The HD Domain of the Escherichia coli tRNA Nucleotidyltransferase Has 2',3'-Cyclic Phosphodiesterase, 2'-Nucleotidase, and Phosphatase Activities*

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In all mature tRNAs, the 3′-terminal CCA sequence is synthesized or repaired by a template-independent nucleotidyltransferase (ATP/CTP):tRNA nucleotidyltransferase; EC 2.7.7.25. The Escherichia coli enzyme comprises two domains: an N-terminal domain containing the nucleotidyltransferase activity and an uncharacterized C-terminal HD domain. The HD motif defines a superfamily of metal-dependent phosphohydrolases that includes a variety of uncharacterized proteins and domains associated with nucleotidyltransferases and helicases from bacteria, archaea, and eukaryotes. The C-terminal HD domain in E. coli tRNA nucleotidyltransferase demonstrated Ni2+-dependent phosphatase activity toward pyrophosphate, canonical 5′-nucleoside tri- and diphosphates, NADP, and 2′-AMP. Assays with phosphodiesterase substrates revealed surprising metal-independent phosphodiesterase activity toward 2',3'-nucleotides, NADP, and 2′-AMP. In the presence of Mg2++, the tRNA nucleotidyltransferase hydrolized 2',3'-cyclic substrates with the formation of 2′-nucleotides, whereas in the presence of Ni2+, the protein also produced some 3′-nucleotides. Mutations at the conserved His-255 and Asp-256 residues comprising the C-terminal HD domain of this protein inactivated both phosphodiesterase and phosphatase activities, indicating that these activities are associated with the HD domain. Low concentrations of the E. coli tRNA (10 nM) had a strong inhibiting effect on both phosphatase and phosphodiesterase activities. The competitive character of inhibition by tRNA suggests that it might be a natural substrate for these activities. This inhibition was completely abolished by the addition of Mg2++, Mn2+, or Ca2+, but not Ni2+. The data suggest that the phosphohydrolase activities of the HD domain of the E. coli tRNA nucleotidyltransferase are involved in the repair of the 3′-CCA end of tRNA.

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In all organisms, the 3′-terminal CCA sequence in all mature tRNAs is required for tRNA aminoacylation reaction and for translation on the ribosome (1, 2). The CCA-adding enzyme (ATP/CTP):tRNA nucleotidyltransferase; EC 2.7.7.25 adds these three nucleotides in the order of C, C, and A to the tRNA nucleotide-73, using CTP and ATP as substrates and producing inorganic pyrophosphate (3). tRNA nucleotidyltransferases (tRNA-NTs) are highly conserved throughout evolution, and they have been identified in all three kingdoms (4). The CCA-adding enzyme is essential in eukaryotes, archaea, and certain eubacteria where some or all tRNA genes do not encode the CCA sequence (5). In these organisms, 3′ trailer sequences are removed from tRNA precursors by nucleases that stop at the discriminator base (position 73), leaving the acceptor stem intact as a substrate for the CCA-adding enzyme (6). In organisms in which tRNA genes encode the CCA sequence (Escherichia coli and many eubacteria), tRNA-NT repairs the tRNA 3′ CCA sequence depleted by exonucleases, and inactivation of the tRNA-NT gene impairs cell growth (7).

The CCA-adding enzyme is unique among nucleotidyltrans-ferases in that it adds an ordered nucleotide sequence to a specific primer without using a nucleic acid template (6, 8). Moreover, the enzyme can monitor and faithfully rebuild tRNAs with incomplete 3′ ends (tRNA-NCC, tRNA-NC, and tRNA-N, where N is the discriminator base). It completes the terminal CCA sequence by adding one nucleotide at a time. The protein consists of a single polypeptide with dual specificity for AMP and CMP incorporation, and a single active site is responsible for addition of both nucleotides (4). Several models have been proposed to explain these properties (9–14), but the molecular details remain unclear.

According to their primary sequence, the CCA-adding en-zymes belong to an ancient superfamily of nucleotidyltrans-ferases, which is divided into two classes (6, 15). Class I contains archaeal CCA-adding enzymes, whereas class II comprises eubacterial and eukaryotic proteins. Class II en-zymes have similar sizes (400–470 amino acids) and share a high degree of sequence identity, and they have been identified in all three kingdoms (6). The C. crescentus CCA-adding enzyme is a member of class II (15). In class II enzymes, the superfamily includes archaeal CCA-adding enzymes, whereas class II comprises eubacterial and eukaryotic proteins. Class II en-zymes have similar sizes (400–470 amino acids) and share a highly conserved 25-kDa N-terminal region with characteristic active-site signature motifs (DxD and RRD), but differ from one another in their C-terminal regions. The E. coli tRNA-NT belongs to the class II enzymes, and its nucleotidyltransferase activity has been characterized (16, 17). This protein has two domains, the N-terminal nucleotidyltransferase domain and the C-terminal phosphatase domain.
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the C-terminal HD domain named after the conserved HD motif (His-255 and Asp-256 in E. coli). The HD motif defines a superfamily of metal-dependent phosphohydrolases that includes a variety of uncharacterized proteins and domains associated with nucleotidyltransferases and helicases from bacteria, archaea, and eukaryotes (18). The HD superfamily is related to a well-characterized eukaryotic 3',5'-cyclic phosphodiesterase (19) that is, however, distinct from the HD superfamily and contains additional conserved regions. The HD domain was predicted to have a metal-dependent phosphodiesterase activity (18).

Here, we report that the E. coli tRNA-NT has 2',3'-cyclic phosphodiesterase, -nucleotidase, and phosphatase activities. Site-directed mutagenesis demonstrated that these three activities are associated with the conserved C-terminal HD motif and probably have partially overlapping active sites. Low concentrations of total E. coli tRNA produced strong competitive inhibition on all three activities, suggesting that tRNA might be a natural substrate for the HD domain-associated activities. Based on our results, we proposed a model for the role of the HD domain-associated activities in the repair of the tRNA 3' end.

MATERIALS AND METHODS

Gene Cloning and Protein Purification—Recombinant proteins were expressed with a His6 fusion tag at the N terminus using a modified pET15b (Novagen) expression vector in E. coli BL21 (DE3) as described previously (20). Purification of the E. coli tRNA nucleotidyltransferase (tRNA-NT) E. coli cultures) was performed using a combination of affinity chromatography on nickel-nitrioltriacetic acid resin (Qiagen) and anion-exchange chromatography on Q-Sepharose (Amersham Biosciences; elution by NaCl gradient 50–200 mm).

Enzymatic Assays—The E. coli tRNA-NT was screened for a variety of catalytic activities as follows. Reactions were performed in 96-well microtiter plates using 0.2-ml reaction mixtures and the following spectrophotometric procedures. After addition of protein samples (0.1–1.0 μg), the plates were incubated for 1–3 h at 37 °C before taking the reading at indicated wavelengths. Positive controls with small amount of commercial enzymes were included in all tests. Phosphatase activity was measured in a reaction mixture containing 50 mM HEPES-K buffer, pH 7.5, 5 mM MgCl2, 0.1 mM MnCl2, and 0.83 mM bis- p-nitrophenyl phosphate (bis-pNPP) with detection at 410 nm. Phosphodiesterase and nuclease activities were measured using 50 mM Tricine buffer, pH 8.5, 5 mM MgCl2, 0.1 mM MnCl2, and 0.83 mM bis-p-nitrophenyl phosphohydrolase (bis-pNPP) with detection at 410 nm. Esterase activity was tested using 1 mM p-nitrophenyl palmitate in 50 mM Tris-HCl, pH 8.0. 0.4% Triton X-100, and 0.1% of Gum Arabic (detection at 410 nm). Protease activity was determined with a mixture of two chromogenic substrates (benzoyl-Arg-p-nitroanilide and Leu-p-nitroanilide; 0.2 mM each) was measured in 50 mM HEPES-K buffer, pH 7.5, 0.5 mM Ca2+, 0.5 mM Zn2+, and 1 mM dithiothreitol (detection at 405 nm). Dehydrogenase and oxidase activities were measured using three pools of different substrates: 20 amino acids (0.2 mM concentrations of each in reaction mixture), eight different alcohols (methanol, ethanol, adonitol, xylitol, dulcitol, mannotol, sorbitol, arabitol; 0.5 mM concentrations of each in reaction mixture), or nine different organic acids (acetic, fumarate, malate, lactate, pyruvate, isocitrate, succinate, oxaloacetate, α-ketoglutarate; 0.5 mM each in reaction mixture). Dehydrogenase activity was measured in a reaction mixture containing 50 mM Tricine buffer, pH 8.5, 0.5 mM NAD, 0.5 mM NADP, the substrate solution (described above), 1 mM Mg2+, and 0.1 mM Mn2+ (detection at 340 nm). Oxidase activity was monitored using the chromogenic reaction of peroxidase and o-dianisidine (21) in a reaction mixture containing 50 mM HEPES-K, pH 8.0, the substrate solution (described above), 0.1 mM o-dianisidine, and 2 μM of peroxidase (detection at 480 nm).

Screening with natural phosphatase substrates was performed in 96-well plates using 160-μl reaction mixtures containing 50 mM HEPES-K, pH 7.5, 0.1 mM substrate (various nucleotides and phosphorylated sugars, amino acids, and organic acids from Sigma), 0.25 mM Mg2+, 0.6 mM Mn2+, or 2–5 μg of protein. After a 30–60 min incubation at 37 °C, the reaction was terminated by the addition of 40 μl of Malachite Green reagent (22), and the production of P2 was measured at 630 nm.

Phosphodiesterase activity of the E. coli tRNA-NT against bis-pNPP was measured in a reaction mixture containing 50 mM HEPES-K, pH 7.0, 3.3 mM bis-pNPP, with or without 0.5 mM Ni2+. After a 30-min incubation at 37 °C, the reaction was stopped by the addition of 0.2 ml of 6 N NaOH and produced p-nitrophenol was determined from the absorbance at 410 nm. Phosphodiesterase activity with natural substrates (various 2',3'- and 3',5'-cyclic nucleoside monophosphates from Sigma) was measured using a quantitative assay based on a measurement of the alkaline phosphatase-sensitive nucleotide product. All incubations (0.4 ml) contained 50 mM HEPES-K, pH 7.0, 1–2 mM substrate, and metal (if indicated). After a 20-min incubation at 37 °C with enzyme (0.2–0.3 μg), the reaction was stopped by the addition of 0.4 ml of 2× alkaline phosphatase buffer (0.2 μl CHES buffer, pH 9.0, and 10 mM MgCl2) and incubated with 1 unit of alkaline phosphatase for 10 min at 37 °C. Liberated P1 was assayed with Malachite Green reagent (22).

For determination of the Km and Vmax, the phosphatase and phosphodiesterase assays contained substrates at concentrations of 0.1–10 mM. Kinetic parameters were determined by non-linear curve fitting from the Lineweaver-Burk plot using GraphPad Prism software (version 4.00 for Windows, GraphPad Software, San Diego, CA).

TLC Analysis—Products of enzymatic digestions, performed as described above, were analyzed by TLC on cellulose plates in solvent A (saturated ammonium sulfate/3 mM sodium acetate/isopropl alcohol; 80:6:2) (23). The reaction products and nucleotide standards were visu-

![Figure 1](https://example.com/figure1.png)

**FIG. 1.** Phosphatase activity of the E. coli tRNA-NT with pNPP as a substrate dependence on metal (A), on Ni2+ concentration (B), on pNPP concentration (C). Experimental conditions were as described under “Materials and Methods.” Reaction mixtures contained 0.5 μg (0.01 nmol) of tRNA-NT and 4 μM pNPP, 5 mM Mg2+ or 0.5 mM concentrations of other metals (A), 8 μM pNPP (B), or 0.1 mM Ni2+ (C).
alized under UV light. 50-μl reaction mixtures contained 50 mM HEPES-K, pH 7.0, 0.1 mM Ni\textsuperscript{2+} (if indicated), 20 mM 2',3'-cAMP (or 2',3'-cGMP), or 2 mM ATP and were incubated at 37 °C without or with E. coli tRNA-NT.

Site-directed mutagenesis was carried out using QuikChange (Stratagene) and mutagenic primers designed to replace the selected amino acid residues by Ala. Wild-type E. coli tRNA-NT gene cloned into pET15b was used as a template for mutagenesis. Plasmids were purified using Qiaprep Spin Mini Prep Kit (Qiagen), and all mutations were verified by DNA sequencing.

RESULTS

tRNA Nucleotidyltransferase Has Phosphatase Activity—General enzymatic screens with pNPP as a substrate identified a new activity in the E. coli tRNA nucleotidyltransferase (tRNA-NT, CCA adding enzyme) (data not shown). The protein was purified in a large scale by a combination of nickel-chelate affinity and anion-exchange chromatography to >95% homogeneity. In solution, most of purified tRNA-NT existed as a monomer (44.8 ± 2.0 kDa) with low levels (up to 20%) of dimers (103.0 ± 10.0 kDa) and oligomers (784.2 ± 33.5 kDa). All three fractions (monomers, dimers, and oligomers) had approximately the same level of phosphatase activity with pNPP. In contrast to the characterized nucleotidyltransferase activity of this protein (16, 17), the phosphatase activity had a neutral pH optimum, pH 7.0, and was

FIG. 2. Hydrolysis of natural phosphatase substrates by the E. coli tRNA-NT. Experimental conditions are described under “Materials and Methods.” Reaction mixtures contained 0.1 mM substrate, 0.1 mM Ni\textsuperscript{2+}, and 1.0 μg of tRNA-NT.

FIG. 3. Phosphohydrolase activity of the E. coli tRNA-NT as a function of substrate concentration. A, ATP; B, PP\textsubscript{i}; C, NADP; D, 2'-AMP; E, 2',3'-cAMP; and F, 2',3'-cGMP. Experimental conditions are described under “Materials and Methods.” Reaction mixtures contained 0.1–0.3 μg of tRNA-NT and 0.1 mM Ni\textsuperscript{2+}. 

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**E. coli tRNA Nucleotidyltransferase**

**Table I**

| Variable Substrate | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) |
|--------------------|------------|-------------------------|---------------------|-------------------------------|
| Phosphatase activity |            |                         |                     |                               |
| pNPP               | 6.20 ± 0.46 | 12.4 ± 0.34             | 10.3 ± 0.28        | 1.66 × 10$^3$               |
| PP                 | 0.10 ± 0.004 | 3.01 ± 0.06             | 2.51 ± 0.05        | 25.1 × 10$^3$               |
| NADP               | 0.15 ± 0.02  | 17.9 ± 0.6              | 14.9 ± 0.5         | 99.3 × 10$^3$               |
| ADP                | 0.19 ± 0.02  | 1.49 ± 0.08             | 1.24 ± 0.07        | 6.53 × 10$^3$               |
| ATP                | 0.18 ± 0.01  | 4.53 ± 0.14             | 3.78 ± 0.12        | 21.0 × 10$^3$               |
| CDP                | 0.53 ± 0.09  | 5.80 ± 0.47             | 4.83 ± 0.39        | 9.11 × 10$^3$               |
| CTP                | 0.13 ± 0.01  | 4.63 ± 0.20             | 3.36 ± 0.17        | 25.9 × 10$^3$               |
| 2′-AMP             | 0.76 ± 0.13  | 3.71 ± 0.17             | 3.09 ± 0.14        | 4.07 × 10$^3$               |
| Phosphodiesterase activity |            |                         |                     |                               |
| 2′,3′-cAMP         | 0.49 ± 0.04  | 3.21 ± 0.1              | 7.63 ± 0.48        | 1.98 × 10$^3$               |
| 2′,3′-cGMP         | 1.60 ± 0.22  | 2.36 ± 0.10             | 1.97 ± 0.08        | 1.23 × 10$^3$               |
| tRNA nucleotidyltransferase activity |        |                         |                     |                               |
| ATP                | 0.03        |                        |                     |                               |
| CTP                | 0.03        |                        |                     |                               |
| tRNA-CC            | 0.015       |                        |                     |                               |
| tRNA-C             | 0.02        |                        |                     |                               |

* Data from Ref. 17.

**Fig. 4.** Cellulose TLC analysis of the reaction products: hydrolysis of ATP (A), 2′,3′-cAMP (B), and 2′,3′-cGMP (C) by the *E. coli* tRNA-NT. Experimental conditions are described under “Materials and Methods.” A, ATP hydrolysis. Sample 1, 6 μl of TLC standards (AMP, ADP, ATP; 3 mM each). Sample 2, no enzyme (20 min incubation). Samples 3–5 contained 2 μg of the *E. coli* tRNA-NT and were incubated at 37 °C for 20 (sample 3), 40 (sample 4), and 60 min (sample 5). B, 2′,3′-cAMP hydrolysis. Sample 1, no enzyme. Sample 2, with enzyme (2 μg) and without metal addition. Sample 3, with enzyme and 0.1 mM Ni$^{2+}$. Sample 4, 6 μl of TLC standards (2′,3′-cAMP, adenosine, 3′-AMP, 2′-AMP; 2.5 mM each). C, 2′,3′-cGMP hydrolysis. Sample 1, no enzyme. Sample 2, with enzyme and without metal addition. Sample 3, with enzyme and 0.1 mM Ni$^{2+}$. Sample 4, 6 μl of TLC standards (2′,3′-cGMP, guanosine, 3′-GMP, 2′-GMP; 2.5 mM each).

greatly stimulated by Ni$^{2+}$ (Fig. 1). With pNPP as a substrate, tRNA-NT showed high affinity to Ni$^{2+}$ ($K_D = 6.0 ± 0.7$ μM in the presence of 8 mM pNPP) and low affinity to pNPP ($K_m = 6.2 ± 0.46$ μM in the presence of 50 μM Ni$^{2+}$) (Fig. 1). However, the specific activity toward pNPP ($V_{max} = 12.4 ± 0.34$ μmol/min/mg of protein) was sufficiently high to produce positive results in general screens without Ni$^{2+}$.

**Phosphatase Activity with Natural Substrates**—Secondary catalytic screens with natural phosphatase substrates (57 compounds) in the presence of Ni$^{2+}$ identified high phosphatase activity of the *E. coli* tRNA-NT against a broad range of canonical nucleoside di- and triphosphates, NADP, and pyrophosphate (Fig. 2). Highest phosphatase activity was found against NADP, ATP, CTP, TTP, and dCTP. Nucleoside diphosphates (ADP, CDP, GDP, and UDP) were also good substrates for the *E. coli* tRNA-NT (data not shown). With all substrates, the protein showed saturation kinetics with highest affinity to pyrophosphate and NADP (Fig. 3 and Table I). Cellulose TLC analysis of the hydrolysis of ATP identified ADP and AMP as reaction products (Fig. 4A), indicating that the *E. coli* tRNA-NT sequentially removes monophosphates from ATP. Enzymatic analysis of the reaction products produced during hydrolysis of ATP or ADP yielded P$_i$, AMP, and ADP as the reaction products (data not shown). With nucleoside monophosphates as substrates, high phosphatase activity was observed against 2′-AMP (Figs. 2 and 3), low activity against 3′-AMP (12.9 nmol/min/mg of protein), and no activity toward 5′-mononucleotides.

As with pNPP, hydrolysis of natural substrates had a neutral pH optimum, pH 7.0, and was greatly stimulated by Ni$^{2+}$ (Fig. 5). With natural substrates, the protein had even higher affinity for Ni$^{2+}$ ($K_D = 1.06 ± 0.1$ μM with pyrophosphate as a substrate). Cu$^{2+}$ stimulated the hydrolysis of pyrophosphate and ATP and completely inhibited the hydrolysis of 2′-AMP, suggesting some difference in the active sites involved in these reactions. Because the nucleotidyltransferase activity of the *E. coli* tRNA-NT (which also involves the hydrolysis of ATP and CTP) had an alkaline pH optimum (pH 9.0–9.5) and required Mg$^{2+}$ (16, 17), it is not related to the Ni$^{2+}$-dependent neutral phosphatase and 2′-nucleotidase activities observed in our experiments.

We also determined the kinetic parameters for the hydrolysis of several natural substrates (Fig. 3, Table I). With all substrates, the protein showed saturation kinetics with highest affinity for pyrophosphate and NADP (Fig. 3, Table I). For the nucleotidyltransferase reaction, 1 mM PP$_i$ caused only 20% inhibition (16). This insensitivity to PP$_i$ can be explained by the presence of pyrophosphatase activity in the *E. coli* tRNA-NT. Whereas the $K_m$ for ATP (0.18 mM) was near the $K_m$ for AMP incorporation (0.33 mM) (17), the $K_m$ for CTP (0.13 mM) was significantly higher than the $K_m$ for CMP incorporation (0.03 mM) (17). The protein showed much higher activity and affinity to NADP (which also has a 2′-phosphate) than to 2′-AMP (Table I), suggesting that it might prefer large substrates like tRNAs.
tRNA-NT Has Phosphodiesterase Activity—Because a number of phosphodiesterases have been shown to possess both nucleotidase and phosphodiesterase activities (24, 25), the *E. coli* tRNA-NT was tested for the presence of phosphodiesterase activity toward 2',3'-cAMP and 2',3'-cGMP, low activity against 2',3'-cCMP, and no activity with 3',5'-cyclic nucleotides (Fig. 3). Like most known 2',3'-cyclic phosphodiesterases, the phosphodiesterase activity of the *E. coli* tRNA-NT had a neutral pH optimum, pH 7.0, and no metal dependence (Fig. 5D). Similar levels of phosphodiesterase activity were observed without metal addition or in the presence of Mg^{2+}, Mn^{2+}, Ca^{2+}, or Ni^{2+}, whereas Co^{2+}, Cu^{2+}, and Zn^{2+} were inhibiting (Fig. 5D). The protein showed highest activity and affinity to 2',3'-cAMP (Table I).

![Fig. 5. Metal dependence for the hydrolysis of various substrates by the *E. coli* tRNA-NT. A, PPi; B, ATP; C, 2'-AMP; D, 2',3'-cAMP. The metal concentrations were 1 mM for Mg^{2+} and 0.1 mM for other metals. Reaction mixtures contained 0.28 μg of tRNA-NT and 0.2 mM PP, (A), 0.5 mM ATP (B), 1 mM 2'-AMP (C), and 1.25 mM 2',3'-cAMP (D). Phosphatase and phosphodiesterase activities were measured as described under “Materials and Methods.”](image-url)

**Fig. 5.** Metal dependence for the hydrolysis of various substrates by the *E. coli* tRNA-NT. A, PPi; B, ATP; C, 2'-AMP; D, 2',3'-cAMP. The metal concentrations were 1 mM for Mg^{2+} and 0.1 mM for other metals. Reaction mixtures contained 0.28 μg of tRNA-NT and 0.2 mM PP, (A), 0.5 mM ATP (B), 1 mM 2'-AMP (C), and 1.25 mM 2',3'-cAMP (D). Phosphatase and phosphodiesterase activities were measured as described under “Materials and Methods.”

**Fig. 6.** Effect of the *E. coli* tRNA on phosphohydrolase activities of tRNA-NT with various substrates (A) and on saturation curves for 2'-AMP (B) and 2',3'-cAMP (C). A, the following concentrations of substrates were used (in the presence of 0.1 mM Ni^{2+} and 0.28 μg of enzyme): 8 mM pNPP, 0.5 mM ATP, 1 mM 2'-AMP, and 0.5 mM 2',3'-cAMP. B, 2'-nucleotidase activity of tRNA-NT (0.28 μg) as a function of 2'-AMP concentration without or in the presence of 1.6 and 4.8 nM *E. coli* tRNA. C, phosphodiesterase activity as a function of 2',3'-cAMP without or in the presence of 3.1 and 9.3 nM *E. coli* tRNA.

**TABLE II**

| tRNA Concentration | K_m (2'-AMP) | K_m (2',3'-cAMP) |
|--------------------|--------------|-----------------|
| No tRNA            | 0.69         | 0.49            |
| 1.6 nM             | 2.06         | ND              |
| 3.1 nM             | ND           | 0.89            |
| 4.8 nM             | 4.21         | ND              |
| 9.3 nM             | ND           | 6.79            |

*E. coli* tRNA-NT had a neutral pH optimum, pH 7.0, and no metal dependence (Fig. 5D). Similar levels of phosphodiesterase activity were observed without metal addition or in the presence of Mg^{2+}, Mn^{2+}, Ca^{2+}, or Ni^{2+}, whereas Co^{2+}, Cu^{2+}, and Zn^{2+} were inhibiting (Fig. 5D). The protein showed highest activity and affinity to 2',3'-cAMP (Table I).

When tested with the general phosphodiesterase substrate bis-pNPP, tRNA-NT had maximal activity at pH 7.0 and reduced activity (25%) at pH 8.5 (used in screens), and it showed a strong requirement for Ni^{2+} (little activity with Mn^{2+} and no activity without metal or in the presence of Mg^{2+}). This strong dependence of the bis-pNPP hydrolysis by the *E. coli* tRNA-NT...
**Table III**

| Protein | Phosphatase activity (with ATP) | 2'-Nucleotidase activity (with 2'-AMP) | Phosphodiesterase activity (with 2',3'-cAMP) |
|---------|-------------------------------|-----------------------------------|-----------------------------------|
| Wild-type | 4.53 | 3.71 | 3.21 |
| H255A | ND | ND | ND |
| D256A | ND | ND | ND |
| H305A | ND | ND | ND |
| D21A | 3.23 | 4.03 | 2.55 |
| D23A | 4.26 | 3.60 | 2.41 |

**Fig. 7.** Effect of Mg\(^{2+}\) on phosphohydrolase activities of tRNA-NT (0.28 µg) in the presence of the E. coli tRNA. A, ATP hydrolysis; B, 2'-AMP hydrolysis; C, 2',3'-cAMP hydrolysis. Reactions were performed as described under “Materials and Methods” and contained 0.5 mM ATP and 7.8 mM tRNA (A); 1 mM 2'-AMP and 15.6 mM tRNA (B); 0.5 mM 2',3'-cAMP and 15.6 mM tRNA (C).

on Ni\(^{2+}\) explains why the general phosphodiesterase screens failed to detect this activity. In addition, our results demonstrated a different metal requirement for the hydrolysis of natural (2',3'-cAMP) and artificial (bis-pNPP) substrates by the E. coli tRNA-NT.

Without metal or in the presence of Mg\(^{2+}\), the E. coli tRNA-NT hydrolyzed 2',3'-cAMP to 2'-AMP as analyzed by cellulose TLC (Fig. 4B). However, in the presence of Ni\(^{2+}\), the protein produced both 2'-AMP and 3'-AMP. Because Ni\(^{2+}\) stimulates 2'-nucleotidase activity of the E. coli tRNA-NT (Fig. 5C), in the presence of this metal, a significant proportion of 2'-AMP product was converted to adenosine (Fig. 4C). As presented on Fig. 4B, in the presence of Ni\(^{2+}\), 2'-AMP was still the main product (~70%) of 2',3'-cAMP hydrolysis. Similar results were obtained with 2',3'-cGMP (Fig. 4C) and 2',3'-cCMP (data not shown) as substrates. Other metals (Mg\(^{2+}\), Mn\(^{2+}\), Ca\(^{2+}\)) and the E. coli tRNA did not affect the hydrolysis of 2',3'-cAMP (data not shown). Thus, the E. coli tRNA-NT has 2',3'-cyclic nucleotide 3'-phosphodiesterase activity in the absence of Ni\(^{2+}\) and 2',3'-cyclic nucleotide 2',3'-phosphodiesterase activity in the presence of Ni\(^{2+}\). At the moment, two groups of 2',3'-cyclic phosphodiesterases are known (BRENDA data base). Members of the first group (EC 3.1.4.16, mostly prokaryotic proteins) convert a 2',3'-cyclic nucleotide exclusively to a 3'-nucleotide, which is further hydrolyzed to a ribonucleotide and P\(_i\). The second group (EC 3.1.4.37, mostly eukaryotic proteins) hydrolyzes the same substrate exclusively to a 2'-nucleotide and then degrades it to a ribonucleotide and P\(_i\). Thus, the E. coli tRNA-NT is the first 2',3'-cyclic phosphodiesterase that can hydrolyze a 2',3'-cyclic phosphodiester at both 2'- and 3'-bonds and produce the corresponding 2'- and 3'-nucleotides.

**Effect of tRNA and Metals**—In the absence of metal or in the presence of Ni\(^{2+}\), low concentrations of total tRNA from E. coli (10 nM) had a strong inhibiting effect on both phosphatase and phosphodiesterase activities of the E. coli tRNA-NT (Fig. 6A). The activities toward natural substrates were especially sensitive to tRNA inhibition (K\(_i\) = 0.29 nM for ATP hydrolysis, 1.08 mM for 2'-AMP hydrolysis, and 1.68 mM for 2',3'-cAMP hydrolysis); the inhibition of pNPP hydrolysis by 30 nM tRNA was minimal (30%) (Fig. 6A). These inhibition constants are at least 10,000 times lower than the K\(_m\) for tRNA in nucleotidyltransferase reaction (15–20 µM) (17). As shown on Fig. 6 (B and C), the E. coli tRNA acted as a competitive inhibitor of the hydrolysis of both 2'-AMP and 2',3'-cAMP and induced a significant increase in K\(_m\) for these substrates (Table II). The inhibition by tRNA was released at high substrate concentrations (Fig. 6, B and C), suggesting that tRNA might be a natural substrate for new phosphohydrolase activities. Total tRNA from yeast had similar inhibiting effects (data not shown). Moreover, we found that the phosphohydrolase activities of the E. coli tRNA-NT were also inhibited in a dose-dependent manner by nanogram quantities of the E. coli total tRNA (with 50% reduction caused by 8.86–22.2 ng of rRNA), genomic DNA (8.95–17.9 ng), or synthetic oligonucleotides poly(A) (36.3–97.6 ng) or poly(C) (14.4–19.1 ng), whereas single nucleotides (like 5'-AMP) had weak inhibiting effect (K\(_i\) = 0.59 mM). This sensitivity to various nucleic acids can be explained by broad substrate specificity of tRNA-NTs that can efficiently recognize all tRNAs regardless of amino acid acceptor specificity, viral RNAs, U2 small nuclear RNAs, synthetic tDNA, and DNA oligonucleotides (10, 26–29).

The inhibitory effect of E. coli tRNA on the tRNA-NT phosphohydrolase activities was suppressed in a concentration-dependent manner by the addition of Mg\(^{2+}\), Mn\(^{2+}\), or Ca\(^{2+}\) (Fig. 7; data shown for Mg\(^{2+}\)). The stimulatory effect of Mg\(^{2+}\) on ATP hydrolysis in the presence of tRNA was observed at a very narrow range of metal concentrations (0.1–1.0 mM), which changed to strong inhibition at Mg\(^{2+}\) concentrations >1 mM (Fig. 7A). The hydrolysis of 2'-AMP (in the presence of tRNA) was stimulated by Mg\(^{2+}\) up to 2.5 mM with significant inhibition (40%) observed at 10 mM Mg\(^{2+}\), whereas no inhibition by Mg\(^{2+}\) (up to 10 mM) was found for the hydrolysis of 2',3'-cAMP (Fig. 7, B and C). In these experiments, the affinity to Mg\(^{2+}\) was highest in the hydrolysis of ATP (apparent K\(_p\) = 0.1 mM), followed by 2'-AMP (K\(_p\) = 0.24 ± 0.1 mM) and 2',3'-cAMP (K\(_p\) = 0.49 ± 0.1 mM). With 2'-AMP as a substrate, it was found that simultaneous presence of Mg\(^{2+}\) and tRNA resulted in a 2-fold time increase in V\(_{max}\) and K\(_m\) (data not shown). In control experiments without tRNA, all three metals (Mg\(^{2+}\), Mn\(^{2+}\), Ca\(^{2+}\); 0.01–1 mM) produced small stimulating effects (10–40%) on both phosphatase and phosphodiesterase activi-
ties of tRNA-NT (data not shown). In conclusion, the three new phosphohydrolase activities of the E. coli tRNA-NT differ in their sensitivities to tRNA and Mg\(^{2+}\), suggesting that the corresponding active sites are different.

**Site-directed Mutagenesis of tRNA-NT**—Analysis of the available sequences of the CCA-adding enzymes (at least 84 entries in EMBL-EBI data base) revealed that at least 40 proteins contain the conserved HD motif located at the C terminus (His-255 and Asp-256 in the E. coli tRNA-NT). All HD motif-containing tRNA-NTs are found in eubacteria, mostly to the \(\alpha\)-subdivision of proteobacteria, and they comprise a highly homologous group (over 50% identity for proteins from 20 or-
SpoT homologues from the breakdown of (p)ppGpp by a Mn$^{2+}$/H$^{11032}$,3-tase, a 2G. Brown, C. H. Arrowsmith, A. M. Edwards, unpublished observations.

Gln-359 (numbers for the $327$, Arg-330, Arg-334, Asp-345, Arg-349, Tyr-357, Pro-358, Gln-359 (numbers for the E. coli tRNA-NT). Using mutagenizing primers, the residues comprising the conserved HD motif were replaced by Ala, creating the H255A and D256A mutant proteins. The proteins were overexpressed and purified using the same purification protocol as for the wild-type protein (see “Materials and Methods”). Both mutant proteins were folded but showed no phosphatase or phosphodiesterase activities indicating that His-255 and Asp-256 are required for both these activities. There is one more HD pair in the C-terminal domain of the E. coli tRNA-NT, His-305 and Asp-306, with His-305 being a conserved residue in the HD domain-containing tRNA-NT (Fig. 8). The H305A mutant also showed no phosphatase or phosphodiesterase activity (Table II), whereas the D306A strain was active (data not shown). Mutations at the conserved nucleotidyltransferase motif DxD (D21A and D23A) had no effect on either phosphatase or phosphodiesterase activities (Table II). These results indicate that the conserved HD motif (H255 and D256), as well as the conserved His-305, are required for both phosphatase and phosphodiesterase activities of the E. coli tRNA-NT and that they are critical for catalysis but not for substrate binding.

**DISCUSSION**

With use of general enzymatic screens and detailed biochemical analysis, we revealed the presence of at least three new activities in the E. coli tRNA-NT: a Ni$^{2+}$-dependent phosphatase, a $2^\prime$-nucleotidase, and a metal-independent $2^\prime,3^\prime$-cyclic phosphodiesterase. These activities have not been reported previously in tRNA nucleotidyltransferases. Site-directed mutagenesis showed that all these activities are associated with the conserved HD domain located at the C terminus of the E. coli tRNA-NT. Moreover, we found neither phosphodiesterase nor phosphatase activities in the purified CCA-adding enzyme from Methanobacterium thermoautotrophicum, which has no HD domain.$^2$ Thus, the new activities observed in the tRNA-NT from E. coli are associated with the conserved HD domain.

To date, only two HD domain-containing proteins have been characterized biochemically, the E. coli dGTPase and RelA/SpoT homologues from E. coli and Streptococcus equisimilis. The E. coli dGTPase (EC 3.1.5.1) catalyzes the Mg$^{2+}$-dependent hydrolysis of dGTP to deoxyguanosine and triphosphate (50, 31). The major role of the E. coli SpoT (EC 3.1.7.2) is the breakdown of (p)ppGpp by a Ms$^{2+}$-dependent (p)ppGpp pyrophosphohydrolase activity (32, 33). The E. coli RelA protein, which is a homolog of SpoT, contains substitutions in the HD domain and has no pyrophosphohydrolase activity (34).

S. equisimilis Rel/Spo enzyme, a bifunctional (p)ppGpp synthetase/hydrolase, catalyzes both synthesis and Mn$^{2+}$-dependent hydrolysis of (p)ppGpp (35). In our work, we identified the presence of $2^\prime,3^\prime$-cyclic phosphodiesterase, $2^\prime$-nucleotidase, and phosphatase activities associated with the HD domain in the E. coli tRNA-NT. Thus, the HD domain is a broad substrate range phosphohydrolase that can catalyze both metal-dependent and -independent phosphomonoesterase and phosphodiesterase reactions.

The HD domain-associated activities of the E. coli tRNA-NT showed different metal dependence and sensitivity to Mg$^{2+}$/tRNA, suggesting that although overlapping on the HD motif, these activities use various residues for catalysis. To date, there is only one three-dimensional structure of the HD-domain containing protein, the N-terminal fragment of the bifunctional Rel/Spo homologue from Streptococcus dysgalactiae (36). All residues of this protein involved in the coordination of Mn$^{2+}$ (His-53, His-77, Asp-78, and Asp-144) are conserved in the HD domain of the E. coli tRNA-NT (His-231, His-255, Asp-256, and Asp-267) suggesting that in the latter, they can be involved in metal coordination. The two available three-dimensional structures of $2^\prime,3^\prime$-cyclic phosphodiesterases (from Arabidopsis thaliana and rat) identified two histidine residues acting without metal to directly activate a water molecule for the nucleophilic attack of a phosphodiester bond (37, 38). These conserved histidines together with two conserved hydroxyamino acids (Ser or Thr) make a signature motif for the $2H$ subfamily of $2^\prime,3^\prime$-cyclic phosphodiesterases (39). Because this motif is not present in the E. coli tRNA-NT, this protein evidently uses a different mechanism for the hydrolysis of the $2^\prime,3^\prime$-cyclic phosphodiester bond.

The nucleotidyltransferase domain of the E. coli tRNA-NT binds various tRNAs and needs Mg$^{2+}$ for catalysis (16, 40). Two Asp residues located near the N terminus are involved in Mg$^{2+}$ coordination and are absolutely required for catalysis (4, 8). Strong competitive inhibition of mononucleotide hydrolysis by tRNA (Fig. 6) suggests that tRNA might be a natural substrate for the HD domain-associated activities. Mg$^{2+}$ itself had small effects on both phosphodiesterase and phosphatase activities, but its addition completely abolished the inhibiting effect of tRNA. Although the mechanism of the protection by Mg$^{2+}$ is unclear at this stage, it indicates that the binding of Mg$^{2+}$ to the nucleotidyltransferase domain can be conveyed in some manner to the HD domain.

The physical association of the HD domain with the E. coli tRNA-NT suggests that the phosphohydrolase activities might be involved in the repair of the tRNA 3′-end. In E. coli and many other bacteria in which tRNA genes encode the CCA sequence, the role of the CCA-adding enzyme is to maintain and repair the tRNA 3′-CCA terminus degraded by intracellular RNases (41, 42). Evidence indicates that bacterial and mammalian ribonucleases produce RNAs containing $2^\prime,3^\prime$-cyclic phosphodiesterases at the 3′ end as true products of the reaction (43–47). The 3′-terminal $2^\prime,3^\prime$-cyclic phosphate in the E. coli tRNAs can also be produced by the activity of 3′-terminal phosphate cyclase, which catalyzes the ATP-dependent con-
version of the 3'-terminal phosphate of tRNA to a 2',3'-cyclic phosphodiester (23). Thus, on the basis of available information and our biochemical data, we can propose the following model for the role of the HD domain in the E. coli tRNA-Nt (Fig. 9). Our model is based on the assumption that the degradation of tRNAs by intracellular RNases produces tRNA molecules with 2',3'-cyclic phosphate at the 3'-end. In the repair process, the phosphodiesterase activity of the HD domain hydrolyzes the cyclic phosphodiester to 2'-monophosphate, which can then be removed by the HD domain 2'-nucleotidase activity (Fig. 9). These activities will eventually produce the unphosphorylated 3'-end of tRNA suitable for the nucleotidyltransferase reaction.

It is interesting that in at least seven archaeal genomes (Sulfolobus, Aeropyrum, Archaeoglobus, Pyrococcus, Methanobacterium, Methanopyrus), the tRNA-NT gene co-occurs in the same operon with a predicted 2',3'-cyclic phosphodiesterase (39). This strong operonic association suggests that in archaea, these predicted phosphodiesterases can also be involved in the process of CCA addition to tRNA, similar to the HD domain in the E. coli tRNA-Nt.

The nucleotidyltransferase reaction is accompanied by the release of inorganic pyrophosphate. The equilibrium of this reaction is shifted in the direction of polymerization if pyrophosphate is removed. Pyrophosphate hydrolysis coupled with polymerization is thought to be catalyzed by a phosphatase that has not yet been definitely identified in any system and was predicted to be performed by the HD domain in the E. coli tRNA-Nt (18). Our observation of high pyrophosphatase activity of the HD domain associated activities of the E. coli tRNA-Nt discovered by general enzymatic screens.

Thus, the E. coli tRNA-Nt is a multifunctional enzyme with 2',3'-cyclic phosphodiesterase, 2'-nucleotidase, phosphatase, and nucleotidyltransferase activities that we suggest act in concert to repair the 3' end of tRNA. Further structural and genetic studies are required to prove this model for the HD-domain associated activities of the E. coli tRNA-Nt discovered by general enzymatic screens.

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Page 36821: An incorrect version of Fig. 3 was inadvertently printed. The correct figure is shown below:

![Graphs showing enzymatic activities](image)

**Fig. 3**

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