THREONYL-tRNA SYNTHETASE FROM YEAST

Aminoacylation of tRNA on its non-accepting 3′-terminal hydroxyl group and its behaviour in enzyme-catalyzed deacylation

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Received 3 March 1978
Revised version received 8 April 1978

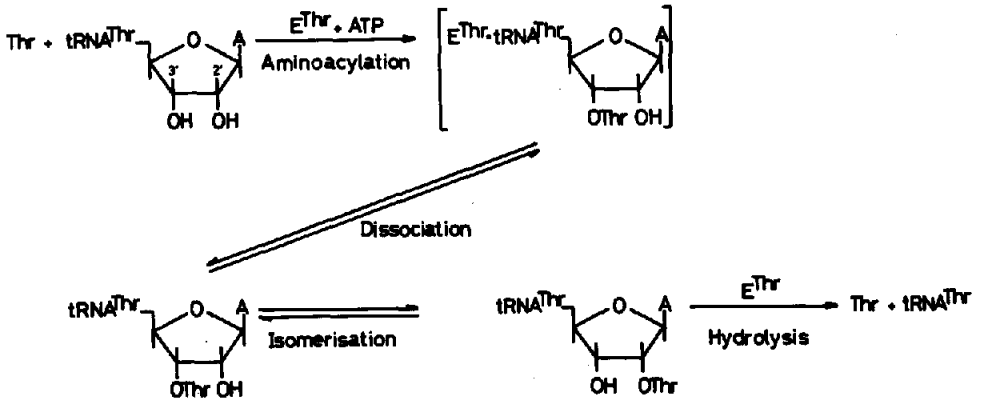
1. Introduction

The hydrolysis of aminoacyl-tRNA by free aminoacyl-tRNA synthetase in the absence of other substrates is well established [1–5], eq. (1):

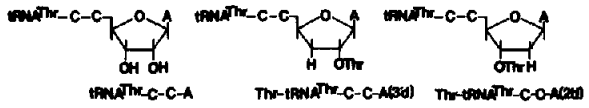
$$\text{Xxx-tRNA} + E^{Xxx} \longrightarrow \text{Xxx} + \text{tRNA} + E^{Xxx} \qquad (1)$$

but is little understood in terms of function or mechanism. In the case of the phenomenologically similar breakdown of aminoacyl-tRNA under aminoacylating conditions, evidence has been obtained for the hydrolysis of the ester bond after transacylation of the aminoacyl residue from the accepting [6] terminal ribose hydroxyl group to the non-accepting vicinal hydroxyl group according to scheme 1 [5]. Whether this pathway also applies to the hydrolysis of aminoacyl-tRNA by free enzyme remains to be determined, but it is in any event of mechanistic interest to study the role of the 3′-terminal ribose hydroxyl groups of tRNA on the free enzyme catalyzed reaction. The detailed investigation of this has been hampered till now by the problem of obtaining a tRNA species bearing its amino acid, in a non-isomerisable form, on the non-accepting ribose site [7]. Implicitly connected with this problem is the high specificity shown by the synthetases for the 2′- or 3′-ribose position of the tRNA during aminoacylation [6]. This difficulty has now been resolved in the case of $tRNA^{Thr}$ from yeast which accepts yeast threonyl-tRNA synthetase activated threonine at the 3′-position under normal con-

Abbreviations: E^{Xxx}, aminoacyl-tRNA synthetase specific for the amino acid Xxx; Thr-$tRNA^{Thr}$–C–C–N, threonine transfer ribonucleic acid aminoacylated with threonine and bearing the 3′-terminal nucleoside adenosine [A] or 3′-deoxyadenosine [A(3′d)] or 2′-deoxyadenosine [A(2′d)] (see scheme 2). Other abbreviations are formed correspondingly



Scheme 1



Scheme 2

ditions. Three approaches have been used to obtain Thr-tRNA^{Thr}–C–C–A(3′d) (scheme 2) and its properties in the hydrolytic reaction have been studied.

2. Materials and methods

The aminoacyl-tRNA synthetases specific for serine, valine, threonine and phenylalanine (EC 6.1.1.11, 6.1.1.9, 6.1.1.3, 6.1.1.20, respectively) from baker's yeast and their corresponding tRNAs were obtained in a pure form with respect to their amino acid acceptance, by the methods in [8–10]. ^{14}C-labelled amino acids were of Stanstar grade from Schwarz (Orangeburg, USA) with spec. act. 50 mCi/mmol. All other reagents were standard commercial preparations. 3′-Terminal nucleotide analysis was performed as in [11].

Aminoacylation:

2.1. *Normal conditions*

For aminoacylation of tRNA^{Thr}, 150 mM Tris–HCl, pH 9, 100 mM KCl, 10 mM $MgSO_4$, 2.5 mM ATP, 40 μM [^{14}C]threonine, 2–5 μM tRNA and the required amount of enzyme were incubated at 37°C. For analytical purposes, in 100 μl incubation volume, the extent of aminoacylation was followed by the filter disc method as in [12]. For preparative aminoacylation, in 1 ml total vol., the aminoacylated tRNA was isolated as in [5].

2.2. *Mischarging conditions*

For the mischarging of tRNAs described in this work the optimal conditions were found to be 10 mM Tris–HCl pH 9, 8 mM $MgSO_4$, 0.5 mM ATP, 50 μM ^{14}C-labelled amino acid, 2–5 μM tRNA, 20% v/v methanol and 10-times more of the appropriate enzyme than was used in the normal aminoacylation experiments, incubated at room temperature. Elevation of incubation temperature and/or substitution of dimethylsulfoxide for methanol was found to decrease the mischarging efficiency.

2.3. *Hydrolysis of Thr-tRNA^{Thr}-C-C-N*

The AMP/PP$_i$-independent hydrolysis of preformed Thr-tRNA^{Thr}–C–C–N species by free threonyl-tRNA synthetase was studied by the method in [5].

3. Results and discussion

3.1. *The synthesis of Xxx-tRNA^{Thr}–C–C–A(3′d)*

The first, and more generally applicable method by which tRNA^{Thr}–C–C–A can be aminoacylated at the normally non-accepting terminal ribose 2′-OH relies on the induced mischarging of tRNA^{Thr}–C–C–A by an enzyme specific for the 2′-OH position. For the mischarging of this tRNA, valyl-tRNA synthetase has been found suitable. tRNA^{Thr}–C–C–A is readily and fully aminoacylated with valine by this enzyme while the 2′-OH bearing tRNA^{Thr}–C–C–A(3′d) is also extensively esterified. The small amount of residual activity associated with the 3′-OH bearing tRNA^{Thr}–C–C–A(2′d) is correlated with the presence of significant quantities of contaminating native tRNA^{Thr}–C–C–A (table 1).

Although there is evidence that the esterase activity of aminoacyl-tRNA synthetases is unspecific with respect to the nature of the amino acid [2] a more rigorous treatment of the hydrolysis would require all tRNA^{Thr}–C–C–N species to be aminoacylated with the same amino acid, namely threonine. The required Thr-tRNA^{Thr}–C–C–A(3′d), which is unobtainable through the threonyl-tRNA synthetase reaction under normal conditions (table 1) is available by two procedures. Firstly, it is well known that valyl-tRNA synthetase will misactivate threonine [13–15]. It is, therefore, not surprising in view of the misacylation described above that the misactivated threonine is transferred to the 2′-OH of tRNA^{Thr}–C–C–A(3′d) (table 1). This reaction is, clearly, only of limited general use since not all synthetases will effectively misactivate the required amino acid [16]. There still remains the possibility of being able to manipulate the 2′,3′ specificity of the enzymes. In the case of the threonine enzyme we observe the first example of such a breakdown in the specificity when mischarging conditions are used (e.g., in the presence of methanol

Table 1
Aminoacylation of $tRNA^{Xxx}$–C–C–N species by aminoacyl-tRNA synthetase (E^{Xxx}) with amino acids Xxx in (nmol/A_{260} unit tRNA) under normal or mischarging conditions

	E^{Thr} with Thr		E^{Val} with Val	
tRNA	Normal[a]	Mischarging	Normal	Mischarging
$tRNA^{The}$–C–C–A	1.42	1.43	n.d.	1.23
$tRNA^{Thr}$–C–C–A(2′d)	1.12	1.2	n.d.	0.16[b]
$tRNA^{Thr}$–C–C–A(3′d)	0.02	0.66	n.d.	0.75[c]
			E^{Val} with Thr	
$tRNA^{Thr}$-C-C-A(3′d)				0.44[c]
	E^{Ser} with Ser		E^{Phe} with Phe	
$tRNA^{Ser}$–C–C–A(3′d)	0.02	0.36[d]		
$tRNA^{Phe}$–C–C–A(2′d)			0.02	<0.05

[a] Data from [6]
[b] $tRNA^{Thr}$-C-C-A(2′d) contained 15% $tRNA^{Thr}$–C–C–A, i.e., approx. 0.21 nmol valine/A_{260} unit tRNA could be accepted thereby
[c] Aminoacylation plateau value was not reached during the incubation period
[d] $tRNA^{Ser}$–C–C–A(3′d) contained 6% $tRNA^{Ser}$–C–C–A, i.e., approx. 0.1 nmol serine/A_{260} unit tRNA could be accepted thereby

and high Mg^{2+}/ATP ratio), where although the 3′-OH can still be aminoacylated, transfer to the 2′-OH also takes place (table 1).

The serine enzyme, another 3′-OH charger behaves identically in that extensive transfer or serine to $tRNA^{Ser}$–C–C–A(3′d) occurs even at suboptimal conditions (i.e., in dimethylsulfoxide at 37°C) (table 1). On the other hand, the 2′-OH specific enzyme tested, that for phenylalanine, showed no signs of loss of specificity with respect to the position of aminoacylation (table 1).

The great sensitivity of this reaction to the conditions used is shown in fig.1. The addition of salts or the absence of methanol enhances the 2′,3′ specificity and reduces the rate and extent of formation of Thr-$tRNA^{Thr}$–C–C–A(3′d). Similar changes in the aminoacylation conditions have no effect on the extent of Thr-$tRNA^{Thr}$–C–C–A(2′d) or Thr-$tRNA^{Thr}$–C–C–A synthesis (table 1) while the kinetics of the reaction leading to the latter two products indicate that the plateau aminoacylation level is reached within 10 min under both normal and mischarging conditions (data not shown, c.f. fig.1). Since the position of attachment of the amino acid to the tRNA may be a function of the enzyme rather than the tRNA, it is possible that

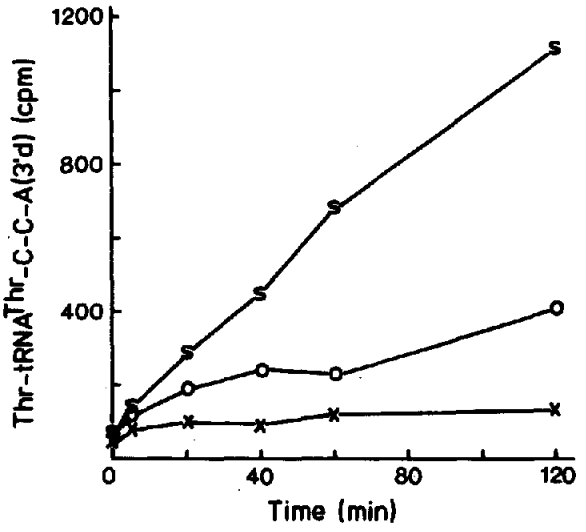


Fig.1. Effect of reaction conditions on the 2′,3′-specificity of E^{Thr}. Aminoacylations were carried out as in section 2 under normal aminoacylation conditions in the presence of 50 mM KCl (x-x), in the absence of KCl (o-o), and under mischarging conditions (s-s).

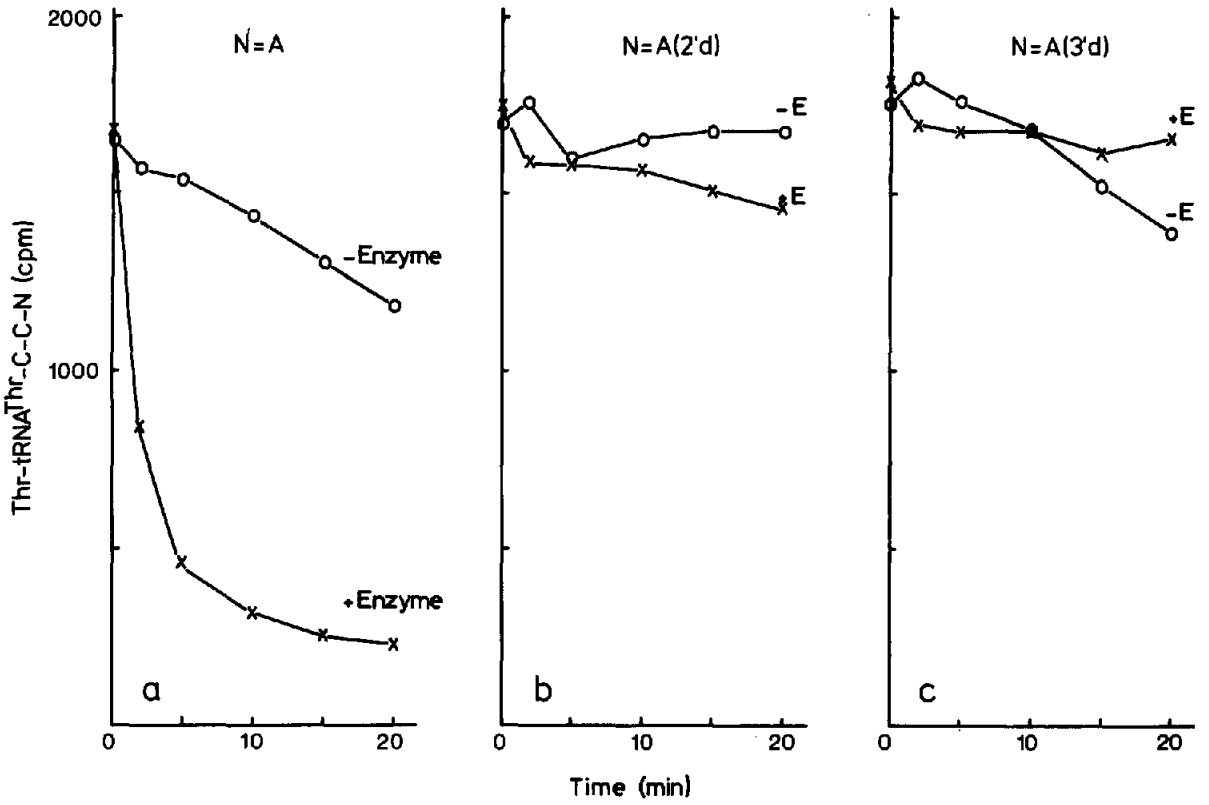


Fig.2. AMP/PP_i-independent hydrolysis of Thr-$tRNA^{Thr}$–C–C–N by free E^{Thr}. The release of [^{14}C]threonine was monitored as in section 2 using 1.7 μM E^{Thr} and 1.5 μM Thr-$tRNA^{Thr}$–C–C–N as indicated.

the effects of organic solvents and of salts observed in this work may be mostly at the level of the protein rather than due to a drastic perturbation of the tRNA structure. The effect of salt on the kinetics of phenylalanyl-tRNA synthetase catalysed aminoacylation has also been suggested to be due to salt-induced changes in the protein [17].

3.2. *The AMP/PP_i-independent hydrolysis of Xxx-$tRNA^{Thr}$–C–C–N*

The AMP/PP_i-independent hydrolysis of Thr-$tRNA^{Thr}$–C–C–N esters by free threonyl-tRNA synthetase was examined. Thr-$tRNA^{Thr}$–C–C–A is rapidly hydrolysed in the presence of the enyzme with a turnover number of 0.19 min^{-1} (fig.2a). From this value it can be seen that this enzyme is somewhat less efficient in the deacylation reaction than some other synthetases (e.g., phenylalanine-, valyl- and isoleucyl-tRNA synthetases with a turnover number in this reaction of 0.67, 0.77 and 1.8 min^{-1}, respectively [5]) but it should be noted that the curve in fig.2a was obtained, at pH 7.6, and not at the pH optimum of threonyl-tRNA synthetase (pH 8.5) in order to minimise the non-enzymatic breakdown of the Thr-$tRNA^{Thr}$–C–C–A.

Figure 2b,c show similar experiments with Thr-$tRNA^{Thr}$–C–C–A(2′d) and Thr-$tRNA^{Thr}$–C–C–A-(3′d). The former was obtained by the standard aminoacylation procedure using the threonyl enzyme while the latter became available by the methods discussed above. It is seen that in neither case is there loss of [^{14}C]threonine from the tRNA. It could be argued that since the aminoacylation level of only 0.73 and 0.54 nmol/A_{260} unit was achieved for the 2′d and for the 3′d ester, respectively, that the non-aminoacylated tRNA would inhibit the hydrolysis

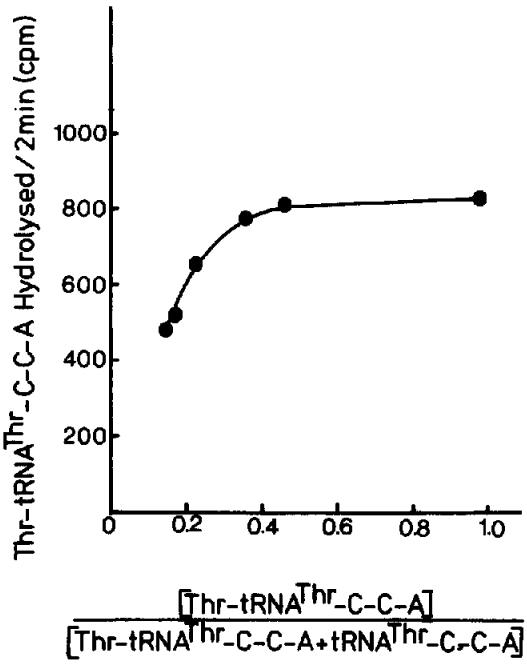


Fig.3. Inhibition of enzymatic hydrolysis of Thr-tRNA^{Thr}–C–C–A by tRNA^{Thr}–C–C–A. The ester hydrolysis was followed as in section 2 at a constant Thr-tRNA^{Thr}–C–C–A concentration (1.5 μM) in the presence of varying concentrations of tRNA^{Thr}–C–C–A using 1.7 μM E^{Thr}.

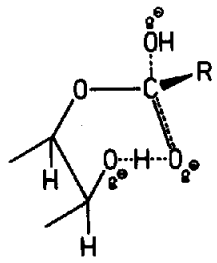


Fig.4. Model for the neighbouring group involvement in the stabilisation of the transition state during non-enzymatic ester hydrolysis (from [20]).

[15] and mask any enzymatic deacylation. However, fig.3 shows that despite the fact that high concentrations of tRNA^{Thr}–C–C–A do inhibit the enzymatic hydrolysis of Thr-tRNA^{Thr}–C–C–A, at the level of aminoacylation of tRNA attained in the cases of the modified tRNAs the inhibition would be negligible. It would appear, therefore, that the neighbouring group effect of the vicinal OH is essential for the enzyme catalysed deacylation. Such a stabilising effect, albeit on the non-enzymatic hydrolysis of aminoacyl-tRNA, by disruption of the *cis* diol moiety by periodate oxidation followed by reduction has been demonstrated in the case of tRNA^{Phe} [18]. Although a number of possible mechanisms exist by which a neighbouring OH might be expected to enhance the non-enzymatic ester hydrolysis rate [19], Bruice and Fife [20] favour The model in which hydrogen binding of the vicinal OH to the carboxyl O brings about the stabilising of the tetrahedral transition state is favoured [20] (fig.4) and thus enhances the rate of the rate determining attack by OH^-. The presence of the synthetase does not aleviate the requirement for the *cis* diol structure but could additionally stabilize the microenvironmental solvation of the transition state. Alternatively the catalytic site functional groups may play a more active role in the hydrolysis. Clearly, however, these experiments do not enable one to define the site on the terminal ribose from which enzymatic hydrolysis occurs; they do indicate the essential nature of the *cis* diol in this process.

4. Summary

Methods have been developed by which tRNA^{Thr} may be aminoacylated at the normally non-accepting 3′-terminal ribose OH. Two of the methods utilize the mischarging ability of the synthetases under special conditions of low salt concentration and presence of organic solvents. The third method demonstrates for the first time that for some synthetases the 2′,3′ specificity may be manipulated by use of similar special conditions. In the case of threonyl-tRNA synthetase, Thr-tRNA^{Thr}–C–C–A(3′d) has been synthesised by this method. The behaviour of threonyl esters of tRNA^{Thr}–C–C–A, tRNA^{Thr}–C–C–A(2′d) and tRNA^{Thr}–C–C–A(3′d) in the free enzyme-catalyzed deacylation has been studied and the results indicate that the *cis* diol functional group is necessary for this hydrolysis. The position on the terminal ribose from which the amino acid is removed in this reaction remains to be identified.

Acknowledgements

We are grateful to Dr F. von der Haar for providing the necessary enzymes and tRNAs used in this work and to Dr M. Sprinzl for help with the nucleotide analysis. A part of this work was presented at the 11th FEBS Meeting, Copenhagen, 1977.

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