

2 The abbreviations used are: ts, temperature-sensitive; DTT, dithiothreitol; FOA, fluoroacetate acid; WT, wild-type.
Structure-Function Analysis of Yeast RNA Triphosphatase

The observed mutational effects are interpretable in light of the Cet1 crystal structure (17). For example, four of the essential glutamates (306, 307, 494, and 496) coordinate directly, or via water, to the essential metal ion, and the essential Lys456 coordinates the sulfate (i.e. the γ phosphate; see Fig. 2). Essential side chains Arg784 and Glu192 form a salt bridge that likely stabilizes the side wall and roof of the tunnel (see Fig. 2). Leu305, Phe310, Val393, and Leu498 are all located on the “outward” face of the β strands and are in no position to directly participate in catalysis. Thus, the lethal or conditionally deleterious effects of mutating these residues reflect the importance of their hydrophobic interactions with the globular protein core upon which the tunnel floor rests.

The Cet1 crystal structure now provides a blueprint for further mutagenesis aimed at identifying all of the functionally important side chains within the triphosphate tunnel. Here we analyzed the contributions of 14 individual charged or polar amino acids in seven of the β strands of the tunnel. The positions chosen for mutation, denoted by dots (•) in Fig. 1, are conserved in the three other known fungal RNA triphosphatases: C. albicans CaCet1, S. cerevisiae Cth1, and S. pombe Pct1. The results of this analysis, together with previous data, identify a total of 15 important side chains in the tunnel cavity. Thus, Cet1 has an unusually complex active site. The active site residues fall into three functional classes: (i) those that participate directly in catalysis via coordination of the γ phosphate or the metal; (ii) those that make critical water-mediated contacts with the γ phosphate or the metal; and (iii) those that function indirectly in catalysis by interaction with other essential side chains and/or stabilization of the tunnel architecture.

We propose a reaction mechanism based on these results.

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 by using the two-stage overlap extension method (18). The mutated genes were digested with NdeI and BamHI and then inserted into the bacterial expression vector pET16b. The presence of the desired mutations was confirmed in every case by DNA sequencing of the fragments. Oligonucleotides were used to completely exclude the acquisition of unwanted mutations during amplification and cloning. The pET plasmids were transformed into Escherichia coli BL21(DE3). Single transformants were inoculated into 100 ml of LB medium containing 0.1 mg/ml of ampicillin and grown at 37 °C until the A600 reached 0.5. Recombinant protein production was induced by placing the culture on ice for 30 min, followed by addition of isopropyl-1-thio-β-D-galactopyranoside to 0.4 mM and ethanol to 2% (v/v) final concentrations of 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 1 mM MgCl2, 20 pmol of γ-[32P]ATP, and enzyme were incubated for 15 min at 30 °C. The reactions were quenched by adding 5 μl of 5 mM formic acid. Aliquots of the mixtures were applied to a polyethyleneimine-cellulose TLC plate, which was developed with 0.75 M potassium phosphate, pH 4.3. The release of [32P]Pi was quantitated by scanning the TLC plate with a phosphorimager.

Mutational Effects on RNA Triphosphatase Function in Vivo—NdeI BamHI fragments encoding mutated versions of Cet1(201–549) were excised from the respective pET16b-CET1 plasmids and inserted into the yeast CEN TRP1 plasmid pCET5–3’ (5) so that expression of the inserted gene is under the control of the natural CET1 promoter. The plasmids were then introduced into S. cerevisiae strain YBS20 (MATa trp1 hi3 ura3 leu2 ade2 can1 cet1::LEU2 p360-CET1) that is deleted at the chromosomal CET1 locus. Growth of YBS20 depends on maintenance of plasmid p360-CET1 (CEN URA3 CET1). Transformants were selected on SD(−Trp) agar. Individual Trp+ isolates were patched onto SD(−Trp) agar and then streaked on agar plates containing 0.75 mg/ml of 5-fluoroorotic acid (5-FOA). Growth was scored after 7 days of incubation at 22 and 30 °C. Lethal alleles were those that failed to form colonies on 5-FOA after 7 days at either temperature. For the viable alleles, individual colonies were picked from the 5-FOA plates and patched on YPD agar. Two isolates of each mutant were streaked on YPD agar at 16, 22, 30, and 37 °C. Growth was assessed as follows: ++ indicates wild-type colony size at all temperatures, and + indicates growth at 16, 22, and 30 °C but no growth at 37 °C (Table 1).

RESULTS

Structure-based Mutational Analysis of Yeast RNA Triphosphatase—Many of the charged and polar amino acids in the β strands of the Cet1 tunnel are conserved in the three other known fungal RNA triphosphatases (Fig. 1). The Cet1 crystal structure shows that these hydrophilic side chains point into the tunnel cavity (Fig. 2) and are thus plausible candidates to participate in substrate binding and reaction chemistry. Here we tested the effects of single alanine mutations at the 14 conserved side chains indicated by dots in Fig. 1. These were as follows: Lys109 in β1; Asp177 in β5; Arg386, Ser387, and Asp397 in β6; Lys403 in β7; Lys427, Asn431, and Glu433 in β8; Arg458 in β9; and Arg469, Asp477, Thr479, and Lys474 in β10. The Ala mutations were introduced into the biologically active domain CET1(201–549), and the mutant polypeptides were expressed as N-terminal His-tagged derivatives in E. coli 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. 3).

Effects of Alanine Mutations on Nucleoside Triphosphatase Activity—The nucleoside triphosphatase activities of the wild-type and mutant proteins were assayed by the release of [32P]Pi 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 [32P], per ng of protein. The specific activities of the 14 Ala mutants, normalized to the wild-type value (defined as 100%), are listed in Table I. Six of the mutations resulted in a severe (at least a 20-fold) decrement in catalytic activity: R393A, E433A, R458A, R469A, and K474A. Three mutant enzymes displayed partial defects: D377A (8%), D397A (21%), and K409A (11%). Five of the mutations had little or no effect on ATP hydrolysis: K309A, S395A, K427A, N431A, and K474A.
Effects of Alanine Mutations on RNA Triphosphatase Activity—The RNA triphosphatase activities of the wild-type and mutant proteins were assayed by the release of \(^{32}\)P from 2 mM \([\text{g}-32\text{P}]\)poly(A) during a 15-min reaction in the presence of 1 mM magnesium. 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 17 pmol of \(^{32}\)Pi per ng of protein. The specific activities of the mutants were normalized to the wild-type value and are listed in Table I. Seven of the mutations resulted in at least a 20-fold activity decrement: R393A, K409A, E433A, R458A, R469A, D471A, and T473A. Two mutants displayed partial defects, D377A (7%) and D397A (24%). Five of the mutations had little or no effect on RNA triphosphatase activity: K309A, S395A, K427A, N431A, and K474A. Note that there was an excellent correlation between the effects of each mutation on the ATPase and RNA triphosphatase activities.

Effects of Alanine Mutations on Cet1 Function in Vivo—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 the cet1Δ strain YBS20, in which the chromosomal CET1 locus has been deleted and replaced by LEU2. Growth of YBS20 is contingent upon maintenance of a wild-type CET1 allele on a CEN URA3 plasmid. Therefore, YBS20 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. Expression of Cet1(201–549) in cet1Δ cells permitted their growth on 5-FOA, whereas expression of the catalytically defective mutants R393A, E433A, R458A, R469A, D471A, and T473A did not. Thus, we conclude that these six mutations were lethal in vivo (Table I).
In contrast, the eight other CET1(201–549)-Ala mutants did support growth of cet1Δ cells on 5-FOA during selection at 25 or 30 °C. The viable CET1(201–549)-Ala strains were then tested for growth on rich medium (YPD) at 16, 25, 30, and 37 °C. K309A, S395A, D397A, K427A, N431A, and K474A cells grew at all temperatures, and their colony sizes were similar to that of wild-type CET1(201–549) cells (scored as + + growth in Table I). D377A and K409A cells displayed a ts phenotype; they grew well at 25 and 30 °C but failed to grow at 37 °C.

The in vivo phenotypes of the alanine mutants correlated with their in vitro activities. The most severe catalytic defects resulted in lethal phenotypes in vivo, whereas catalytically benign mutations had no effect on cell growth. Even the D397A mutation, which reduced RNA triphosphatase activity to one-fourth the wild-type level in vitro, had no apparent effect in vivo (Table I). The marginally functional mutants D377A and K409A, with 7 and 5% of wild-type RNA triphosphatase activity, respectively, were nonetheless capable of sustaining growth at 25 and 30 °C but failed to grow at 37 °C.

The in vivo phenotypes of the alanine mutants correlated with their in vitro activities. The most severe catalytic defects resulted in lethal phenotypes in vivo, whereas catalytically benign mutations had no effect on cell growth. Even the D397A mutation, which reduced RNA triphosphatase activity to one-fourth the wild-type level in vitro, had no apparent effect in vivo (Table I). The marginally functional mutants D377A and K409A, with 7 and 5% of wild-type RNA triphosphatase activity, respectively, were nonetheless capable of sustaining growth at 25 and 30 °C. These findings underscore the suggestion from earlier mutational analyses (7) that yeast cells require a threshold level of RNA triphosphatase activity for growth and can tolerate at least a 5-fold reduction before growth is overtly affected.

Mutants D377A and K409A Are Thermolabile in Vitro—The thermal stability of wild-type Cet1(201–549) and the D377A and K409A mutants was tested by preincubation of the purified enzyme preparations 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 levels of input WT, D377A, and K409A enzyme in the assay mixtures were adjusted to achieve similar extents of ATP hydrolysis in the control reaction mixtures containing unheated enzyme. 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. 4. The activity of WT Cet1(201–549) was stable to preincubation at 30 °C and reduced only modestly by treatment at 35 and 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). D377A and K409A were clearly thermolabile. The inactivation curve for K409A was shifted almost

| Strand | Mutation | ATPase % of WT | RNA Triphosphatase % of WT | In Vivo Phenotype |
|--------|----------|----------------|---------------------------|------------------|
| β1     | K309A    | 78             | 70                        | ++               |
| β5     | D377A    | 8              | 7                         | ts               |
|        | D377N    | 96             | 110                       | ++               |
|        | D377E    | 97             | 98                        | ++               |
| β6     | R393A    | <0.5           | <0.5                      | lethal           |
|        | R393K    | 2              | 2                         | lethal           |
|        | R393Q    | 2              | 2                         | lethal           |
|        | S395A    | 98             | 97                        | ++               |
|        | D397A    | 21             | 24                        | ++               |
| β7     | K409A    | 11             | 5                         | ts               |
|        | K409R    | <0.5           | <0.5                      | lethal           |
|        | K409Q    | 1              | <0.5                      | lethal           |
| β8     | K427A    | 93             | 89                        | ++               |
|        | N431A    | 90             | 94                        | ++               |
|        | E433A    | <0.5           | <0.5                      | lethal           |
|        | E433D    | 1              | <0.5                      | lethal           |
|        | E433Q    | 1              | <0.5                      | lethal           |
| β9     | R458A    | 2              | 2                         | lethal           |
|        | R458K    | 1              | 1                         | lethal           |
|        | R458Q    | <0.5           | <0.5                      | lethal           |
| β10    | R469A    | 3              | 1                         | lethal           |
|        | R469K    | <0.5           | <0.5                      | lethal           |
|        | R469Q    | 1              | <0.5                      | lethal           |
|        | D471A    | 1              | 1                         | lethal           |
|        | D471E    | <0.5           | <0.5                      | lethal           |
|        | D471N    | <0.5           | <0.5                      | lethal           |
|        | T473A    | 1              | 1                         | lethal           |
|        | T473S    | 25             | 17                        | ++               |
|        | T473V    | 1              | 1                         | lethal           |
|        | K474A    | 100            | 125                       | ++               |

Fig. 4. D377A and K409A are thermolabile in vitro. Aliquots (20 μl) of WT Cet1(201–549), D377A, and K409A were preincubated for 10 min at 30, 35, 40, 45, or 50 °C and then quenched on ice. Control aliquots were kept on ice throughout the pretreatment. ATPase reaction mixtures contained control or pre-heated enzymes as follows: WT, 75 ng; D377A, 500 ng; and K409A, 500 ng. The amounts of unheated control WT and mutant enzymes were sufficient to hydrolyze between 30 and 60% of the input ATP during the 15-min ATPase reaction at 22 °C. The extent of ATP hydrolysis by pre-heated enzyme was normalized to that of the unheated control enzyme (defined as 1.0). The normalized activities are plotted as a function of preincubation temperature. Each datum is the average of two separate thermal inactivation experiments.
15 °C to the left relative to the WT enzyme, and a shift to the left of 10 °C was observed for D377A.

**Structure-Activity Relationships at Essential Amino Acids**

Two conservative substitutions were introduced at each of the 6 residues defined by alanine scanning as essential for function in vitro and in vivo (Arg393, Glu433, Arg458, Arg469, Asp471, and Thr473). The 12 recombinant proteins were purified from soluble bacterial extracts by nickel-agarose chromatography (Fig. 3). The manganese-dependent ATPase and magnesium-dependent RNA triphosphatase activities of the conservative mutants were determined by protein titration (Table I). The mutant alleles were also cloned into a CEN TRP1 vector under the transcriptional control of the natural CET1 promoter and tested by plasmid shuffle for functional complementation of the cet1Δ mutant (Table I).

Substitution of any of the three essential arginines (393, 458, and 469) by lysine or glutamine failed to restore the NTPase and RNA triphosphatase activities above the levels seen for the respective alanine-substituted proteins. Moreover, the R393K, R393Q, R458K, R458Q, R469K, and R469Q mutations were lethal in vivo. We surmise that Cet1 function requires a bidentate arginine side chain at each position and not merely positive charge.

Replacing the essential Glu453 side chain by either aspartate or glutamine had no salutary effect on the NTPase and RNA triphosphatase activities in vitro, and the E433D and E433Q alleles were lethal in vivo. Similarly, changing the essential Asp471 residue to either glutamate or asparagine yielded catalytically defective proteins that were nonfunctional in vivo. Thus, an acidic moiety is strictly essential at both positions 433 and 471, and the distance of the carboxylate from the main chain is also critical for Cet1 activity.

The essential Thr473 residue was replaced by serine and valine. Whereas the T473V protein was as defective in γ phosphate hydrolysis as T473A (1% of WT activity) and RNA triphosphatase to 17% of the WT level, the T473S protein complemented the cet1Δ mutation at all temperatures. We conclude that the hydroxyl moiety at position 473 is required for Cet1 function.

**Structure-Activity Relationships at Asp377 and Lys409**—Conservative changes were also introduced at positions Asp377 and Lys409, where alanine mutations had resulted in partial loss of function in vitro and a ts growth defect. The D477A, D477E, K409R, and K409Q proteins were purified from soluble bacterial extracts by nickel-agarose chromatography (Fig. 3). The ATPase and RNA triphosphatase activities were restored to WT levels when Asp377 was replaced by asparagine or glutamate, and the D377N and D377E strains grew normally at all temperatures (Table I). The sufficiency of asparagine suggests that a polar side chain with hydrogen bonding potential is the relevant functional group. The noteworthy findings at position Lys409 were that the conservative changes to arginine and glutamine were at least an order of magnitude more deleterious than the alanine mutation (Table I). Consequently, K409R and K409Q were lethal in vivo.

**DISCUSSION**

Using the crystal structure of Cet1 as a guide for mutational analysis, we have identified 6 side chains within the triphosphatase tunnel that are essential for RNA triphosphatase activity in vitro and in vivo. Five of the six critical amino acids (Arg393 in β6, Glu433 in β8, Arg458 in β9, and Asp471 in β10) have identical counterparts in C. albicans CaCet1, S. cerevisiae Cth1, and S. pombe Pct1 (Fig. 1). The sixth essential position, Arg469 in β10, is occupied by lysine or histidine in the other fungal enzymes. We also identified Asp377 in β5 and Lys409 in β7 as being important for function, albeit not essential, insofar as their replacement by alanine resulted in partial loss of function in vitro and a ts growth defect in vivo. Lys409 has an identical counterpart in all three other fungal triphosphatases, whereas Asp377 is either an aspartate or a glutamine (Fig. 1). Several other residues were nonessential for Cet1 function in vitro and in vivo, including Lys397, Ser398, Lys427, and Lys474, which are conserved in the other fungal RNA triphosphatases.

The present findings, together with earlier mutational analyses (6, 7, 9), reveal an unusually complex active site for the yeast RNA triphosphatase, in which a total of 15 individual side chains in the tunnel cavity are essential or important for catalysis, and each of the 8 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 (see Table I and Ref. 7). Interpretation of the mutational results in light of the Cet1 crystal structure (17) engenders a plausible model for catalysis by the fungal RNA triphosphatase family.

First, we can group the tunnel residues into three functional classes (Fig. 5). Class I residues participate directly in catalysis via coordination of the γ phosphate (Arg393, Lys409, and Arg458) or the essential metal (Glu305, Glu307, and Glu494). Class II residues make water-mediated contacts with the γ phosphate or the essential metal. Class III residues function indirectly via their interactions with other essential side chains and/or stabilization of the tunnel architecture.

How do these functional groups contribute to catalysis? We postulate a one-step in-line mechanism whereby the metal ion (coordinated by residues on the tunnel floor) plus the Arg393, Arg458, and Lys409 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

| β Strand | Amino Acid | Contacts / Function |
|----------|------------|---------------------|
| 1        | Glu365     | metal               |
|          | Glu369     | metal               |
| 6        | Arg393     | γPO₄ (bidentate)    |
| 8        | Lys435     | γPO₄ (bidentate)    |
|          | Arg458     | γPO₄ (bidentate)    |
| 11       | Glu466     | metal               |
| 5        | Asp377     | water to γPO₄       |
| 8        | Glu433     | water to γPO₄       |
| 10       | Asp471     | water to metal      |
| 11       | Glu484     | water to metal      |
| 7        | Lys409     | Glu365 (metal)      |
| 9        | Arg454     | Glu466              |
| 10       | Arg456     | Asp471 (water to γPO₄) |
|          | Thr473     | Arg458 (γPO₄)      |
| 11       | Glu482     | Arg454              |

**FIG. 5.** Three functional classes of active site residues. Class I amino acids directly coordinate the γ phosphate or the essential metal. Class II amino acids make water-mediated contacts with the γ phosphate or the metal. Class III residues function indirectly via their interactions with other essential side chains and/or stabilization of the tunnel architecture.
attacking water is apical to the β phosphate leaving group. We speculate further that the substrate is bound within the tunnel such that the β and α phosphates are located on the entrance side of the tunnel (i.e. anterior to the sulfate in Fig. 2), in which case the water molecule situated posterior to the γ phosphate (Fig. 2) would be poised to act as the attacking nucleophile, and the Glu433 side chain coordinating this water molecule would serve as a general base catalyst.

How does the proposed mechanism fit to the available mutational data? The critical role of the enzyme-bound metal ion is clearly underscored by that fact that Cet1 is intolerant of virtually any perturbations of the metal coordination sphere. None of the three glutamates that contact the metal directly (Glu305, Glu307, or Glu496) can be functionally substituted by either glutamine or aspartic acid (7). In addition to their contacts with the metal, Glu305 and Glu307 are engaged in a network of interactions with other side chains projecting from the tunnel floor and lateral wall (Fig. 2). Of these, only the Glu305–Lys409 ion pair is functionally relevant, as surmised from the deleterious effects of Lys409 mutations. Conceivably, Lys409 helps position the Glu305 side chain with respect to the metal whereas Glu305 helps tether the β strand to the tunnel wall via Lys409. Glu307 interacts with the conserved Lys309 in strand β1, but the elimination of Lys309 has no apparent functional consequences, nor does the loss of Asn431, which makes a bifurcated hydrogen bond to both Glu305 and Glu307 (Fig. 2).

A most remarkable feature of the octahedral metal complex is the requirement for two residues, Asp471 and Glu494, to position the same metal-bound water (Fig. 2). RNA triphosphatase and NTPase activity in vitro and Cet1 function in vivo are abolished when Asp471 is replaced by either asparagine or glutamate. In addition to its water-mediated metal interaction, Asp471 forms a hydrogen bond with its essential neighbor Arg469 in β10. Arg469 is located on the exit side of the tunnel and is not in proximity to the γ phosphate. Thus, we suspect that Arg469 is not a direct catalyst and that its essentiality reflects its role in positioning Asp471. Note that although Arg469 also engages in a hydrogen bond to Ser463 in strand β9 (Fig. 2), the fact that the S460A mutation has only a modest effect on activity in vitro and no effect on yeast cell growth (7) (i.e. S460A does not phenocopy R469A) would argue against an important role for the hydrogen bond to serine. Instructive mutational effects occur at position Glu494, which interacts exclusively with the metal-bound water in the crystal structure. The E494Q mutation abolishes phosphohydrolase activity in vitro and is lethal in vivo (7). However, although the E494D mutation also abrogates the magnesium-dependent RNA triphosphatase function and is accordingly lethal in vivo, the E494D change spares the manganese-dependent ATPase activity, E494D being one-fourth as active as the wild-type enzyme (7).

The mutational and structural data are consistent with the hypothesis that Glu433 serves as a general acid to promote the attack of water on the γ phosphorus. Glu433 coordinates a water in the crystal structure, which is in turn coordinated by the sulfate at a distance of 3.7 Å from the sulfur center. Cet1 activity is reduced by 2 orders of magnitude by the E433Q mutation, which suggests that the side chain must be able to accept a proton from the water, not merely serve as a hydrogen bonding partner. Although Asp377 also coordinates a water in the tunnel cavity, the loss of this side chain is less deleterious than the loss of Glu433, and the function of Asp377 is restored fully by asparagine, which would not be able to abstract a proton from the water. Consequently, Asp377 and its associated water are not attractive candidates for the roles of general acid and nucleophile, respectively.

There are likely to be additional important interactions of active site amino acids with the β and α phosphates of the substrate, and perhaps with a second metal ion bound to the 5′ triphosphate, that cannot be appreciated from the available crystal structure. Lys409 and Arg454 are candidates to interact with the β or α phosphates based on their location anterior to the sulfate in the product complex. The observation that mutations of Arg454 result in a 30-fold increase in the Kₘ for ATP is consistent with such a role (7), although it remains to be shown that the effect is a consequence of a direct contact between Arg454 and the substrate. Arg454 forms a salt bridge to Glu392 and this interaction appears to be important in stabilizing the tunnel structure (6, 7).

In summary, the interior of the Cet1 triphosphatase tunnel has a distinctive baroque architecture supported by an intricate network of hydrogen bonds and electrostatic interactions, of which a surprisingly high proportion are required for phosphohydrolase activity. A more complete picture of the enzyme mechanism and the interactions supporting the tunnel structure should emerge as the mutational analysis is extended to the remaining residues of the component strands and as Cet1 is crystallized with a 5′ triphosphate bound in the active site.

REFERENCES

1. Shuman, S. (2000) Prog. Nucleic Acids Res. Mol. Biol. 66, 1–40
2. Takagi, T., Moore, C. R., Diehn, F., and Buratowski, S. (1997) Cell 88, 867–873
3. Martins, A., and Shuman, S. (2000) J. Biol. Chem. 275, 35070–35076
4. Evans, S. V. (1993) J. Mol. Graph. 11, 134–158
5. Ho, C. K., Schwer, B., and Shuman, S. (1998) Mol. Cell. Biol. 18, 5189–5198
6. Ho, C. K., Pei, Y., and Shuman, S. (1998) J. Biol. Chem. 273, 34151–34156
7. Pei, Y., Ho, C. K., Schwer, B., and Shuman, S. (1999) J. Biol. Chem. 274, 19860–19874
8. Rodriguez, C. R., Takagi, T., Cho, E., and Buratowski, S. (1999) Nucleic Acids Res. 27, 2183–2188
9. Pei, Y., Lehman, K., Tian, L., and Shuman, S. (2000) Nucleic Acids Res. 28, 1885–1892
10. Pei, Y., Schwer, B., Hausmann, S., and Shuman, S. (2001) Nucleic Acids Res. 29, 387–390
11. Jin, J., Dung, W., and Guarino, L. A. (1998) J. Virol. 72, 10011–10019
12. Gross, C. H., and Shuman, S. (1998) J. Virol. 72, 10020–10028
13. Ho, C. K., Martins, A., and Shuman, S. (2000) J. Virol. 74, 5456–5469
14. Ho, C. K., Gong, C., and Shuman, S. (2001) J. Virol. 75, 1744–1750
15. Ho, C. K., and Shuman, S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3050–3055
16. Tsukamoto, T., Shibagaki, Y., Imajoshi-Omih, S., Murakoshi, T., Suzuki, M., Nakamura, A., Gotoh, H., and Mizumoto, K. (1997) Biochem. Biophys. Res. Commun. 239, 116–122
17. Lima, C. D., Wang, L. K., and Shuman, S. (1999) Cell 99, 533–543
18. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 51–59
Structure-Function Analysis of the Active Site Tunnel of Yeast RNA Triphosphatase
Martin Bisaillon and Stewart Shuman

J. Biol. Chem. 2001, 276:17261-17266.
doi: 10.1074/jbc.M100980200 originally published online February 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100980200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 18 references, 9 of which can be accessed free at http://www.jbc.org/content/276/20/17261.full.html#ref-list-1