Characterization of the Adenylation Site in the RNA 3′-Terminal Phosphate Cyclase from Escherichia coli*

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RNA 3′-terminal phosphate cyclases are a family of evolutionarily conserved enzymes that catalyze ATP-dependent conversion of the 3′-phosphate to the 2′,3′-cyclic phosphodiester at the end of RNA. The precise function of cyclases is not known, but they may be responsible for generating or regenerating cyclic phosphate RNA ends required by eukaryotic and prokaryotic RNA ligases. Previous work carried out with human and Escherichia coli enzymes demonstrated that the initial step of the cyclization reaction involves adenylation of the protein. The AMP group is then transferred to the 3′-phosphate in RNA, yielding an RNA-N3′pp5′A (N is any nucleoside) intermediate, which finally undergoes cyclization.

In this work, by using different protease digestions and mass spectrometry, we assign the site of adenylation. In this work, by using different protease digestions and mass spectrometry, we assign the site of adenylation. In this work, by using different protease digestions and mass spectrometry, we assign the site of adenylation. In this work, by using different protease digestions and mass spectrometry, we assign the site of adenylation. In this work, by using different protease digestions and mass spectrometry, we assign the site of adenylation.

The properties of the human and bacterial cyclases are very similar. Both enzymes catalyze conversion of the 3′-terminal phosphate to a 2′,3′-cyclic phosphodiester in a reaction dependent on ATP, other nucleoside triphosphates being much less active co-factors. With both enzymes, the cyclization of the 3′-phosphate at the 3′-end of RNA occurs by a three-step mechanism (2–4, 20, 23, 24) as follows.

(i) Enzyme + ATP → enzyme-AMP + PPi
(ii) RNA-N3′p + enzyme-AMP → RNA-N3′pp5′A + enzyme, where N3′ is any nucleoside, and p is a phosphate group.
(iii) RNA-N3′pp5′A → RNA-N>p + AMP, where N>p is nucleoside 2′,3′-cyclic phosphate.

Evidence for step (i) comes from identification by either SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or gel filtration of the covalent cyclase-AMP complex. Step (ii) is supported by the ability of 3′-phosphorylated RNA but not 3′-OH-terminated RNA to release AMP from the preformed adenylylated cyclase complexes and by accumulation of the RNA-N3′pp5′A molecules when the ribose at the RNA 3′-terminus is replaced with the 2′-deoxy- or 2′-O-methylribose (2, 3, 20, 23, 24). Step (iii) probably takes place nonenzymatically as the result of nucleophilic attack by the adjacent 2′-OH on the phosphorus in the phosphodiester linkage.

Mechanistically, with respect to formation of the covalent protein-nucleoside monophosphate intermediate and transfer of nucleoside monophosphate to the terminal phosphate (or pyrophosphate) in nucleic acid, the cyclase resembles RNA and DNA ligases and capping enzymes (reviewed in Ref. 25). In all the later cases, neucleotidyl transfer occurs through a covalent lysyl-nucleoside monophosphate phosphoamide intermediate; the active-site lysine is present in a conserved short sequence motif, KXDG. RNA ligases, ATP-dependent DNA ligases, and capping enzymes also contain several additional conserved motifs (25). Neither KXDG nor these additional sequence motifs are identifiable in cyclases.

In this work, we determined the adenylation site of the E. coli cyclase. The adenylated amino acid His-309 is conserved in a large subfamily of cyclases encompassing all bacterial and archaeal proteins and also some metazoan proteins. Mutations common with any proteins of known function. However, data base searches indicated that genes encoding proteins with a significant similarity to the human cyclase are conserved among eucarya, bacteria, and archaea. When the protein encoded in the Escherichia coli genome was overexpressed, it showed RNA 3′-phosphate cyclase activity. The E. coli cyclase gene forms part of a previously uncharacterized operon, expression of which is controlled by an alternative sigma factor, σ54 (20, 24).

The cyclase has been purified from HeLa cell extracts, and its cDNA had been cloned (20, 23). The enzyme is expressed in all mammalian tissues and cell lines investigated, and has a nucleoplasmic localization, consistent with its postulated role in RNA processing (20). The cyclase has no apparent motifs in

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The abbreviations are as follows: N, nucleoside; DTT, dithiothreitol; ESIMS, electrospray ionization mass spectrometry; LC-ESIMS, high performance liquid chromatography interfaced with ESIMS; MSMS, tandem mass spectrometry; NanoeSI, nanoelectrospray ionization; PAGE, polyacrylamide gel electrophoresis; Cam, carboxyamidomethyl.
of His-309 in the \( E. \) coli cyclase abrogate formation of the AMP-cyclase intermediate and cyclization of the 3\(^{\prime}\)-phosphate in RNA.
Overexpression was performed in the *E. coli* strain BL21(DE3)pLysS as described before (20). For purification, the bacterial pellet was resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 0.3 mM NaCl, 10 mM imidazole, 1 mM DTT) supplemented with 0.5% Triton X-100 and protease inhibitors (complete protease inhibitors-EDTA mixture, Roche Molecular Biochemicals). The pellet was lysed by sonication, and a lysate, cleared by centrifugation, was applied to a Ni-NTA column (Qiagen) pre-equilibrated with buffer A. The protein was more than 95% pure as judged by SDS-PAGE (20). Protein concentration was determined using the Brad-ley Coomassie Blue protein assay kit (Bio-Rad). The purified protein was suspended in buffer A (50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 2 mM MgCl2, 1 mM CaCl2, 1 mM DTT) containing 20 μM [α-32P]ATP. After incubation for 3 h at 25 °C, the sample was divided into three equal aliquots, which were submitted to digestion with either trypsin, endoproteinase Glu-C (both from Promega, Madison, WI), or Lys-C protease (Wako, IG Instrumenten Gesellschaft, Zurich, Switzerland) overnight at 30 °C (Lys-C) or 37 °C (trypsin and endoproteinase Glu-C). A 10% aliquot of each digestion reaction was analyzed by SDS-PAGE using a Tris/Tricine/urea system described by Schagger and Von Jagow (28) after boiling the samples in the presence of SDS and DTT. Separated peptides were visualized by silver staining and autoradiography. In the remaining 90% of each digest, was resolved in the same electrophoresis system, and the peptides were subjected to sequence analysis by Edman degradation using an Applied Biosystems (Foster City, CA) model 477A sequencer and following the manufacturer’s recommendations.

**RESULTS**

**Known Cyclases and Cyclase-like Proteins Can Be Grouped into Two Subfamilies**—A dendrogram of the *E. coli* and human cyclases and cyclase-like proteins encoded in different organisms indicated that they can be subdivided into two classes (Fig. 1A). Members of class I include all prokaryotic proteins, the *Dictyostelium discoideum* enzyme, and one of the two proteins expressed in *Drosophila* and humans. The previously characterized *E. coli* and human cyclases belong to this class of enzymes. To the class II belong proteins encoded in genomes of budding and fission yeast, *Caenorhabditis elegans*, and also second forms of proteins expressed in *Drosophila* and humans.

Alignment of the members of the cyclase class I subfamily is shown in Fig. 1B. Analyzed proteins and also class II (data not shown) have no apparent motifs in common with other proteins in various data bases. The N-terminal halves and C-proximal parts of class I cyclases are relatively highly conserved at the 5 μg of trypsin followed by a 1-h cleavage with 5 μg of endoproteinase Glu-C. Liquid chromatography-purified adenylated peptide was digested with 0.2 μg of subtilisin (Sigma) for 90 min at 25 °C in 50 mM NH4HCO3. Peptides were separated on a 1 × 250-mm Vydac C8 column (Hesperia, CA) equilibrated in 98% solvent A (25 mM ammonium acetate, pH 6.2, 2% CH3CN) and solvent B (25 mM ammonium acetate, pH 6, 80% CH3CN), and a linear gradient was developed from 5 to 50% solvent B in 60 min at a flow rate of 50 μl/min. The LC-ESIMS system was as described previously (29). NanoESI-MSMS was performed according to the published method of Wilm and Mann (30). The mass spectra were acquired on an API 300 mass spectrometer (PE Sciex, Toronto, Ontario, Canada) equipped with a NanoESI source (Protana, Odense, Denmark).

**Model Building**—A homology-based model of the human cyclase was obtained using the coordinates of the *E. coli* enzyme.2 The two enzymes were aligned using the program Bestfit (Genetics Computer Group, Madison, WI). The optimal alignment (34% identity with 5 gaps) was used by the program MODELER (Ref. 31; BIOSYM/Molecular Simulations, San Diego, CA) to build and refine 6 human cyclase models. Human cyclase is 27 residues longer than the *E. coli* enzyme. No attempt was made to model this part of the polypeptide chain. The best model, selected on the lowest violations of the probability density functions, was evaluated using the program Profiles, 3D (Ref. 32; BIOSYM/Molecular Simulations). Its overall self compatibility score (162.2) was slightly better than that expected for a polypeptide chain of this length (157.6), indicating the reliability of the model.

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to delineate the protein region containing the reaction mixtures, since attempts to purify the adenylated complex by gel filtration under non-denaturing conditions resulted in loss of incorporated label. Hence, as observed previously for the human enzyme (3, 23), the *E. coli* cyclase-AMP complex may undergo hydrolysis in the absence of SDS. The peptides resulting from protease digestion of the cyclase-AMP complex were separated by SDS-PAGE, located by autoradiography, and analyzed by microsequencing. This analysis demonstrated that the adenylated amino acid is located between residues 258 and 324 (data not shown; see Fig. 1B).

To identify the adenylated amino acid residue, the enzyme adenylated in the presence of cold ATP and nonadenylated control enzyme were digested with trypsin followed by endoprotease Glu-C. The peptides were separated by reverse-phase LC-ESIMS. Comparison of digests of the adenylated and nonadenylated proteins indicated the presence in the former of one additional peptide with a measured mass of 2436 Da (data not shown), suggesting that it corresponds to FTVAHPSCHLLTNIAVVER. This peptide was detected (Fig. 2). Its mass, corresponding to an anhydro-AMP residue. As a result, the adenylated amino acid residue, since the AMP residue was lost under the conditions of the experiment, is located between residues 258 and 324 (data not shown; see Fig. 1B).

**Protease Mapping of the Adenylation Site Region in the *E. coli* Cyclase**—To delineate the protein region containing the adenylation site, protease mapping of the adenylated *E. coli* cyclase was performed. The protein was adenylated in the presence of [γ-32P]ATP and treated with either trypsin, endoprotease Glu-C, or Lys-C protease. In this and other experiments it was essential to add proteases directly to adenylation sequence level, with central regions of proteins being highly variable. The two most outstanding regions of similarity are glycine-rich sequences corresponding to residues 9–27 and 155–166 of the *E. coli* protein. Interestingly, the sequence of the human Hs1 protein is more related to the *D. discoideum* protein than to the *Drosophila* Dm1 protein (Fig. 1). It is possible that human and *Drosophila* proteins are not orthologs and that additional cyclase genes are expressed in vertebrates and/or insects.

Previous demonstration that the covalent human cyclase-AMP complex is unstable when heated in 0.1 N HCl or when treated with hydroxylamine at pH 4.7, but insensitive to heating in 0.1 N NaOH, has suggested that AMP is linked to the protein via a phosphoamide bond, possibly involving the lysine ε-amino group (3, 23). The *E. coli* cyclase-AMP complex is likewise resistant to treatment with alkali (0.1 N NaOH, 1 min at 95 °C) but is unstable in 0.1 N HCl (1 min at 95 °C), consistent with the phosphoamide linkage (data not shown). As evident from the alignment shown in Fig. 1B, no conserved lysine residue is present in class I cyclases.

**FIG. 4. Analysis of the subtilisin-digested adenylated peptide.** The presence of the AMP residue was directly examined by NanoESI-MSMS of the precursor ion 869 shows two major fragments. The mass of 345.5 is interpreted as deprotonated AMP, and the one of 327.5 as deprotonated anhydro-AMP.

**FIG. 5. Activity of the wild-type and mutant *E. coli* cyclases in adenylation (A) and 3'-phosphate cyclization (B) assays.** A, over-expressed and purified wild-type (WT) and mutant cyclase (H309N and H309A) preparations (20 ng/assay) were incubated with [γ-32P]ATP, and the resulting complexes were analyzed by SDS-PAGE and autoradiography. (B) Cyclization of the 3'-terminal phosphate in AAAA-CAAAAp*, measured by the Norit assay. Assays were performed as described under “Experimental Procedures.” The amounts of added cyclase are indicated.
assigned to AMP - H⁺ (345.5 Da) and AMP - H⁺ - H₂O (327.5 Da).

The active-site peptide, CHLL, could in principle be adenylated on Cys-308 or His-309, and carboxymidomethylation could also have taken place on either residue (33, 34). That the AMP moiety is present at Cys-308 can, however, be excluded. From the difference in the m/z value for the y11 and y12 ions (160.5 Da) in the tandem mass spectrum of the original 2493-Da peptide (Fig. 3), it can be concluded that Cys-308 is carboxymethylated. Furthermore, the y13 ion (m/z 1512) demonstrates that His-309 is not carboxymidomethylated. This was confirmed by Edman degradation of this peptide, which yielded phenylthiohydantoin-Cam-Cys in cycle 8 (data not shown). These results leave His-309 as the only possible site of adenylation. This conclusion is strengthened by two observations. (i) The first is the stability of the cyclase-AMP complex in 0.1 M NaOH and its sensitivity to acidic pH. This is consistent with a P-N rather than a P-S linkage (35). The acid lability of the P-N bond also explains why phenylthiohydantoin-His, rather than a modified residue, was observed in cycle 9 during Edman degradation of the 2493-Da peptide (data not shown). (ii) Second, the histidine residue is conserved in all class I cyclases, whereas the cysteine residue is only found in the E. coli enzyme (Fig. 1B).

Activity of the Cyclase Mutants—To directly assess the importance of His-309 for activity of the E. coli cyclase, two single-amino acid mutant enzymes were engineered, overexpressed in E. coli, and purified. Inspection of the three-dimensional structure of the enzyme revealed that replacement of His-309 by either Asn or Ala would most likely not disturb the structure of the protein.

Mutant proteins were tested for activity as acceptors in the adenylation reaction and for their ability to catalyze cyclization of the 3’-phosphate in the model oligoribonucleotide substrate, AAAACAAAAGp* (the asterisk indicates a radiolabeled phosphate). Both of the His-309 mutations completely abolished adenylation of the protein (Fig. 5A) and its activity to catalyze cyclization of the 3’-phosphate (Fig. 5B). The recombinant C308A mutant protein was also engineered, overexpressed, and purified. Although less efficiently that the wild-type protein, this mutant underwent adenylation and catalyzed cyclization of the 3’-phosphate (data not shown). These results provide further evidence for His-309 acting as an adenylate acceptor.

DISCUSSION

In this work we assigned the site of adenylation in the E. coli cyclase to His-309 by using protease digestions and mass spectrometry. Consistent with this histidine residue acting as an AMP acceptor, its replacement with asparagine or alanine abrogated both formation of the enzyme-AMP complex and the cyclization of the 3’-terminal phosphate in a model RNA substrate. Based on the crystal structure of the E. coli cyclase, the introduced His-309 mutations should not interfere with the local structure of the protein, since this residue is largely exposed to solvent. Furthermore, changing it into a smaller residue (Ala or Asn) circumvents the problem of steric hindrance (see Fig. 6). Hence, it is unlikely that these mutations exerted their effect on enzyme activity indirectly by modifying folding of the protein rather than its catalytic site.

The human class I cyclase has 34% identity and 43% similarity with the E. coli enzyme. Modeling of the human sequence using the coordinates of the E. coli enzyme showed an overall folding that is very similar (data not shown). Importantly, the human enzyme contains a histidine residue at position 329, which corresponds to His-309 of the E. coli enzyme. Moreover, as expected for a catalytic-site residue, its immediate neighborhood in the three-dimensional model also appears to be highly conserved (Fig. 6). In particular, the presence of Glu-14 and Gln-18 is noteworthy, since these residues have been found to form hydrogen bonds with the histidine in the E. coli enzyme. Alignment of proteins belonging to the class II subfamily did not identify a conserved histidine residue in the C-terminal region or anywhere else in this group of proteins. It remains to be established whether class II proteins have cyclase activity.

To the best of our knowledge, the E. coli cyclase is the only established example of a protein adenylated on a histidine and using ATP as a co-factor. However, three other proteins are known to undergo modification of a histidine residue with nucleotidyl groups other than adenyllyl. Galactose-1-phosphate uridylyltransferase, an enzyme involved in the Leloir pathway for galactose metabolism, is transiently uridylated at the Nε2 position of the imidazole ring of a histidine residue; UDP-glucose is a uridylyl group donor in this reaction (36, 37). A second example is the gag protein of the S. cerevisiae double-stranded RNA L-A virus. His-154 of this protein makes a co-

3 E. Billy, D. Hess, J. Hofsteenge, and W. Filipowicz, unpublished observation.

Fig. 6. Structure of the neighborhood of His-309 in the E. coli and human cyclases.
valent complex with m\(^7\)GMP (m\(^7\)G is 7-methylguanosine), following a nucleophilic attack by the imidazole nitrogen on the α-phosphate of the m\(^7\)GpppN cap structure in mRNA. The capture of m\(^7\)GMP by the viral protein results in decapping of cellular mRNAs (38). Recently, Cartwright and McLennan (39) reported that the brine shrimp GTP:GTP guanylyltransferase, the enzyme responsible for synthesis of diguanosine tetraphosphate (Gp\(_2\)G), forms a histidyl-GMP reaction intermediate via Nε2 of a histidine residue. Although only few proteins are known to be nucleotidylated on a histidine, phosphoryl transfer reactions involving a phosphohistidine residue are more common and found in many proteins that are members of two-component signaling systems in both prokaryotes and eukaryotes (40).

Amino acid sequences flanking the adenylated His-309 in the E. coli cyclase and corresponding histidines in other members of the class I family of cyclases do not resemble sequences found in other proteins that undergo nucleotidylation on histidine or other amino acid residues. For example, the bacteriophage T4 RNA ligase, like many other RNA and DNA ligases, forms a covalent protein-adenyl intermediate and transfers AMP to the 5′-terminal phosphate in nucleic acid to form the 5′-phosphoanhydride ligation intermediate (reviewed in Ref. 19). Notably, in the absence of the physiological 5′-phosphorylated substrate, T4 RNA ligase can inefficiently transfer AMP to 3′-terminal phosphate, resulting in 3′-5′ phosphoanhydride formation and 3′-phosphate cyclization, via a mechanism probably very similar to that of the RNA cyclase (41, 42). However, in RNA and DNA ligases, the nucleotidyl transfer occurs to the lysine in a conserved sequence motif, KXDG; this motif is not present in cyclase class I or class II families. We have previously tested whether, in the absence of the 3′-phosphorylated end, the human cyclase has a potential to activate the 5′-terminal phosphate in RNA. No evidence of \(A^\text{β}^\text{pp}^\text{N}\) formation was found, even when a large excess of the enzyme was used. In addition, no evidence of cyclase-catalyzed inter- or intramolecular ligation of either 5′- or 3′-phosphorylated oligoribonucleotides was obtained (20). Taken together, these data argue that RNA ligases and RNA 3′-phosphate cyclases are very distinct enzymes.

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