PROPERTIES OF THE 5'-TERMINUS OF $tRNA^{His}$: KINETICS OF POLYNUCLEOTIDE KINASE CATALYZED EXCHANGE AND EFFECT OF DEPHOSPHORYLATION ON THE AMINOACYLATION REACTION

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SUMMARY: Bacteriophage T_4 induced polynucleotide kinase was found to be ineffective in transferring ^{32}P from [γ-^{32}P]ATP to the 5'-terminus of 5'-phosphorylated E. coli $tRNA^{His}$ using the ADP mediated exchange reaction. However, prior dephosphorylation with alkaline phosphatase allowed polynucleotide kinase catalyzed phosphorylation of $tRNA^{His}$. Contrary to reports for other tRNA species, alkaline phosphatase catalyzed 5'-terminus dephosphorylation destroys the amino acid accepting ability of $tRNA^{His}$. Aminoacylation competency of the $tRNA^{His}$ is restored after phosphorylation with polynucleotide kinase.

Mature histidyl-tRNA is a negative effector acting in regulation of histidine operon expression in Salmonella typhimurium (1). Histidyl-tRNA also has been shown to bind tightly to the first enzyme of histidine biosynthesis, ATP phosphoribosyltransferase [EC 2.4.2.17], to form a complex that might be involved in histidine operon regulation (2,3). E. coli and the identical S. typhimurium $tRNA^{His}$ is unique among sequenced tRNA species (4) in that the CCA stem contains an eighth base pair arising from an additional 5'-base leaving only the CCA nucleotide residues at the 3'-hydroxyl end unpaired (5,6). We have found that the 5'-terminus of $tRNA^{His}$ displays some unusual properties that may be related to this unique CCA stem structure.

Phage T_4 induced polynucleotide kinase catalyzes the transfer of the γ-phosphate from ATP to the 5'-hydroxyl terminus of polynucleotides and oligonucleotides (7). With deoxyoligonucleotides, the enzyme can catalyze the reverse reaction to act as an ADP dependent phosphomonoesterase (8). Chaconas, et al. (9) used polynucleotide kinase to catalyze an exchange reaction with [γ-^{32}P]ATP to ^{32}P-label the 5'-terminus of E. coli $tRNA^{Val}$ and crude yeast tRNA.

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We report here that E. coli $tRNA^{His}$ will not undergo the exchange reaction.

The effect of 5′-terminus dephosphorylation on the amino acid accepting ability has been examined for a number of tRNA species. Bulk E. coli tRNA has been tested for its ability to accept a mixture of [^{14}C]amino acids and to accept [^{14}C]phenylalanine after dephosphorylation (10). No decrease in the accepting ability was observed indicating that the 5′-terminal phosphate is not essential for aminoacylation of most tRNA species. Yeast $tRNA^{Ser}$ has been shown to retain its amino acid accepting ability after dephosphorylation (11), and it has been reported that dephosphorylated E. coli $tRNA_f^{Met}$, $tRNA_m^{Met}$, and $tRNA^{Phe}$ accept the same amount of amino acids as the phosphorylated tRNA species (12). We report here that dephosphorylated $tRNA^{His}$ is unable to accept histidine.

MATERIALS AND METHODS

Nuclease-free polynucleotide kinase from T_4 XF-1 infected E. coli B [EC 2.7.1.78] was purchased from PL Biochemicals. A unit of kinase transfers 1 nmole of phosphate from ATP to yeast RNA (PL Biochemicals, catalog number 3700) in 30 min at 37°. E. coli alkaline phosphatase [EC 3.1.3.1] was purchased from Worthington (Grade BAPF) and further purified on DEAE-agarose (13). A unit liberates 1 μmole of p-nitrophenol from p-nitrophenylphosphate per min at 25° in 1.0 M Tris·Cl, pH 8.0. Pure histidyl-tRNA synthetase [EC 6.1.1.21] was a generous gift of Dr. Bruce Ames (14).

The $tRNA^{His}$ was purified from bulk E. coli B tRNA (Plenum) to a histidine accepting ability of 1200-1600 pmoles per A_{260} unit (S. M. Brooks, L. Meyers and S. M. Parsons, unpublished results). An A_{260} unit is that amount of material that, when dissolved in 1 ml of solvent, gives an absorbance of 1 with a 1 cm pathlength. The [γ-^{32}P]ATP was prepared as described (15).

To test for amino acid accepting capacity, $tRNA^{His}$ was incubated at 37° with 200 nmoles of ATP, 2 nmoles of L-[2,5-^{3}H]histidine (4800 dpm/pmole, Amersham/Searle), and 1.7 units of histidyl-tRNA synthetase in 40 μl of 0.1 M sodium cacodylate, 8 mM $MgCl_2$, 0.25 M dithiothreitol, pH 7.5 buffer. Aliquots (15 μl) were periodically spotted onto 2.1 cm GF/C filters (Whatman), and each filter was placed immediately into ice-cold 10% trichloroacetic acid and washed as described (16). After drying, the filters were placed in a toluene scintillation cocktail for radioactivity determination. For each reaction, a blank prepared without tRNA was carried through the same procedure. To test for ^{32}P incorporation into tRNA, an aliquot of the reaction mixture was spotted onto a GF/C filter that was processed as described above for the aminoacylation assay.

RESULTS

Polynucleotide kinase reaction with $tRNA^{His}$. A preparation of $tRNA^{His}$,

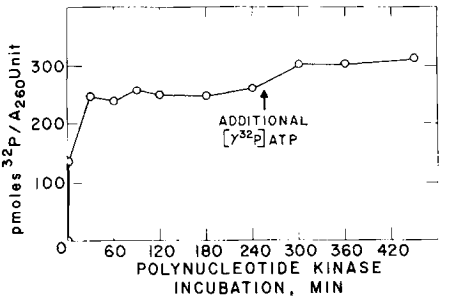

Figure 1.
Polynucleotide kinase catalyzed exchange reaction. An aliquot of $tRNA^{His}$ (0.84 A_{260} unit) was incubated with 3.3 nmoles of [γ-^{32}P]ATP (3,000 cpm/pmole), 1.5 nmoles of ADP, and 21 units of polynucleotide kinase at 37° in 100 µl of 50 mM Tris·Cl, 5 mM $MgCl_2$, 10 mM dithiothreitol, pH 7.6 buffer containing 0.25 mg/ml bovine serum albumin. At various time intervals, 3 µl aliquots were removed and tested for trichloroacetic acid precipitable radioactivity. After 255 min, 2.5 nmoles of [γ-^{32}P]ATP (3 µl) was added to the remaining reaction mixture (79 µl). The incorporation of ^{32}P is calculated assuming that the exchange reaction freely occured so that the original [γ-^{32}P]ATP specific activity was diluted by the tRNA 5′-terminal phosphate. If the exchange did not occur the actual incorporation of ^{32}P would be less than indicated.

which was estimated to contain approximately 80% $tRNA^{His}$, was incubated with [γ-^{32}P]ATP, ADP, and polynucleotide kinase. Figure 1 shows that the incorporation of ^{32}P radioactivity was rapid and that a plateau was reached that corresponded to an apparent labeling of approximately 20% of the tRNA. Addition of more [γ-^{32}P]ATP did not increase the extent of labeling significantly. A similar experiment using a 10 fold molar excess of [γ-^{32}P]ATP and ADP and using 95% pure $tRNA^{His}$ resulted in labeling of 7% of the total tRNA. The ^{32}P-labeled material was ethanol precipitable and chromatographed on a Sephadex G-100 column as expected for tRNA (not shown).

The [^{3}H]histidine accepting ability of a preparation of $tRNA^{His}$ that had an apparent incorporation of ^{32}P into 6% of the tRNA was not affected by the kinase treatment, showing that the $tRNA^{His}$ had not been degraded by nuclease. This kinase-reacted [^{3}H]histidyl-tRNA was chromatographed on an RPC-5 column (17), and the effluent was monitored for tritium and ^{32}P radioactivity. From the elution profile shown in Figure 2, it is apparent that most of the ^{32}P did

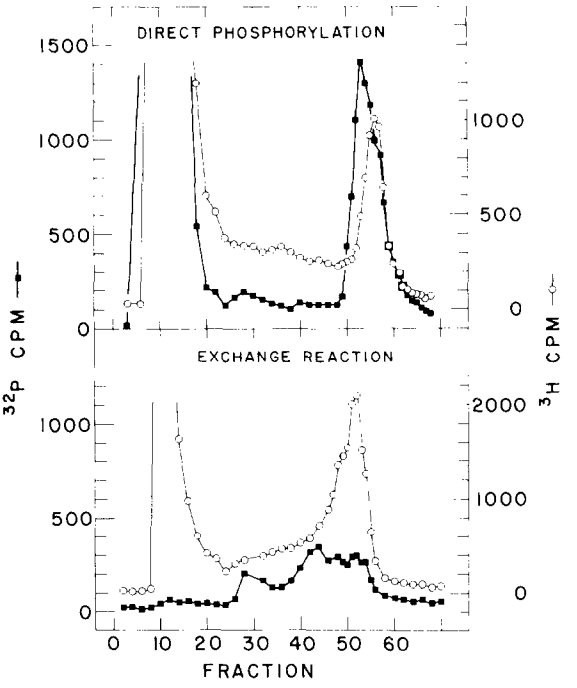

Figure 2.
RPC-5 column of polynucleotide kinase-reacted $tRNA^{His}$ after aminoacylation. Exchange reaction. An aliquot of $tRNA^{His}$ (3 A_{260} units) was reacted similarly to that of Figure 1 with 12 nmoles [γ-^{32}P]ATP, 10 nmoles ADP, and 6 units of polynucleotide kinase in 250 µl. After a 60 min incubation, the mixture was applied to a 0.9 x 5 cm DEAE-agarose column at 4° equilibrated with the kinase incubation buffer 0.1 M in NaCl. The column was eluted with this buffer until the bulk of the [γ-^{32}P]ATP had eluted. The tRNA then was eluted with the same buffer containing 1.0 M NaCl. The tRNA containing fractions were pooled and the tRNA precipitated with two volumes of ethanol. The $tRNA^{His}$ was aminoacylated with [^{3}H]histidine. The reaction mixture was adjusted to pH 4.5 with acetic acid and applied to an RPC-5 column (0.9 x 55 cm) equilibrated with 10 mM sodium acetate, 10 mM magnesium acetate, 2 mM sodium thiosulfate, 0.4 M NaCl, pH 4.5 buffer. The column was eluted with a linear gradient of 0.65 to 0.8 M NaCl (200 ml each) and 2.1 ml fractions were collected and the radioactivity determined. Fractions 49-56 were pooled and the tRNA precipitated by addition of two volumes of ethanol. The precipitate was washed with 1 ml of a solution of 10 mM sodium acetate, 10 mM $MgCl_2$, 1 M NaCl, pH 4.5 buffer mixed with 2 volumes of 95% ethanol. The precipitant was dried and then dissolved in 1 ml of the above buffer without ethanol and the A_{260} and radioactivity were determined. Direct phosphorylation. A preparation of tRNA (0.60 A_{260} unit) was dephosphorylated with alkaline phosphatase before reaction with [γ-^{32}P]ATP and polynucleotide kinase as described in Figure 3. The ^{32}P-labeled tRNA was aminoacylated without separation from the kinase reaction, and chromatographed on the RPC-5 column as described above. Fractions 51-62 were pooled, and the tRNA was precipitated, washed, redissolved, and analyzed for ^{32}P and A_{260} as described above.

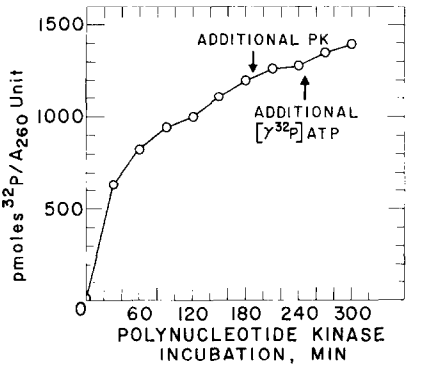

Figure 3.
Polynucleotide kinase catalyzed phosphorylation of dephosphorylated tRNA. An aliquot of $tRNA^{His}$ (0.82 A_{260} unit) was dephosphorylated by incubation with 1.3 units of alkaline phosphatase for 2.5 hr at 37° in 360 µl of 50 mM Tris·Cl, 8 mM $MgCl_2$, pH 8.0 buffer. The mixture was applied to a 0.9 x 5 cm DEAE-agarose column at 4° equilibrated with 10 mM Tris·Cl, 0.1 M NaCl, pH 7.5 buffer. After all of the alkaline phosphatase activity had eluted the column was washed with 10 mM sodium acetate, 10 mM $MgCl_2$, 1.0 M NaCl, pH 4.5 buffer. Fractions containing tRNA were pooled and precipitated with 2 volumes of 95% ethanol. For the labeling reaction, 0.60 A_{260} unit of this dephosphorylated tRNA was incubated with 17 nmoles of [γ-^{32}P]ATP (455 dpm/pmole) and 21 units of polynucleotide kinase in 220 µl of 50 mM Tris·Cl, 5 mM $MgCl_2$, 10 mM dithiothreitol, pH 8.0 buffer. At the indicated time intervals, 5 µl aliquots were tested for trichloroacetic acid precipitable radioactivity. At the points shown, 7 units (2 µl) of polynucleotide kinase or 14 nmoles (40 µl) of [γ-^{32}P]ATP were added.

not co-chromatograph with [^{3}H]histidyl-tRNA. After RPC-5 chromatography the apparent ^{32}P incorporation into $tRNA^{His}$ was less than 1%. Thus material other than $tRNA^{His}$ was selectively labeled with the exchange reaction, and $tRNA^{His}$ was labeled to only a very small extent or not at all.

A preparation of $tRNA^{His}$ was incubated with alkaline phosphatase to remove the 5′-terminal phosphate (10), and the enzyme was removed by chromatography. The results of incubation of this dephosphorylated $tRNA^{His}$ with [γ-^{32}P]ATP and the kinase are shown in Figure 3. The maximum incorporation of ^{32}P represented labeling of approximately 90% of the tRNA and a plateau had not yet been reached.

A preparation of $tRNA^{His}$ that had been ^{32}P labeled by this direct phosphorylation procedure was aminoacylated with [^{3}H]histidine and chromatographed on an RPC-5 column. As shown in Figure 2, the majority of the ^{32}P chromatographed

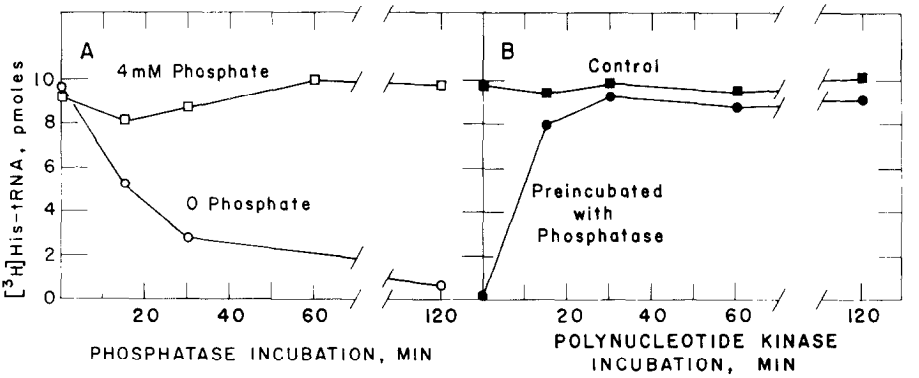

Figure 4.
The effect of the 5′-terminal phosphate on $tRNA^{His}$ amino acid accepting ability. A. Alkaline phosphatase inactivation. An aliquot of $tRNA^{His}$ (0.14 A_{260} unit) was incubated at 37° with 0.21 unit of alkaline phosphatase in 110 µl of 50 mM Tris·Cl, 8 mM $MgCl_2$, pH 8.0 buffer. One reaction also contained 4 mM sodium phosphate. At the indicated time, 20 µl aliquots were tested for [^{3}H]histidine accepting ability. The data points shown are the average of two determinations. B. Polynucleotide kinase reactivation. An aliquot of $tRNA^{His}$ (0.14 A_{260} unit) was dephosphorylated as described above in a total volume of 60 µl. A control was incubated in the same manner in the absence of alkaline phosphatase. After 120 min, 0.5 µmole of ATP and 10 units of polynucleotide kinase were added to each incubation mixture, and the buffer was made 50 mM Tris·Cl, 5.5 mM $MgCl_2$, 4 mM phosphate, pH 7.6 in a final volume of 110 µl. At the times shown, 20 µl aliquots were removed and tested for [^{3}H]histidine accepting ability except that the aminoacylation assay was made 4 mM in phosphate. The data points shown are the average of two determinations.

closely with [^{3}H]histidyl-tRNA. The lack of coincidence of the peaks is caused by deacylated tRNA migrating ahead of histidyl-tRNA on RPC-5. Deacylated $tRNA^{His}$ is present due to incomplete aminoacylation in the synthetase reaction and hydrolysis of [^{3}H]histidyl-tRNA during chromatography. After RPC-5 chromatography, the $tRNA^{His}$ had retained 1300 pmoles of ^{32}P per A_{260} unit. Hence, the direct polynucleotide kinase catalyzed phosphorylation of the 5′-terminus of $tRNA^{His}$ proceeds nearly quantitatively.

Effect of 5′-terminus dephosphorylation on the histidine accepting ability of $tRNA^{His}$. We found that alkaline phosphatase, even after purification reported to remove nuclease contaminants (13), rapidly destroyed the histidine accepting ability of $tRNA^{His}$ as shown in Figure 4A. However, if phosphate, an inhibitor of alkaline phosphatase (18), was included in the phosphatase

incubation, $tRNA^{His}$ was unaltered in histidine accepting ability (Figure 4A).

A preparation of $tRNA^{His}$ that had lost its ability to accept histidine after incubation with alkaline phosphatase could be reactivated completely by phosphorylation with polynucleotide kinase and ATP as shown in Figure 4B. Consequently, we conclude that the loss in accepting ability upon incubation with alkaline phosphatase is due to 5′-terminus dephosphorylation. We also tested E. coli bulk tRNA for an increase in histidine acceptance upon treatment with polynucleotide kinase. There was no apparent increase indicating that the $tRNA^{His}$ from E. coli exists chiefly in the 5′-phosphorylated state.

DISCUSSION

Whether the inability of $tRNA^{His}$ to participate in the polynucleotide kinase catalyzed exchange reaction is unique or is characteristic of other tRNA species as well is not clear. The conditions we used were similar to those reported to successfully label E. coli $tRNA^{Val}$ and yeast bulk tRNA (9). An equilibrium constant of about 50 favoring phosphorylated deoxyoligonucleotide has been reported (8). Consequently, with the exchange conditions used here the maximal labeling observed should have represented fully phosphorylated tRNA if the exchange reaction had occured. However, recent reports on the phosphorylation of double-stranded DNA (19) and $tRNA^{Phe}$ (20) with the kinase indicate that a true thermodynamic equilibrium often is not established. Thus, quantitation of the extent of ^{32}P-labeling by the kinase exchange reaction might be complicated by mechanistic features of this enzyme, such as formation of a dead-end complex (20), that currently are not understood.

The finding that the 5′-terminal phosphate of $tRNA^{His}$ is critical for its aminoacylation appears to be unique. However, the 5′-terminal phosphate of other tRNA species has been shown to be important in other functions. Dephosphorylation of Phe-tRNA and of $Met\text{-}tRNA_m$ severely reduces the ability of these tRNAs to complex with elongation factor Tu (12). Dephosphorylation also has been found to reduce the rate of hydrolysis of N-acetylphenylalanyl-tRNA by

peptidyl-tRNA hydrolase (21). Thus, the 5′-terminal phosphate is often important.

In addition to $tRNA^{His}$, E. coli $tRNA_f^{Met}$ has a unique CCA stem with the normally paired 5′-terminal base unpaired (4). This unpaired base is important in preventing binding of methionyl-$tRNA_f^{Met}$ to elongation factor Tu (12) and in resistance of N-formylmethionyl-$tRNA_f^{Met}$ to peptidyl-tRNA hydrolase (21). The importance of this unusual CCA stem in $tRNA_f^{Met}$ suggests that the unusual CCA stem in $tRNA^{His}$, containing eight base pairs, might likewise have a special function.

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