Volume 226, number 2, 227–231 FEB 05408 January 1988

Stronger affinity of reticulocyte release factor than natural suppressor $tRNA^{Ser}$ for the opal termination codon

Takaharu Mizutani and Teruaki Hitaka

Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467, Japan

Received 21 October 1987

Animal natural suppressor tRNA did not affect the release reaction of reticulocyte release factor (RF) at the same concentration of tRNA (both estimated as being present at a similar level of $3–5 \times 10^{-8}$ M in vivo); even at a 10-fold greater concentration the tRNA did not prevent the release reaction with RF. In order to confirm this result, the K_a values were determined. The K_a value between RF and UGA was 1.26×10^6 M^{-1} and that between the suppressor tRNA and UGA amounted to 8×10^3 M^{-1}. This result showed that RF had a 150-fold stronger affinity than suppressor tRNA for the opal termination codon. Incorporation of phosphoserine into phosphoprotein via phosphoseryl-tRNA was inhibited by addition of RF to the reaction mixture. These results suggest that animal natural suppressor tRNA in the normal state does not perform its suppressor function, except in special cases where mRNA has the context structure near the opal termination codon (UGA).

Suppressor tRNA; Release factor; Seryl-tRNA; Opal termination codon; Reticulocyte lysate

1. INTRODUCTION

It has been reported that one $tRNA^{Ser}$ in higher vertebrates corresponds to the opal termination codon (UGA) as a natural suppressor tRNA [1]. This seryl-tRNA was phosphorylated with tRNA kinase to yield phosphoseryl-tRNA (Ps-tRNA) [2–4]. We showed that a small amount of ^{32}P on Ps-tRNA was incorporated into proteins in a protein-synthesizing system of reticulocyte lysates [5]. Meanwhile, a proposal for the role of Ps-tRNA as an intermediate in the metabolic pathway from 3-phosphoglycerate to glycine was made [6]. However, it has been clarified that the opal suppressor, Ps-tRNA, is not a substrate for Ps aminotransferase [7]. This result is reasonable from the standpoint of the compartmentalization of aminoacyl-tRNA synthetase and tRNA pools, separating the metabolism of amino acids.

The suppressor tRNA content is about 1/50 of the total serine tRNAs in liver. It is well known that recessive genes frequently do not produce functional products. Therefore, the presence of this natural suppressor shows that this tRNA must play a significant role. Thus, the amount of suppressor tRNA is not negligible and suppressor tRNA should carry out this inevitable function in the normal state of eukaryotes. It is possible that Ps-tRNA might serve as one of the active regulators in the protein-synthesizing system, especially during the termination of protein synthesis by competition of the termination codon with the release factor (RF) [8]. It is necessary to investigate the affinities of RF or opal suppressor tRNA for the termination codon UGA in order to gain an understanding of the role of suppressor tRNA. Here, we describe some results on the influence of suppressor tRNA on the release reaction and on the K_a values for the association of sup-

Correspondence address: T. Mizutani, Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467, Japan

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

pressor tRNA to the termination codon compared to that for RF to the codon.

2. EXPERIMENTAL

Suppressor $tRNA^{Ser}$, initiator $tRNA^{Met}$, and tRNA kinase were prepared from bovine liver according to [2,9,10]. [^{32}P]ATP was synthesized from carrier-free [^{32}P]phosphate [11]. [^{14}C]Serine, [^{14}C]leucine and [^{3}H]methionine were products from Amersham. Met-RS was prepared from the supernatant obtained from centrifugation at 105000 × *g* of mouse liver extracts by means of standard chromatography methods on DEAE-cellulose. UpG(pA)$_n$ was prepared from UpG (Sigma) and ADP using polynucleotide phosphorylase (Pharmacia) according to [12] and purified on DEAE-cellulose in 7 M urea. The mean chain length of the oligonucleotide was estimated as n = 30 from the elution position with NaCl (0.4 M) from the column. The oligonucleotide was used after dialysis against 20 mM Tris-HCl, pH 7.8, 50 mM KCl, 1 mM mercaptoethanol and 0.1 mM EDTA. Part of the oligonucleotide was radiolabeled by phosphorylation with T_4 polynucleotide kinase (Miles) for use in determining association constants.

Rabbit reticulocyte lysates were prepared as in [13] and protein synthesis with Ps-tRNA was performed [5]. Ribosomes were prepared from the lysates [8]. Preparation of Ps-tRNA was also carried out according to [5]. RF was purified from rabbit reticulocyte lysates by ultracentrifugation, fractional precipitation, and chromatography on DEAE-cellulose, phosphocellulose and Sephacryl S-200 [8]. The purity of RF was assessed by standard SDS-PAGE. RF activity in eluates from chromatography was measured by the release of Met from the [^{3}H]Met-tRNA·ribosome intermediate. The rate of the release reaction was estimated from measurements of radioactivity from the unreacted [^{3}H]Met-tRNA·ribosome intermediate on a Millipore membrane (0.45 μm) [14] or free [^{3}H]Met, which was liberated from the intermediate, in ethyl acetate extracts obtained under acidic conditions [8]. The results obtained using both methods were consistent with each other. The practical release reaction was carried out as follows: the intermediate was a mixture of 20 μl of 30 A_{260} units/ml ribosomes, 3 μl of 13 μM [^{3}H]Met-tRNA, 3 μl of 5 mM GTP and 10 μl of 20 A_{260} units/ml UG(A)$_n$. (One A_{260} unit is defined as the amount of material giving an absorbance reading of 1.0 at 260 nm when dissolved in 1.0 ml and measured with a light path of 1.0 cm.) After incubation for 10 min at 26°C, the intermediate was mixed with 30 μl RF (30 μg/ml). The reaction proceeded at 26°C for 15 min, [^{3}H]Met release subsequently being measured by the two methods described above.

The K_a value for association of RF or suppressor tRNA to UG(A)$_n$ was determined by the filtration method with membranes. Binding of RF to [^{32}P]UG(A)$_n$ was performed as follows: [^{32}P]-UG(A)$_n$ (2 μl of 6.4 A_{260} units/ml; 1000 cpm/μl) was mixed with 48 μl RF (2.2×10^{-8}–2.2×10^{-7} M in 20 mM Tris-HCl, pH 7.4, 60 mM KCl, 11 mM $MgCl_2$). The mixture was incubated for 10 min at various temperatures. Bound [^{32}P]-UG(A)$_n$ was collected on a nitrocellulose membrane (Sartorius SM 11307, pore size 0.2 μm) and determined using a scintillation counter (Aloka LC 1000). Binding of [^{3}H]Ser-$tRNA^{su+}$ to UG(A)$_n$ was carried out as follows: [^{3}H]Ser-tRNA (0.2 μM) and UG(A)$_n$ (10 μM) were incubated in 0.05 ml of 20 mM Tris-HCl, pH 7.4, 60 mM KCl, 11 mM $MgCl_2$, for 10 min at 0, 20 and 40°C. After dilution of the mixture with 0.2 ml of 0.2 M acetic acid, the solution was rapidly applied in the apparatus within a few seconds and the bound [^{3}H]Ser-tRNA collected on a Millipore filter (HA type 0.45 μm).

3. RESULTS

Fig.1 shows the influence of suppressor Ser-tRNA and Ps-tRNA on the release reaction of RF. In fig.1, the concentrations of tRNA and RF were 1.4×10^{-8} and 2×10^{-8} M, respectively. The results showed that the suppressor Ser-tRNA exerted no influence on the release reaction of RF at a similar concentration. This concentration of tRNA and RF was also an approximation to those estimated in vivo (RF, 5×10^{-8} M; suppressor tRNA, 3×10^{-8} M). In other experiments, a 16-fold greater suppressor tRNA level over RF concentration had no effect on the release reaction. These results showed that suppressor tRNA at the same concentration as RF had no effect on the release reaction and suggested that RF had a

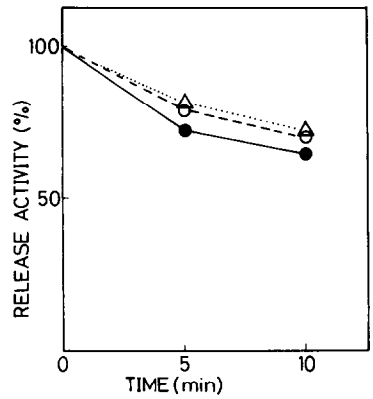

Fig.1. Influence of suppressor Ser-tRNA on the release reaction of RF. (○) RF only; (●) RF + suppressor Ser-tRNA; (△) RF + Ps-tRNA.

stronger affinity to $UG(A)_n$ than that of suppressor tRNA.

We then determined the association constants for Ser-$tRNA^{su+}$ and $UG(A)_n$ compared to that for RF and $UG(A)_n$. The results are depicted in fig.2. The association constant (K_a) for suppressor Ser-tRNA with $UG(A)_n$ at 20°C amounted to $8 \times 10^3\ M^{-1}$, although this value was obtained using acidic conditions (see section 2). This value was consistent with that of $tRNA^{Phe}$ with UUC [15]. This low affinity of tRNA to the codon correlates with the idea that deacylated tRNA must release easily from the codon of mRNA on ribosomes. The K_a for RF and $[^{32}P]UG(A)_n$ at 20°C was found to be $1.26 \times 10^6\ M^{-1}$, showing that RF has a stronger affinity than suppressor tRNA for $UG(A)_n$. This strong affinity of RF for $UG(A)_n$ is reasonable because most of the opal termination codons on normal mRNAs are not suppressed in vivo by suppressor tRNA but must be terminated with RF, under conditions of similar RF and suppressor tRNA concentrations in the cytosol. Thus, the termination codon (UGA) should not be naturally recognized by suppressor tRNA but by RF, since RF has a stronger affinity (150-fold) than suppressor tRNA for $UG(A)_n$.

From fig.2, the following thermodynamic parameters ($kcal \cdot M^{-1}$ at 20°C) were obtained:

System	dF	dH	TdS
Ser-tRNA:$UG(A)_n$	−5.21	−4.17	1.04
RF:$UG(A)_n$	−7.75	−3.16	4.59

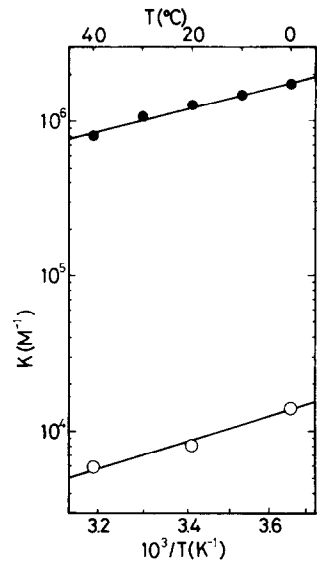

Fig.2. Association constants for $[^3H]$Ser-$tRNA^{su+}$:$UG(A)_n$ (○) and RF:$[^{32}P]UG(A)_n$ (●) complexes as a function of the reciprocal absolute temperature.

The errors are between 1 and 2 $kcal \cdot M^{-1}$. It is seen that the free energy of binding of RF:$UG(A)_n$ is slightly greater than that of Ser-tRNA:$UG(A)_n$. This change in dF results from dH and TdS. However, a detailed understanding of these changes in enthalpy and entropy cannot be gained without including the contributions from water of solvation. The fact that TdS is slightly positive in both cases means that before binding occurred, the anticodons were surrounded by well-ordered water.

Fig.3 shows the influence of RF (0.05 μM) on the incorporation of phosphoserine into phosphoproteins (hot trichloroacetic acid insoluble) through Ps-tRNA. RF added at 0.05 μM (basal concentration of RF in the lysate system ~0.01 μM) inhibited the incorporation of Ps at a level of 50% of that for conditions without additions. The level of suppressor tRNA used was about 2 μM in the lysates. These RF and suppressor tRNA concentrations demonstrated competition between RF and $tRNA^{su+}$ as obtained from the K_a values in fig.2. It is a simple matter to understand the value of 50% inhibition of Ps incorporation by RF. Similar results were obtained with the *E. coli* system [16].

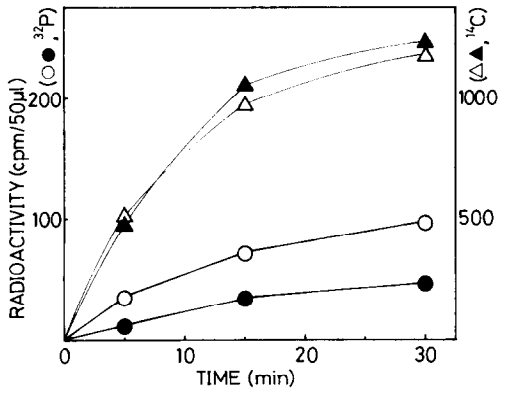

Fig.3. Influence of RF on the incorporation of Ps into phosphoproteins (hot trichloroacetic acid insoluble) from [^{32}P]Ps-tRNA (○,●). Protein synthesis was carried out using rabbit reticulocyte lysates [5]. (○, △) Absence of RF; (●, ▲) presence of RF; (△, ▲) incorporation of [^{14}C]leucine in control experiments (no inhibition by RF).

4. DISCUSSION

This study was performed to gain a better understanding of animal natural suppressor tRNA, which may participate in regulation of the release reaction of RF during protein synthesis. However, it has been shown that suppressor tRNA at a concentration similar to that in vivo does not inhibit RF function. In order to confirm these results, the association constant between suppressor tRNA and UGA was compared with that for RF and UGA. The results showed that RF had an about 150-fold stronger affinity than suppressor tRNA for UGA. These results suggest that natural suppressor tRNA does not play a role in the normal state and only rarely exerts a significant effect on mRNAs which have a specific structure showing context effects at or near the opal termination codon [17] like the dyad structure for the case of RNA synthesis termination. Another possibility for regulation by suppressor tRNA at the level of protein synthesis might be to cause changes in concentration. One of these modes of regulation has been demonstrated with estradiol, i.e., the effect of estradiol on the amino acid-accepting activity was investigated during protein synthesis by restoration with tRNA nucleotidyl transferase [18]. Some forms of regulation have been observed in protein synthesis systems, for example, inhibition by phosphorylation of IF [19] and activation by phosphorylation of S6 protein [20]. Therefore, unknown factors which accelerate the suppressor function of suppressor tRNA may be present in the cytosol.

Recently, the opal termination codon was discovered in the structural frame of proteins. The codon, UGA, was used as selenocysteine in the active center of eukaryotic glutathione peroxidase [21]. Another example is presented by formate dehydrogenase [17], in which selenocysteine is present in the active center, similarly to glutathione peroxidase. The nucleotide sequence of mRNA surrounding the UGA codon of selenocysteine of these enzymes formed dyad-like structures by base pairing (context effects) and can effectively accept selenocysteyl-tRNA or a precursor on the UGA codon of mRNA. This specific structure supports the context effects of suppressor tRNA and helps selenocysteyl-tRNA or the precursor to play a role through context mechanisms. This structure including context effects may also prevent the function of that activity of RF which recognizes the termination codon (UGA), even though this study showed that RF has a stronger affinity for the opal termination codon than does suppressor tRNA.

Recently, a study of suppressor tRNA has shown that the anticodon arm, anticodon loop and D arm on suppressor tRNA are important in the suppressor function of the tRNA [23–25]. These studies suggest that a particular conformation of tRNA is necessary for tRNA to recognize such codon-anticodon interactions. This as yet unelucidated but nevertheless important conformation may enable tRNA, corresponding to UGA, to recognize UGA at the position of selenocysteine on glutathione peroxidase mRNA. From the standpoint of the natural suppressor tRNA role, it is influential in the introduction of Su^+ tRNA genes. However, ochre Su^+ activity was not found to be detrimental to monkey cells [26] and the presence of the Su^+ tRNA genes did not alter the normal growth rates of some murine cell lines [27]. These studies suggest that some readthrough proteins may be produced with suppressor tRNA under normal conditions, not as a hindrance but as normal constituents, even though the amount is low.

At equimolar or slightly greater concentrations of suppressor tRNA than RF, the tRNA was not effective in carrying out a suppressor function with

respect to UGA. This suggests that production of the readthrough protein is very low. However, some reports in which suppressor tRNA was used showed that the readthrough proteins were present in greater amounts than non-readthrough proteins (normal terminated proteins). In those experiments (in vitro), readthrough proteins were produced using suppressor tRNA at very high concentrations (about 10^3-fold that of RF). For example, Diamond et al. [28] and ourselves [5] have performed protein synthesis at 10–30 μM suppressor tRNA and confirmed production of readthrough protein. Moreover, some suppressor tRNAs have a high affinity for the termination codon in protein synthesis systems, such as reticulocyte lysates or a yeast cell-free system [29,30], and effective suppression has been observed. Thus, it is necessary for the condition under which the suppressor activity of the tRNA is determined to be normalized when the activity of suppressor tRNA is being measured or compared.

REFERENCES

[1] Hatfield, D. (1985) Trends Biochem. Sci. 10, 201–204.
[2] Mizutani, T. and Hashimoto, A. (1984) FEBS Lett. 169, 319–322.
[3] Sharp, S.J. and Stewart, T.S. (1977) Nucleic Acids Res. 4, 2123–2136.
[4] Hatfield, D., Diamond, A. and Dudock, B. (1982) Proc. Natl. Acad. Sci. USA 79, 6215–6219.
[5] Mizutani, T. and Tachibana, Y. (1986) FEBS Lett. 207, 162–166.
[6] Mäenpää, P.H. and Bernfield, M.R. (1970) Proc. Natl. Acad. Sci. USA 67, 688–695.
[7] Mizutani, T., Kanbe, K., Tachibana, Y., Kimura, Y. and Hitaka, T. (1986) Nucleic Acids Res. Symp. Ser. 17, 179–182.
[8] Caskey, C.T., Beaudet, A.L. and Tate, W.P. (1974) Methods Enzymol. 30, 293–303.
[9] Narihara, T., Fujita, Y. and Mizutani, T. (1982) J. Chromatogr. 236, 513–518.
[10] Mizutani, T., Narihara, T. and Hashimoto, A. (1984) Eur. J. Biochem. 143, 9–13.
[11] Walseth, T.F. and Hohnson, R.A. (1979) Biochim. Biophys. Acta 526, 11–31.
[12] Leder, P., Singer, M.F. and Brimacombe, R.L.C. (1965) Biochemistry 4, 1561–1567.
[13] Suzuki, H. and Hayashi, Y. (1975) FEBS Lett. 52, 258–261.
[14] Nirenberg, M. and Leder, P. (1964) Science 145, 1399–1407.
[15] Eisinger, J. (1971) Biochem. Biophys. Res. Commun. 43, 854–861.
[16] Beaudet, A.L. and Caskey, C.T. (1970) Nature 227, 38–40.
[17] Zinoni, F., Birkmann, A., Stadtman, T.C. and Böck, A. (1986) Proc. Natl. Acad. Sci. USA 83, 4650–4654.
[18] Lutz, W.H. and Barker, K.L. (1986) J. Biol. Chem. 261, 11230–11235.
[19] Ochoa, S. and DeHaro, C. (1979) Annu. Rev. Biochem. 48, 549–580.
[20] Thomas, G., Siegman, M. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 3952–3956.
[21] Chambers, I., Franpton, J., Goldfarb, P., Affara, N., McBain, W. and Harrison, P.R. (1986) EMBO J. 5, 1221–1227.
[22] Lipmann, F. (1984) Annu. Rev. Biochem. 53, 1–33.
[23] Winey, M., Mendanhall, M.D., Cummins, C.M., Culbertson, M.R. and Knapp, G. (1986) J. Mol. Biol. 192, 49–63.
[24] Yarus, M., Cline, S., Raftery, L., Wier, P. and Bradley, D. (1986) J. Biol. Chem. 261, 10496–10505.
[25] Raftery, L.A., Bermingham, J.R. and Yarus, M. (1986) J. Mol. Biol. 190, 513–517.
[26] Laski, F., Belagaje, R., Hudziak, R., Capecchi, M., Norton, G., Norton, G., Palese, P., RajBhandary, U. and Sharp, P. (1984) EMBO J. 3, 2445–2452.
[27] Hudziak, R., Laski, F., RajBhandary, U., Sharp, P. and Capecchi, M. (1982) Cell 31, 137–146.
[28] Diamond, A., Dudock, B. and Hatfield, D. (1981) Cell 25, 497–506.
[29] Kohli, J., Kwong, T., Altruda, F., Soll, D. and Wahl, G. (1979) J. Biol. Chem. 254, 1546–1551.
[30] Tuite, M.F., Bower, P.A. and McLaughlin, C.S. (1986) Biochim. Biophys. Acta 866, 26–31.