***In vitro* reconstitution of anticodon nuclease from components encoded by phage T4 and *Escherichia coli* CTr5X**

Michal Amitsur, Ilan Morad and Gabriel Kaufmann

Biochemistry Department, Tel Aviv University, Tel Aviv 69978, Israel

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During phage T4 infection of *Escherichia coli* strains containing the *prr* locus the host tRNA^{Lys} undergoes cleavage–ligation in reactions catalyzed by anticodon nuclease, polynucleotide kinase and RNA ligase. Known genetic determinants of anticodon nuclease are *prr*, which restricts T4 mutants lacking polynucleotide kinase or RNA ligase, and *stp*, the T4 suppressor of *prr* encoded restriction. The present communication describes an *in vitro* anticodon nuclease assay in which the specific cleavage of tRNA^{Lys} is driven by an extract from *E.coli prr*⁺ (restrictive) cells infected by phage T4. The *in vitro* anticodon nuclease reaction requires factor(s) encoded by *prr*, is stimulated by a synthetic Stp polypeptide and appears to require additional T4 induced factor(s) distinct from Stp.

Key words: tRNA^{Lys}/polynucleotide kinase/RNA ligase/*Escherichia coli prr* gene/synthetic T4 Stp polypeptide

Introduction

Bacteriophage T4 induces the cleavage–ligation (reprocessing) of two host tRNAs during infection of *Escherichia coli* strains which restrict T4 mutants deficient in 3'-phosphatase polynucleotide kinase or RNA ligase (David *et al.*, 1982a,b; Kaufmann and Amitsur, 1985). In this pathway, tRNA^{Lys} is cleaved 5' to the wobble base by anticodon nuclease (ACNase), yielding 2':3'-P> and 5'-OH cleavage termini. Subsequently, the cleavage termini are converted through the triple activities of polynucleotide kinase into a 3'-OH and 5'-P pair. Finally, RNA ligase joins the latter intermediate to restore tRNA^{Lys}, apparently unchanged (Amitsur *et al.*, 1987).

Two genes are implicated with the ACNase function: *prr* and *stp* (Kaufmann *et al.*, 1986). The host locus *prr* dictates restriction of T4 mutants lacking polynucleotide kinase (termed interchangeably *pseT*[−] or *pnk*[−]) or RNA ligase (*rli*[−]). *prr* which resides in an optional *E.coli* DNA element has been transduced from a restrictive (*prr*⁺) strain into various permissive (*prr*⁰) strains (Abdul Jabbar and Snyder, 1984). More recent deletion analysis of the *prr* locus has shown that it encodes both a latent ACNase and factors masking this latent activity (R.Levitz, D.Chapman, M. Amitsur, L.Snyder and G.Kaufmann, in preparation).

Inactivating mutations in the T4 *stp* gene alleviate the *prr* encoded restriction (Depew and Cozzarelli, 1974; Sirotkin *et al.*, 1978; Runnels *et al.*, 1982) and abolish the manifestation of ACNase in T4 infected *E.coli prr*⁺ cells (Kaufmann *et al.*, 1986). *stp* coincides with a basic open

reading frame (ORF) of 29 codons, presumably encoding a polypeptide (Stp) needed for ACNase activity (Chapman *et al.*, 1988).

We describe here an *in vitro* ACNase reaction which depends on *prr* encoded product(s) (Prr), is stimulated by a synthetic polypeptide of the predicted Stp sequence and appears to require additional factors induced by phage T4. The data also suggest coupling of ACNase activity with protein synthesis. A preliminary account of these results has been reported elsewhere (Kaufmann *et al.*, 1989).

Results

Detection of ACNase activity *in vitro*

In vivo, ACNase cleaves a tRNA^{Lys} species into fragments spanning residues 1–33 and 34–76. These products feature 2':3'-P> and 5'-OH cleavage termini, respectively (Amitsur *et al.*, 1987). The same reaction served to monitor ACNase in cell extracts. The standard tRNA^{Lys} substrate employed was labeled internally in the cleavage junction. This was accomplished by ligating the 5'-³²P-labeled tRNA^{Lys} fragment 34–76 with the non-labeled fragment 1–33 (see Materials and methods). ACNase was expected to convert this substrate into fragment 1–33 with a labeled 2':3'-P> end-group whose identity could be established by nuclease P1 mediated release of labeled pUp> (Figure 1). Similarly sized, non-specific cleavage products of tRNA^{Lys} would have yielded the labeled wobble nucleotide 5-methylamino-methyluridine 5'-monophosphate (pU8).

The standard source of ACNase was a crude (S-30) extract prepared from *E.coli* CTr5X (*prr*⁺) infected with a T4 *pnk*[−] strain. The *pnk* mutation prevented further processing of the cleavage product. Where indicated, the T4 strain also carried an *stp* mutation. Depending on the *stp* allele we term the extracts *stp*⁺ or *stp*[−]. The method of extract preparation and the composition of the reaction mixture were adapted from an *in vitro* translation protocol because previous *in vivo* studies had indicated that ACNase is sensitive to chloramphenicol (I.Morad and G.Kaufmann, unpublished). A synthetic polypeptide of the predicted Stp sequence (synthetic Stp, see Materials and methods) found to stimulate ACNase was also included in the standard assay mixture.

When internally labeled tRNA^{Lys} was incubated in the standard ACNase reaction mixture it yielded a major product which migrated in polyacrylamide gel electrophoresis identical with tRNA^{Lys} fragment 1–33 from T4 *pnk*[−] infected *E.coli* CTr5X (Figure 2). The reaction proceeded at 25°C with a lag of 15–30 min and levelled off after 2 h, reaching in the experiment shown an extent of ~20% of the input tRNA^{Lys}. When the unreacted substrate was isolated and incubated in a fresh ACNase reaction mixture it was cleaved like the original tRNA^{Lys} substrate (not shown) indicating that the limited progress of the reaction was due to dissipation of an enzymatic activity rather than heterogeneity of the substrate.

Fig. 1. *In vitro* ACNase assay and product analysis. The ACNase substrate is tRNA^{Lys} labeled with ³²P in between U33 and the wobble base (U8) at position 34. The reaction yields tRNA^{Lys} fragment 1–33 with a labeled 2':3'-P> end and non-labeled fragment 34–76. The labeled end-group is released by nuclease P1 and identified by further digestion with RNase A alone or in combination with nuclease P1.

Fig. 2. Time course of tRNA^{Lys} cleavage by ACNase. The internally labeled [³²P]tRNA^{Lys} substrate (Figure 1) was incubated in an ACNase reaction mixture supplemented with synthetic Stp. Aliquots were separated at the indicated time points by polyacrylamide gel electrophoresis, as described in Materials and methods. The tRNA^{Lys} fragment 1–33 marker was isolated from *E.coli* CTr5X infected with T4 *pseTΔI* (*pnk*[−]). The extent of cleavage was determined densitometrically.

Fig. 3. End-group analysis of the ACNase reaction product. The ACNase reaction product (Figure 2) was eluted from the gel and digested with nuclease P1. The digest was chromatographed on a PEI–cellulose thin layer as described in Materials and methods. The labeled product (lane a) was eluted from the chromatogram and rechromatographed after digestion with RNase A alone (lane b) or in combination with nuclease P1 (lane c).

The *in vitro* product resembling tRNA^{Lys} fragment 1–33 (Figure 2) was isolated and digested with nuclease P1. This converted virtually all the radioactivity into a pUp>-like compound while pU8 could not be detected (Figure 3, lane a). The pUp>-like compound was identified as pUp> by digestion with RNase A alone (Figure 3, lane b) or in combination with nuclease P1 (Figure 3, lane c). These treatments yielded labeled pUp or P_i, respectively. Nuclease P1 diagnosis served subsequently to ascertain the authenticity of ACNase activities.

Uniformly labeled tRNA^{Lys} was employed as a substrate to monitor both the 5' and 3' cleavage products. It yielded two labeled product bands of comparable intensities. Their electrophoretic mobilities were identical to those of *in vivo* derived tRNA^{Lys} fragments 1–33 and 34–76 (Figure 4). Taken together, the data indicated that tRNA^{Lys} was converted *in vitro* into fragment 1–33 with a uridine 2':3'-P> end-group and fragment 34–76 which probably had a 5'-OH end. Thus, in terms of reaction specificity, the *in vitro* cleavage of tRNA^{Lys} was indistinguishable from the *in vivo* ACNase activity.

Requirements of ACNase which suggest coupling to translation

Table I summarizes some requirements of the *in vitro* ACNase reaction. Omission of ATP, GTP or of both from the ACNase reaction mixture inhibited the cleavage of tRNA^{Lys} by >90%. ACNase activity was detected neither in the S-150 supernatant fraction nor in the crude ribosome fraction. However, it could be partially restored by recombining them. These properties distinguish ACNase from known *E.coli* ribonucleases and suggest that it depends on protein synthesis.

The *in vitro* ACNase activity depends on factor(s) encoded by the host *prf* gene and on factors induced by the phage

Crude S-30 extracts from uninfected *E.coli* CTr5X seemed devoid of ACNase activity even when supplemented with

Fig. 4. *In vitro* cleavage of a uniformly labeled tRNA^{Lys}. Uniformly labeled [³²P]tRNA^{Lys} species L1 (Materials and methods) was digested for 1 h at 25°C with or without synthetic Stp. The digest was separated as in Figure 2. tRNA^{Lys} fragments 1–33 and 34–76 from T4 *pnk*[−] infected *E. coli* CTr5X served as a marker.

Table 1. Requirements of the *in vitro* ACNase reaction

Reaction mixture ^a	ACNase activity ^b	Relative activity
complete	2.92	100
−ATP	0.08	3
−GTP	0.01	0
−ATP, −GTP	0.22	8
complete	2.03	100
S-150 supernate	0.03	1
crude ribosomes ^c	0.02	1
S-150 supernate + crude ribosomes	0.94	46

^aThe complete mixture containing the internally labeled tRNA^{Lys} was as described in Materials and methods.

^bExpressed as % input tRNA^{Lys} converted into fragment 1–33.

^cNo tRNA was added to the crude ribosome fraction. Hence, the specific activity of the tRNA^{Lys} substrate was higher in this reaction mixture but even under these conditions ACNase could not be detected.

synthetic Stp (Figure 5a). Hence, in addition to Stp, other T4-induced factors could be required for the *in vitro* ACNase activity. An extract from T4 *pnk*[−] infected *E. coli* K10 (*prp*^D) was also deficient in ACNase (Figure 5b), suggesting a requirement for Prr. In fact, mixing the two inactive extracts restored ACNase activity (Figure 5c) to levels similar to those of the standard T4 infected *prp*^r system (Figure 5d).

Synthetic Stp stimulates the *in vitro* ACNase reaction

The cleavage of uniformly labeled tRNA^{Lys} was stimulated 4-fold by synthetic Stp (Figure 4). This effect was studied more closely using internally labeled tRNA^{Lys} (Figures 5 and 6). In the absence of synthetic Stp the extract supported a low level of ACNase activity (henceforth termed basal ACNase activity). Addition of synthetic Stp up to 2 μM did not increase the basal ACNase activity. Between 2 and 8 μM Stp stimulated ACNase increasingly while further addition had no effect. It is noteworthy that the saturating

Fig. 5. ACNase complementation assays. The ACNase reaction mixtures contained extracts of: (a) uninfected *E. coli* CTr5X (*prp*^r), (b) *E. coli* K10 (*prp*^D) infected with T4 *pse*Δ1, (c) a mixture of (a) and (b) at a 1:1 ratio, (d) *E. coli* CTr5X infected with T4 *pse*Δ1. Synthetic Stp was included as indicated.

Fig. 6. ACNase stimulation versus Stp concentration. Reaction mixtures containing the standard *stp*⁺ extract (■) or buffer III instead of the extract (○) were incubated for 1 h at 25°C with the indicated amounts of synthetic Stp.

concentration of Stp exceeded that of the tRNA^{Lys} substrate by more than an order of magnitude. Finally, by itself Stp had no ACNase activity.

Experiments aimed to determine if endogenous Stp contributes to the basal ACNase activity were inconclusive. Extracts of *E. coli* CTr5X cells infected by a T4 *pnk*[−] and *stp*[−] double deletion mutant exhibited variable levels of basal ACNase activity, ranging from barely detectable to levels seen with the *stp*⁺ system. Moreover, an extract from the T4 *stp*[−] infected *E. coli* K10 cells exhibited basal ACNase activity when complemented with the inactive extract of uninfected *E. coli* CTr5X (not shown). The possible meaning of this ‘Stp-free’ ACNase activity is discussed below.

Discussion

Previous *in vivo* studies indicated that T4 induced anticodon nuclease cleaves the host tRNA^{Lys} 5' to the wobble base to generate 2':3'-P> and 5'-OH termini (Amitsur *et al.*, 1987). The manifestation of the *in vivo* activity depends on the host locus *prp* and on the T4 *stp* gene (Kaufmann *et al.*, 1986). In the Results we described a specific *in vitro* cleavage of tRNA^{Lys}, identical with that catalyzed by ACNase *in vivo*. The *in vitro* ACNase reaction is also characterized by

dependence on Prr and the ability of synthetic Stp to stimulate it. The data also invoke the existence of T4 induced ACNase factors distinct from Stp and suggest that ACNase activity is somehow coupled to protein synthesis.

The involvement of Prr in the *in vitro* ACNase reaction was demonstrated by two facts. First, extracts of T4-infected *E.coli prr^P* cells failed to catalyze the ACNase reaction. Second, ACNase activity could be restored when the Prr deficient extract was complemented with the inactive extract of uninfected *E.coli prr^F* cells. The latter was probably a source of Prr. The same complementation experiment suggested the existence of T4 induced ACNase factor(s) that are distinct from Stp. This entity was present in the first extract but missing from the second and could not be replaced by Stp. The nature of Prr and the additional T4 induced ACNase factor(s) is not known. Future attempts to isolate and characterize these factors will depend on the *in vitro* ACNase complementation assay.

Physiological Stp has not been detected yet, nor could its activity be demonstrated by comparing ACNase levels in *stp⁺* and *stp⁻* extracts. Yet, ACNase stimulation by synthetic Stp strongly argues for the existence of a physiological counterpart related in structure and function. This idea is reinforced by amino acid replacements in synthetic Stp which abolish ACNase stimulation (our unpublished results). The failure to demonstrate a dependence of ACNase on endogenous Stp and the ability to stimulate the enzyme with synthetic Stp indicate that endogenous Stp was either missing or limiting. This deficiency may be attributed to the instability of endogenous Stp or to its dilution below a critical threshold needed for activity. The latter possibility is hinted at by the sigmoidal shape of the ACNase stimulation versus Stp concentration curve. This behavior suggests that Stp acts cooperatively and indicates that its absolute concentration is an important parameter. The same considerations may explain why synthetic Stp became effective only at a vast excess over the tRNA^{Lys} substrate. Thus, while the effective concentration of synthetic Stp could reflect a physiological situation (it corresponds roughly to 10³ copies per cell, cf. Neidhardt, 1987) the tRNA^{Lys} substrate was diluted many fold compared to its concentration *in vivo*. Nevertheless, the apparent inefficiency of synthetic Stp can be ascribed to other causes. For example, endogenous Stp could differ in structure or mode of integration into the ACNase enzyme. Another possibility is that below the effective threshold Stp is neutralized by nonspecific binding or proteolysis. At the saturating concentration of Stp other ACNase factors may become rate-limiting.

A basal ACNase reaction was sustained by the *stp⁻* extract, contrary to the absolute *in vivo* dependence of ACNase on *stp* (Kaufmann *et al.*, 1986). A possible solution to this problem has been offered by a deletion analysis of the cloned *prr* locus. This analysis indicated that *prr* encodes both a latent ACNase and factors masking this activity. Inactivation of the latter elicits ACNase activity without T4 infection. Hence, a likely role of Stp and perhaps the other T4 encoded factors is to activate the latent ACNase by antagonizing the masking factors (R.Levitz, D.Chapman, M.Amitsur, L.Snyder and G.Kaufmann; in preparation). In a similar vein, the Stp-free basal ACNase activity seen *in vitro* may be due to a selective loss of *prr*-encoded masking factors and potentiation of the latent ACNase.

The requirements of ACNase activity for ATP and GTP and the failure to detect it in the separate S-150 supernatant and crude ribosome fractions suggests a dependence of ACNase on protein synthesis. Such a dependence may signify coupling between ACNase activity and a translation step in which the vulnerable tRNA^{Lys} takes part. Alternatively, a short lived ACNase protein is replenished.

The time-lag preceding the ACNase reaction may be needed for the interaction of several macromolecular components involved in ACNase activation. Likely to participate in such a process are Prr, Stp and the additional T4 induced ACNase factor(s). However, the apparent coupling of ACNase to protein synthesis raises the possibility that yet other factors are involved in ACNase activation. Such complexities indicate that the isolation of ACNase as one entity may not be feasible. More promising seems to be the analysis of individual subunits and auxiliary factors of ACNase by gene cloning and complementation assays.

Materials and methods

Materials

The complete sequence of the polypeptide referred to as synthetic Stp is: NH₂-Met-Ser-Asn-Phe-His-Asn-Glu-His-Val-Met-Gln-Phe-Tyr-Arg-Asn-Asn-Leu-Lys-Thr-Lys-Arg-Leu-Arg-Thr-Pro-Val-Arg-Lys-Ile-COOH. In most of the experiments shown we have used a preparation of the full-length peptide synthesized at the Biological Services, Weizmann Institute of Science, Rehovot, Israel. Derivatives containing residues 2-29 and 3-29 were equally effective in ACNase stimulation. The identity of these preparations was not ascertained by sequencing due to impurities which were difficult to remove. A new batch of the Stp residues 2-29 polypeptide was manufactured by Multiple Peptide Systems Inc., San Diego, CA, USA. The product was purified by HPLC RP-18 column (Lichrosorb, 5 µM, Merck) chromatography using a linear gradient of water and 70% acetonitrile both containing 0.1% of trifluoroacetic acid. Its sequence was ascertained by sequential Edman degradations on an automated protein sequenator. This defined batch of the Stp 2-29 polypeptide was as effective in ACNase stimulation as the previous preparations whose sequence was not verified. Enzymes were purchased from P-L Pharmacia Inc., Sweden, and radiochemicals from Amersham Ltd, UK.

Strains

E.coli and T4 strains used in this study are listed in Table II. The *saΔ3* deletion (Depew *et al.*, 1975) was introduced into T4 *pseTΔ1* by marker rescue from an M13-T4 clone with the *saΔ3* deletion (Chapman *et al.*, 1988).

Preparation of cell extracts

E.coli S-30 cell extracts were prepared essentially as described by Chambliss *et al.* (1983). Bacteria were harvested at 4 × 10⁸ cells/ml as such or following infection at m.o.i. of 8 with the T4 strain of choice for 8 min at 37°C. Cultures of 750 ml were poured over 250 ml of frozen medium. The cells were pelleted, washed successively in buffer I [10 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 1 M KCl, 5 mM EDTA, 10% glycerol and 10 mM 2-mercaptoethanol] and buffer II (buffer I with only 50 mM KCl), quickly frozen and thawed in 2 ml/g wet wt of buffer III (10 mM Tris-HCl, 15 mM MgCl₂, 60 mM NH₄Cl, 10% glycerol, 5 mM 2-mercaptoethanol, 3.3 mM phenylmethyl sulfonylfluoride and 0.2 mM diisopropylfluoro-

Table II. *E.coli* and bacteriophage T4 strains

Strain	Relevant property	Reference
<i>E.coli</i>		
CTr5X	<i>prr^F</i>	Depew and Cozzarelli (1974)
K10	<i>prr^P</i>	Garen and Otsuji (1964)
Bacteriophage T4		
<i>pseTΔ1</i>	<i>pnk⁻</i>	Snyder <i>et al.</i> (1976)
<i>pseTΔ1,saΔ3 pnk⁻ + stp⁻</i>	(deletion)	This study

phosphate). After two passages at 18 000 p.s.i. in a 4 ml Aminco pressure cell, the lysates were centrifuged twice at 27 000 g for 15 min and the supernatants stored at -80°C .

Preparation of labeled tRNA^{Lys} substrates

Uniformly labeled [³²P]tRNA^{Lys} was isolated from total *E. coli* CTr5X tRNA by hybridization with the cognate M13 clone mRL8601, followed by electrophoresis on non-denaturing polyacrylamide gel. Out of the two tRNA^{Lys} bands thus resolved, only the species that is cleaved during T4 infection and later restored (L1) was used. Lysine tRNA labeled specifically in the cleavage junction was synthesized by ligating the 5'-³²P-labeled fragment 34–76 to non-labeled fragment 1–33, both fragments resulting from an *in vivo* anticodon nuclease cleavage. This entailed three gel electrophoresis steps and resulted in highly purified tRNA^{Lys}. Both procedures were previously described (Amitsur, *et al* 1987).

In vitro anticodon nuclease assay

The anticodon nuclease reaction mixture contained per 10 μl : ~ 5000 c.p.m. of [³²P]tRNA^{Lys} ($\sim 10^4$ c.p.m./ng), 5 μl of S-30 extract (containing 80 μg of protein), 0.5–1 μg of synthetic Stp, 4% polyethylene glycol (mol. wt 8000), 7.5 mM MgCl₂, 60 mM NH₄Cl, 75 mM Tris–HCl buffer (pH 7.5), 2 mM ATP, 0.1 mM GTP, 5 mM phosphoenolpyruvate, 5 U pyruvate kinase, 1.5 mM phenylmethylsulfonylfluoride (PMSF) and 0.1 mM diisopropylfluorophosphate (DIFP). The reaction mixture contained ~ 5 μg of endogenous *E. coli* tRNA, 100 ng of which were estimated to be intact tRNA^{Lys} species L1 and its fragments, in the case of extracts from T4 infected cells. The reaction was started by adding labeled tRNA^{Lys} to the remaining mixture at 0°C . Incubation was at 25°C for 1 h or as indicated. The reaction was terminated by adding 50 μl of 5 $\mu\text{g}/\text{ml}$ proteinase K in 0.1% SDS, 10 mM Na–EDTA and 100 mM Tris–HCl buffer (pH 8.0). Following 15 min at 25°C , RNA was extracted with aqueous phenol, precipitated with ethanol and dissolved in 10 μl of 10 M urea and 0.01% of xylene cyanol. Aliquots were separated by electrophoresis on 15% polyacrylamide–7 M urea gel in Tris–borate EDTA. tRNA^{Lys} fragments from T4 *pnk*[–] infected *E. coli* CTr5X served as markers. The gel was autoradiographed at -80°C with pre-flashed Agfa Curix RP2 film and Rarex blue III intensifiers (Wolf X-ray). Product bands were quantitated by comparison to standards of known radioactivity, using an Ultrosan Laser Densitometer (LKB Inc., Bromma, Sweden).

RNA end-group analysis

The tRNA fragment produced by ACNase cleavage was extracted from the gel and digested with nuclease P1. The reaction mixture (5 μl) contained the labeled tRNA fragment, 1 μg of carrier yeast tRNA, 0.1 μg nuclease P1 and 10 mM ammonium acetate buffer, pH 5.0. Following 30 min incubation at 37°C the digest was chromatographed on polyethylenimine (PEI)–cellulose thin layer (Polygram CEL 300, PEI-UV₂₅₄ Macherey-Nagel, GmbH; Düren, FRG) using 0.25 M ammonium bicarbonate as a developer. The pUp>-like nucleotide shown in Figure 1 (lane a) was eluted and aliquots of it incubated in 10 μl reaction mixtures containing 1 μg carrier tRNA, 10 mM sodium acetate buffer (pH 5.0) and 0.1 μg of pancreatic RNase alone or in combination with 0.1 μg of nuclease P1 and the digests rechromatographed.

The pUp> and pU8 markers were derived, in respective order, from uniformly labeled [³²P]tRNA^{Lys} fragment 1–33 from T4 *pnk*[–] infected *E. coli* CTr5X and from 5'-³²P-labeled tRNA^{Lys} fragment 34–76 (Amitsur *et al.*, 1987), by nuclease P1 cleavage.

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