# Interaction of cinnamyl-tRNA$^{Phe}$ with *Escherichia coli* elongation factor Tu

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The products of nitrous acid mediated-deamination of Phe-tRNA$^{Phe}$ from *E. coli* were analyzed and their capability to interact with elongation factor Tu from *E. coli* was investigated. Thin-layer chromatography as well as HPLC analysis revealed the existence of at least two deamination products, 3-phenyllactyl-tRNA$^{Phe}$ and cinnamyl-tRNA$^{Phe}$. It could be shown that the aminoacyl-tRNA analogues were active in the formation of the ternary complex with EF-Tu·GTP, although with a lower efficiency than native Phe-tRNA$^{Phe}$. For both modified acyl-tRNAs the dissociation constant was determined to be $3 \times 10^{-5}$ M.

*Phenylalanyl-tRNA* *Phenyllactyl-tRNA* *Cinnamyl-tRNA*
*Elongation factor Tu* *Nucleic acid–protein interaction*

## 1. INTRODUCTION

The treatment of esters of phenylalanine with nitrous acid leads to the formation of several reaction products. The most prominent ones are 3-phenyllactic-, 3-hydroxy-3-phenylpropionic- and tropic acid esters, $C_6H_5$–$CH_2$–CH(OH)–COOR, $C_6H_5$–CH(OH)–$CH_2$–COOR and $C_6H_5$–CH($CH_2$OH)–COOR, respectively [1]. Depending on the reaction conditions the ester of cinnamic acid, $C_6H_5$–CH=CH–COOR, is also formed in substantial amount [1]. Analogous reaction products are expected to originate from phenylalanyl-tRNA$^{Phe}$ by treatment with nitrous acid. In the past such deaminated phenylalanyl-tRNA was utilized for the investigation of the substrate requirements in in vitro protein synthesizing systems [2–4]. Hervé and Chapeville reported the incorporation of 3-phenyllactic acid into the N-terminal position of the growing peptide chain [2]. Fahnestock and Rich could later demonstrate that 3-phenyllactic acid from Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ can also be incorporated into an internal position of the polypeptide chain via an ester linkage [5]. Whereas Fahnestock et al. showed that Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ from *E. coli* interacts with the *E. coli* elongation factor Tu·GTP complex [3], such an interaction was not detected with eukaryotic elongation factor 1 [4]. In all above cited communications dealing with the activity of nitrous acid-treated phenylalanyl-tRNA$^{Phe}$ no attention was paid to the fact that depending on the reaction conditions this substrate consists of several components [1].

Here, we performed a more careful analysis of the products of nitrous acid-treated Phe-tRNA$^{Phe}$ from *E. coli* and investigated the ability of the acyl-tRNAs formed to participate in ternary complex formation with *E. coli* elongation factor Tu and GTP. We can demonstrate that at least one of the hydroxyacyl-tRNA$^{Phe}$ derivatives, the 3-phenyllactyl-tRNA$^{Phe}$, forms the ternary complex with EF-Tu·GTP. In addition cinnamyl-tRNA$^{Phe}$

*Abbreviations:* HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-ethane sulfonic acid; EF-Tu, elongation factor Tu from *E. coli*; tRNA$^{Phe}$, phenylalanine-specific tRNA from *E. coli*; tRNA$^{Phe}_{HONO}$, nitrous acid-treated tRNA$^{Phe}$; Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$, nitrous acid-treated Phe-tRNA$^{Phe}$; HPLC, high performance liquid chromatography

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which occurs in the reaction mixture after nitrous acid treatment of Phe-tRNA$^{Phe}$, in ~40% yield also binds to EF-Tu·GTP. The dissociation constant of this interaction has a value of $3 \times 10^{-5}$ M for both modified acyl-tRNA species.

## 2. MATERIALS AND METHODS

tRNA$^{Phe}$ from *E. coli*, RNase A from beef pancreas (EC 3.1.27.5) and pyruvate kinase from rabbit muscle (EC 2.7.1.40) were products of Boehringer (Mannheim). EF-Tu·GDP was prepared essentially according to [6] and had spec. act. 22000 units/mg protein. One unit of EF-Tu is the capacity of protein to bind 1 pmol [$^3$H]GDP. Phenylalanyl-tRNA synthetase from *E. coli* was a gift from Dr E. Holler (Regensburg). [$^3$H]Phenylalanine and [$^{14}$C]phenylalanine were from Amersham Buchler (Braunschweig). Cinnamic acid, L-3-phenyllactic acid and proteinase K were obtained from Serva (Heidelberg).

Phenylalanine specific tRNA from *E. coli* was enzymatically aminoacylated with *E. coli* phenylalanyl-tRNA synthetase and isolated after phenol extraction by gel filtration as in [7]. The treatment of [$^{14}$C]Phe-tRNA$^{Phe}$ with nitrous acid was performed according to [3]: The reaction mixture (0.3 ml) contained 10 $A_{260}$ units Phe-tRNA$^{Phe}$, 0.25 M sodium acetate (pH 4.3), 2 M sodium nitrite and 0.01 M magnesium acetate. After incubation, 30 min at 25°C, the Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ was desalted on a Biogel P2 column using water as eluant and concentrated by evaporation under reduced pressure. In an analogous way tRNA$^{Phe}_{HONO}$ was prepared and aminoacylated to Phe-tRNA$^{Phe}_{HONO}$.

Chromatographic analysis of the acyl residues attached to the tRNA was performed as follows: The [$^{14}$C]acyl-tRNA$^{Phe}$ (2 $A_{260}$ units) was incubated in 0.1 ml 1 M triethylamine for 45 min at 37°C. Aliquots of the hydrolysate were chromatographed on silicagel plates (Macherey und Nagel, Düren) using a solvent containing *n*-butanol : acetic acid : water (78 : 5 : 17, by vol.). Authentic samples of L-3-phenyllactic acid and cinnamic acid were used as standards. The distribution of the radioactivity on the plates was determined with a Berthold thin-layer chromatography scanner.

High-performance liquid chromatography was carried out on the Du Pont LC 850 system, equipped with a Du Pont Zorbax ODS column (0.46 × 25 cm). Elution was performed with 0.01 M potassium phosphate buffer (pH 6.0) containing 30% methanol. The absorption of the eluant was monitored at 254 nm. For the analysis of the acyl residues from [$^{14}$C]acyl-tRNA$^{Phe}$ hydrolysates, the $^{14}$C-radioactivity in adequate fractions of the eluant was determined by scintillation counting. Samples of authentic cinnamic acid (60 $\mu$l of 0.1% cinnamic acid in 10% aqueous methanol) were treated with: (a) 40 $\mu$l 1% aqueous potassium permanganate; or (b) 40 $\mu$l saturated aqueous bromine solution. These reagents lead to fast formation of: (a) 2,3-dihydroxy-3-phenylpropionic acid [8]; or (b) 2,3-dibromo-3-phenylpropionic acid [9]. The products of both reactions were analyzed on HPLC and the retention times were compared with the retention times of the products resulting from identically treated [$^{14}$C]acyl-tRNA$^{Phe}$ hydrolysates.

The acyl-tRNA·EF-Tu·GTP ternary complex formation was monitored by gel filtration on Ultrogel AcA 54 columns [7] or by RNase protection assay [10]. The conversion of EF-Tu·GDP to EF-Tu·GTP was performed as in [7].

Isolation of the ternary complex Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$·EF-Tu·GTP on Ultrogel AcA 54 column: In 200 $\mu$l buffer containing 150 mM Tris–HCl (pH 7.6), 150 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 29.6 $\mu$M EF-Tu·GTP and 12.2 $\mu$M [$^{14}$C]Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ (spec. act. 71 Ci/mol) ternary complex was formed by incubation for 3 min at 37°C. The mixture was applied onto the Ultrogel AcA 54 column and gel filtration performed as in fig.2. [$^{14}$C]Acyl residues referred to as Phe$_{HONO}$ originating from the [$^{14}$C]Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$·EF-Tu·GTP ternary complex were analyzed by thin-layer chromatography as follows: Ternary complex-containing fractions (see fig.2) were pooled and the protein digested by incubation with 2.5 $\mu$g proteinase K/ml in the presence of 3% SDS for 1.5 h at 37°C. The Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ remained unaffected by this treatment and was isolated by gel filtration on a Biogel P2 column. Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$-containing fractions were evaporated to dryness, the pellet dissolved in 7 $\mu$l 1 M aqueous triethylamine and incubated at 37°C for 45 min. Aliquots of the hydrolysate were spotted on silicagel thin-layer plates and chromatographed as above.

## 3. RESULTS AND DISCUSSION

Phenylalanine specific tRNA from *E. coli* was deaminated by nitrous acid treatment either in its phenylalanylated or not phenylalanylated form. Since this tRNA contains the modified nucleoside, 3-(3-amino-3-carboxypropyl)uridine, $acp^3U$, in position 47 of its sequence [11], there are two potential sites for the reaction with nitrous acid: The primary amino group of the modified base $acp^3U$ and the primary $\alpha$-amino group of phenylalanine attached to the 3'-end of the tRNA. In line with the published data, the modification of the side chain of the $acp^3U$ 47 in $tRNA^{Phe}$ from *E. coli* does not affect the enzymatic aminoacylation reaction [12]. Therefore in addition to $Phe_{HONO}$-$tRNA^{Phe}_{HONO}$ obtained by nitrous acid treatment of Phe-$tRNA^{Phe}$, also Phe-$tRNA^{Phe}_{HONO}$ could be prepared by enzymatic aminoacylation of $tRNA^{Phe}_{HONO}$.

By high-performance liquid chromatography of the alkaline hydrolysates of [$^{14}$C]$Phe_{HONO}$-$tRNA^{Phe}_{HONO}$ we could demonstrate that ~40% of the radioactivity was associated with the compound having identical retention time as an authentic cinnamic acid sample. In addition, this compound isolated after alkaline hydrolysis from [$^{14}$C]$Phe_{HONO}$-$tRNA^{Phe}_{HONO}$ reacted with aqueous bromine or potassium permanganate solutions and, as judged from HPLC analysis it provided the same products as an identically treated cinnamic acid standard. The possibility that the cinnamic acid was formed not during the nitrous acid treatment of Phe-$tRNA^{Phe}$ but during the alkaline hydrolysis of $Phe_{HONO}$-$tRNA^{Phe}_{HONO}$ or the work-up procedure, could be excluded by appropriate control experiments. We therefore conclude that the major products of the nitrous acid-mediated deamination of Phe-$tRNA^{Phe}$ are the 3-phenyllactic-$tRNA^{Phe}$ or its isomers (60%) and cinnamyl-$tRNA^{Phe}$ (40%). In this work we did not attempt to perform the identification of other hydroxylated compounds as 3-hydroxy-3-phenylpropionic acid or tropic acid, which also could be formed by nitrous acid treatment of Phe-$tRNA^{Phe}$ [1].

Since we were not able to separate the different acylated tRNAs in the $Phe_{HONO}$-$tRNA^{Phe}_{HONO}$ preparation by chromatographic procedures, the investigation of their activity in respect to acyl-tRNA·EF-Tu·GTP ternary complex formation

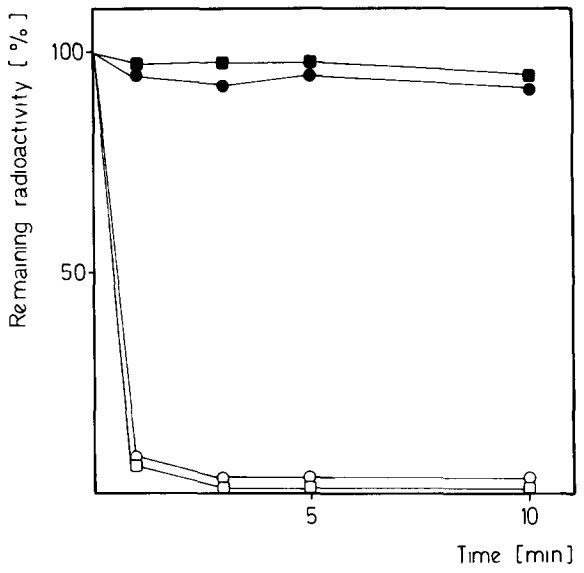


Fig.1. Protection of aminoacyl-$tRNA^{Phe}$ species against RNase digestion by EF-Tu·GTP. The ternary complex was formed in a 100 $\mu$l mixture of [$^{14}$C]Phe-$tRNA^{Phe}_{HONO}$ (spec. act. 57 Ci/mol), 2.1 $\mu$M, [$^3$H]Phe-$tRNA^{Phe}$ (spec. act. 930 Ci/mol), 1.8 $\mu$M, and EF-Tu·GDP 11.8 $\mu$M, in a buffer containing 50 mM Tris–HCl (pH 7.6), 50 mM $NH_4Cl$, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 $\mu$M GTP, 5 mM phosphoenolpyruvate and 20 $\mu$g pyruvate kinase. After 10 min at 0°C the mixture was treated with 0.025 $\mu$g RNAase for time indicated. The $^3$H- (●) and $^{14}$C- (■) radioactivities precipitable in 5% aqueous trichloroacetic acid from 18 $\mu$l aliquots were determined. The control experiment without EF-Tu·GTP is indicated by open symbols.

was performed directly with their mixture. As expected, the deamination of the $acp^3U$ 47 does not affect the ability of Phe-$tRNA^{Phe}$ to form ternary complexes. In the experiment shown in fig.1 a full protection of both the native Phe-$tRNA^{Phe}$ as well as the Phe-$tRNA^{Phe}_{HONO}$ against RNase digestion in the presence of EF-Tu·GTP was observed. In the absence of EF-Tu·GTP both phenylalanylated tRNAs were degraded rapidly. By variation of the aminoacyl-tRNA concentration in experiments like that shown in fig.1 an apparent dissociation constant for the respective phenylalanyl-tRNA could be determined [10]. Both species, Phe-$tRNA^{Phe}_{HONO}$ and Phe-$tRNA^{Phe}$, possess an identical $K_d^{app}$ of $1 \times 10^{-7}$ M.

Gel filtration experiments as well as an assay using the protection of aminoacyl-tRNA against nuclease digestion by EF-Tu·GTP were employed to monitor the interaction of nitrous acid treated Phe-tRNA$^{Phe}$ with *E. coli* EF-Tu·GTP. In both cases double-labelling was used for direct comparison of two acyl-tRNAs species in one experiment. A typical gel filtration shown in fig.2, demonstrates the preferential formation of the ternary complex (appearing as the first peak) with the [$^{14}$C]Phe-tRNA$^{Phe}_{HONO}$ if almost equimolar amounts of EF-Tu·GTP, [$^{14}$C]Phe-tRNA$^{Phe}_{HONO}$ and [$^{3}$H]Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ were present in the reaction mixture. Nevertheless, this experiment demonstrates that also [$^{3}$H]Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ forms a ternary complex, although with a lower efficiency than Phe-tRNA$^{Phe}_{HONO}$ directly reflecting the contribution of the $\alpha$-aminogroup of phenylalanine to this interaction. Using the nuclease protection assay we determined the dissociation constant for the interaction of Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ with EF-Tu·GTP to be $3 \times 10^{-5}$ M.

In order to show, which components from the mixture of [$^{14}$C]Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ form ternary complexes with EF-Tu·GTP, we isolated a substantial amount of this complex by gel filtration chromatography. The ternary complex eluted in the early fractions of the column was subjected to alkaline hydrolysis as in section 2 and the mixture of the acyl residues formed was analyzed by thin layer chromatography. The result shown in fig.3 revealed that Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ appearing in the ternary complex was acylated to 53% with 3-phenyllactic acid, to 44% with cinnamic acid and to 3% with phenylalanine (fig.3a). Phe-tRNA$^{Phe}$ eluting with the ternary complex from the AcA-54 column was not detected in the analysis of a hydrolysate derived from Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ starting material (fig.3b). Obviously an undetectable

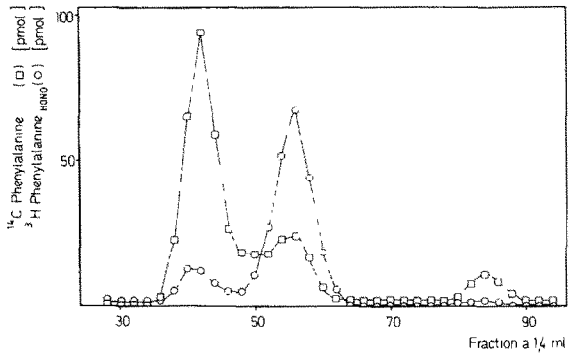


Fig.2. Gel filtration of the acyl-tRNA·EF-Tu·GTP ternary complexes on Ultrogel AcA 54 column. A 200 $\mu$l mixture containing 4.3 $\mu$M [$^{14}$C]Phe-tRNA$^{Phe}_{HONO}$ (spec. act. 57 Ci/mol), 4.5 $\mu$M [$^{3}$H]Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ (spec. act. 930 Ci/mol), and 5.9 $\mu$M EF-Tu·GTP was applied onto the AcA 54 column (70 × 2 cm) which was developed by a buffer containing 50 mM HEPES (pH 7.0), 50 mM $NH_4Cl$, 50 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol and 2% glycerol. The $^{3}$H- and $^{14}$C-radioactivity in 0.3 ml-aliquots of the fractions was determined. The ternary complex was eluted in the first peak, the second and third peak correspond to not complexed acyl-tRNA$^{Phe}$ and free acids, respectively.

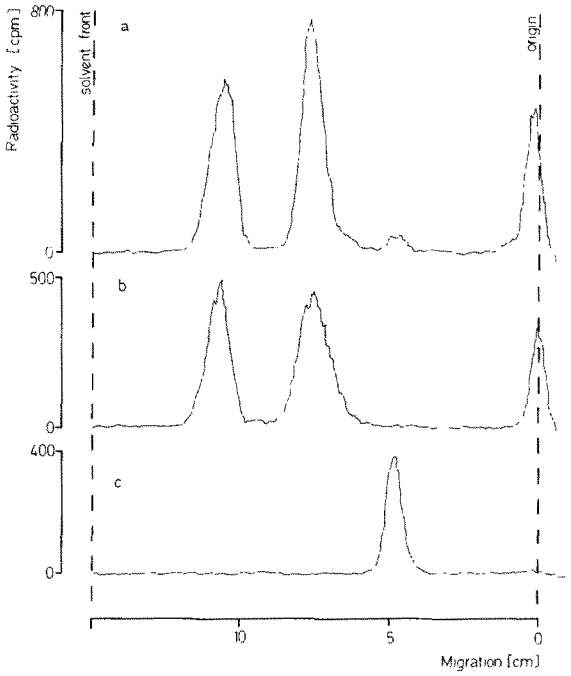


Fig.3. Analysis of the tRNA-bound acyl residues resulting from nitrous acid treatment of Phe-tRNA$^{Phe}$. The preparation of the samples and the chromatography procedure are described in section 2: (a) 7 $\mu$l of a sample containing [$^{14}$C]acyl residues isolated by gel filtration of the ternary complex Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$·EF-Tu·GTP; (b) 190 pmol [$^{14}$C]Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ hydrolysate (spec. act. 57 Ci/mol); (c) a standard of 60 pmol [$^{14}$C]phenylalanine (spec. act. 57 Ci/mol). The peaks were identified by comigration with authentic standards of phenylalanine, 3-phenyllactic acid and cinnamic acid at 4.7 cm, 7.5 cm and 10.8 cm, respectively.

amount of intact [$^{14}$C]Phe-tRNA$^{Phe}$ was present in the starting mixture of Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$, which then became concentrated in the ternary complex peak during the gel filtration reflecting the much stronger interaction of native Phe-tRNA$^{Phe}$ with EF-Tu·GTP as compared to the Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ mixture. Nevertheless, this experiment clearly demonstrates that the interaction of both 3-phenyllactic-tRNA$^{Phe}$ as well as cinnamyl-tRNA$^{Phe}$ with EF-Tu·GTP takes place. Judging from the ratio of cinnamic to 3-phenyllactic acid before and after the gel filtration (fig.3a,b) the affinity of both modified acyl-tRNA$^{Phe}$ species to EF-Tu·GTP is about equal. This result implies that further possible isomers of hydroxyphenylpropionyl-tRNA$^{Phe}$ were either not present in Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ preparation or these acyl-tRNAs are also involved in the formation of ternary complexes and were not resolved during the chromatographic analysis applied.

From the determined dissociation constant of $3 \times 10^{-5}$ M for Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ and from the results in fig.3 we conclude that the replacement of the $\alpha$-amino group by a hydroxyl group as well as the complete elimination of the amino group and formation of an $\alpha,\beta$-unsaturated tRNA acyl ester, reduce the ability of these acyl-tRNAs to interact with EF-Tu·GTP ~300-fold as compared to native Phe-tRNA$^{Phe}$. It is remarkable that the unsaturated $\alpha$-carbon and the absence of the $\alpha$-hydroxyl group in cinnamyl-tRNA$^{Phe}$ do not affect this interaction more severely. Evidently, the presence of the free $\alpha$-amino group [13] and the ester function of the aminoacyl-residue [14] are the most prominent factors determining an efficient aminoacyl-tRNA·EF-Tu·GTP complex formation. In the course of the recent reinvestigation of the interaction between formyl-Met-tRNA$_f^{Met}$ and EF-Tu·GTP $K_d^{app} = 3 \times 10^{-5}$ M was reported [15]. This value is identical with our $K_d^{app}$ for Phe$_{HONO}$-tRNA$^{Phe}_{HONO}$ indicating that the *N*-acylation, replacement by a hydroxyl group, or complete elimination of the $\alpha$-amino group lead to an identical reduction of the affinity of aminoacyl-tRNA to EF-Tu·GTP. Thus the presence of the $\alpha$-hydroxyl group in the 3-phenyllactyl-tRNA$^{Phe}$ does not have any stabilizing influence on the formation of the ternary complex. Such an effect was implied in [3].

Beside the additional information on the substrate requirements for ternary complex formation, our observation that cinnamyl-tRNA$^{Phe}$ can form ternary complexes with EF-Tu·GTP may also be useful for the development of affinity labelling reagent derived from cinnamic acid.

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