Volume 3 no.4 April 1976 Nucleic Acids Research

Anticodon conformation and accessibility in wild-type and suppressor tryptophan tRNA from E.coli.

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Received 22 January 1976

A B S T R A C T

The association between Trp-tRNA and Pro-tRNA, which have complementary anticodon sequences, has been used as a probe of anticodon conformation. It is unaffected, however, by the base change in the D-stem present in UGA-suppressor Trp-tRNA. This does not support the hypothesis that UGA suppression depends upon a conformational change induced in the anticodon.

The stable denatured form of wild-type Trp-tRNA no longer interacts with Pro-tRNA ; the structure of the anticodon region must therefore be quite different in the denatured form.

I N T R O D U C T I O N

UGA nonsense codons are suppressed by a mutant tryptophan tRNA which has the same anticodon CCA as the wild type tRNA (1). The two species differ in a single base in position 24, such that the base pair b2 is G.U in the wild type and A.U in the suppressor tRNA (1). It is not understood how this base change alters the specificity of codon recognition by $tRNA^{Trp}$. One possibility is that the conformation of the anticodon loop is affected by the mutation in such a way as to alter the interaction with codons. NMR studies of the two species indicate that a conformational difference in the anticodon loop does exist (Jones, C.R., Kearns, D.R. and Buckingham, R.H., unpublished results), though it cannot be assumed that this will directly affect the decoding specificity of the tRNA. An investigation of UpGpA binding to $tRNA^{Trp}$ failed to detect any difference between the wild type and suppressor species (2).

We decided to use the technique of complementary anticodon association introduced by Eisinger (3, 4) to investigate this problem further since it could be adapted to reveal very small differences in association constants. It seemed useful to do this because it is probable that ribosomes must possess

some mechanism for improving discrimination at the level of codon-anticodon association (5).

A second reason for these experiments is related to the denaturation of $tRNA^{Trp}$. The wild type tRNA has a stable denatured form (6, 7) not found in the mutant tRNA (1, 8) which must be exposed to much lower ionic strength to be denatured (8). The structure of denatured $tRNA^{Trp}$ is of considerable interest, in particular because it retains the ability to interact with $EFT_u GTP$ (9). Some models have been proposed for the denatured form (8, 10). One aspect of these models which it is possible to test is the availability of the anticodon sequence for complementary binding.

We report here that the association constants between wild type or Su^+ $tRNA^{Trp}$ and the complementary anticodon in $tRNA^{Pro}$ are closely similar and differ by less than 2 % under a wide range of conditions of temperature and ionic strength. However, the denatured wild-type $tRNA^{Trp}$ shows no detectable interaction with $tRNA^{Pro}$. A preliminary account of this work was given recently (11).

MATERIALS AND METHODS

tRNA

$tRNA^{Trp}$ (Su^-) was prepared as previously described (11) from *E. coli* B tRNA (General Biochemicals). tRNA from the UGA suppressor strain CAJ64 was prepared as before (8) and $tRNA^{Trp}$ (Su^+) was purified by the same method as for the wild-type species, except for a preliminary chromatography on Sephadex A50 according to Nishimura et al (13). The suppressor species accepted 1700 pmol tryptophan/A_{260} unit and was estimated to be more than 95 % pure ; two preparations of the wild-type tRNA were used, which from tryptophan acceptance were estimated to be 80 % and 90 % pure respectively. $tRNA^{Pro}$ was prepared on a column of $tRNA^{Trp}$ (Su^+) (35 A_{260} units) covalently linked to Biogel P200 (0.5 ml, see below). A sample containing 650 A_{260} units of total K12 tRNA (except for $tRNA^{Trp}$ and $tRNA^{Met}$) in 0.6 ml of 1M-NaCl, 10 mM-$MgCl_2$, 20 mM sodium cacodylate pH 7.4 was passed at 0.5 ml/h 4°C, through the

column, which had been equilibrated with the same solution. The column was then washed with 4 ml of this solution, and eluted with 20 mM-sodium cacodylate, pH 7.4, at 20°C. About 12 A_{260} units of tRNA were recovered in a single eluted peak and showed $tRNA^{Pro}$ acceptor activity. The cycle could be repeated many times to accumulate the product. To estimate the purity of this preparation it was chromatographed on BD cellulose as described by Nishimura et al (13), and eluted with a linear NaCl gradient in 50 mM sodium acetate buffer, pH 5, 10 mM-$MgCl_2$ at 4°C. A single sharp peak was eluted at 0.6 M-NaCl and contained more than 95 % of the applied tRNA.

Digestion and labelling of tRNA

$tRNA^{Trp}$ was digested with snake venom phosphodiesterase (Worthington VPH) as described by Miller et al (14). The extent of digestion was followed by loss of tryptophan acceptance measured with purified Trp-tRNA synthetase (for A removal) or crude synthetase (for CA removal). Reconstitution with labelled CTP alone or CTP plus labelled ATP was performed under the conditions of Miller et al (14) with tRNA-nucleotidyl-transferase (a kind gift from Dr S. Litvak, Institut de Biologie Moléculaire, Université Paris VII). Reconstitution in the first case was continued long enough to replace most of the cytidine removed, so that the labelled sequence was mostly $pCpC^{*}OH$ with little $pCpC^{*}pC^{*}OH$.

Aminoacylation of tRNA

$tRNA^{Trp}$ was charged as previously described (12) either with purified tryptophanyl tRNA synthetase or the crude enzyme eluted from DEAE cellulose (15). Proline acceptance was measured as described by Muench and Berg (15).

Preparation and use of polyacrylamide-bound tRNA

tRNA was periodate-oxidised under mild conditions (16) and linked to hydrazine-activated P200 (Bio-Rad Laboratories -400 mesh) as described by Grojean et al (17). About 50 A_{260} units of tRNA were fixed per ml of wet packed beads with about 95 % efficiency. Analytical columns generally contained

0.2 ml of packed beads and were of polythene, 4.5 mm internal diameter, with water jackets. Flow was controlled by a Varioperpex peristaltic pump (L.K.B.) with 1 : 250 ratio gearbox. Fractions of 0.2 ml were collected and counted for radioactivity with 0.4 ml H_2O and 5 ml Triton X-100 : toluene scintillation fluid (1 : 2 v/v). For samples containing charged tRNA, the fraction collector tubes contained 50 µl of 2 M sodium acetate buffer pH 5 ; after collection, the fractions were precipitated with 10 % TCA using bovine serum albumin as carrier, collected on GF/C glass fibre filters (Whatman), washed with 1 % TCA and then ethanol, dried and counted with toluene-based scintillation fluid.

Radioisotopes

[2-^{3}H] ATP (550 Ci/mol), [U-^{14}C] ATP (500 Ci/mol), [5-^{3}H] CTP (18 Ci/mmol) and [U-^{14}C] CTP (260 Ci/mol) were from the Radiochemical Centre, Amersham, U.K. and were used without dilution except for [^{3}H] CTP which was diluted to 2 Ci/mmol. [2.3-^{3}H]-tryptophan (8 Ci/mmol) was from CEA, Saclay France. Radioactivity was counted in an Intertechnique SL 30 liquid scintillation counter.

Treatment of ^{3}H/^{14}C ratio

For two peaks of radioactivity slightly displaced with respect to each other a simple approximate relation can be derived between the slope of the ratio $r = {}^3H/{}^{14}C$, considered as a linear function of the elution volume v close to the centre of the peaks, and the peak displacement Δv. By considering the peaks to the Gaussian in form, with the same width $V_{\frac{1}{2}}$ at half height, it is easily shown that $\Delta v = V_{\frac{1}{2}}^2 \frac{dr}{dv} H/8C\ln 2$, where H and C are the total amounts of ^{3}H and ^{14}C activity in the respective peaks. This was applied to the data presented in Fig. 1.

RESULTS

Consideration of the genetic code shows that the tRNA with an anticodon complementary to the CCA anticodon of $tRNA^{Trp}$ should

be one of the $tRNA^{Pro}$ isoaccepting species. In the absence of a simple procedure for their isolation the complementary anticodon association was itself used on a preparative scale. Use was made of the fixation by a hydrazone linkage of the CCA end of the tRNA to polyacrylamide beads (17, 18) to make an affinity column containing immobilised Su^+ $tRNA^{Trp}$. This column preferentially fixed $tRNA^{Pro}$ and yielded a preparation more than 95 % pure as estimated by subsequent chromatography on BD-cellulose.

The procedure was then used in the reverse sense, i.e. with $tRNA^{Pro}$ as the immobilised tRNA, as an analytical tool in order to look for differences between the wild type and mutant $tRNA^{Trp}$ in their association with $tRNA^{Pro}$. Since CCA occurs both as the anticodon and at the 3'OH terminus, samples of $tRNA^{Trp}$ lacking the terminal adenosine and radioactively labelled were prepared by digestion with venom phosphodiesterase followed by repair with tRNA nucleotidyltransferase using (^{3}H)- or (^{14}C)-CTP. Other samples were repaired in the presence of CTP and (^{3}H)- or (^{14}C)-ATP. It was thus possible to compare wild type and suppressor tRNA in a single chromatographic operation by using mixed isotopes, or to see the effect of removal of the terminal adenosine.

The behaviour of a mixture of (^{14}C)Su^+tRNA^{Trp}(CC*) and (^{3}H)Su^-tRNA^{Trp} (CC*) on a column containing immobilised $tRNA^{Pro}$ is shown in Fig. 1 (a). Two peaks of tritium can be seen ; the first, which emerges from the column in the void volume, contains other species of tRNA present as impurities in the sample of wild type $tRNA^{Trp}$ and also some denatured wild type tRNA, as discussed further below. The second tritium-containing peak is eluted in about 20 column volumes of solution, a retardation which results from the complementary anticodon interaction and is quantitatively related to the association constant. The (^{14}C) radioactivity (Su^+) is eluted essentially as one peak, coincident with the main tritium peak. The ratio $^3H/_{14C}$ is constant over the peaks indicating that they are not displaced with respect to each other. The precision of the experiment is such that the association constants can be said to differ by not more than 2 %. The absolute value of the association constant that can be calculated is relatively much less precise, mostly because of uncertainty about

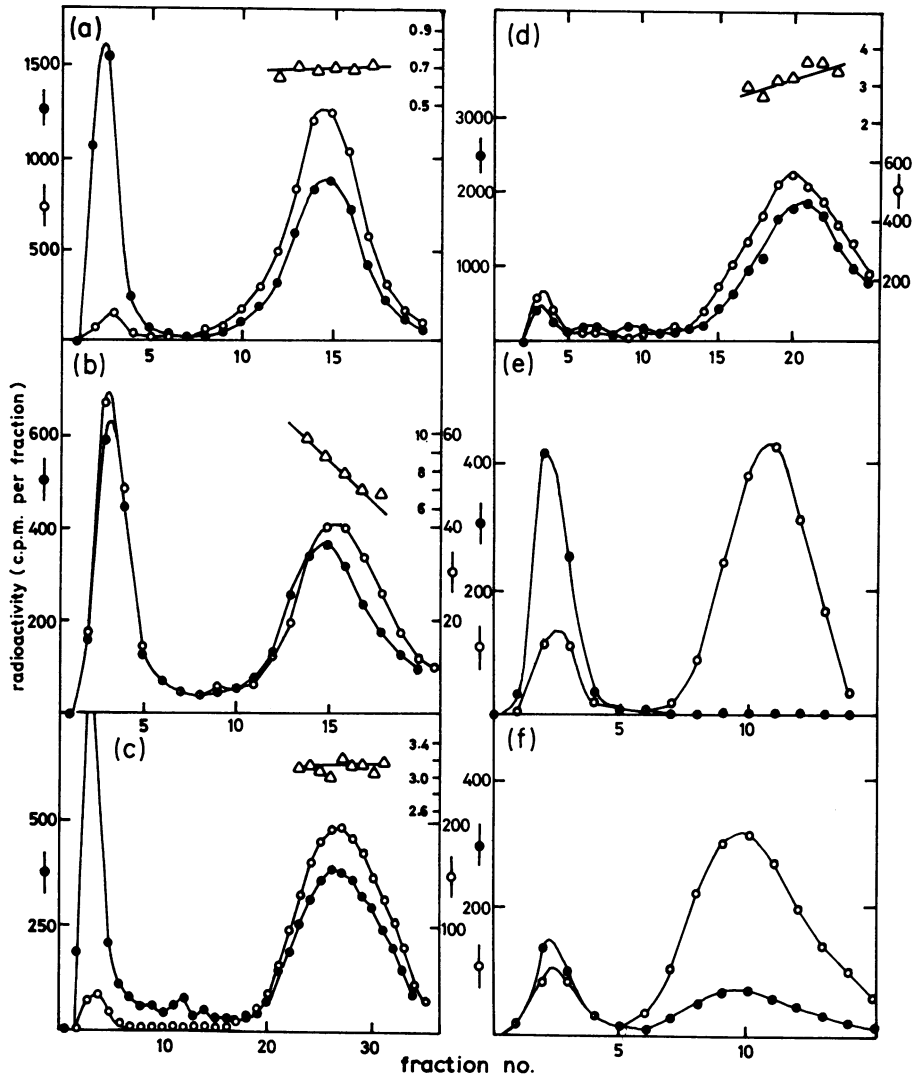

Fig. 1 Chromatography on immobilised $tRNA^{Pro}$ of $tRNA^{Trp}$ as follows :
a) (^{3}H) Su^- tRNA (CC*) and (^{14}C) Su^+ tRNA (CC*) ; (b) (^{3}H) Su^- tRNA (CC*) and (^{14}C) Su^- tRNA (CCA*) ; (c) (^{3}H) Su^- tRNA (CCA*) and (^{14}C) Su^+ tRNA (CCA*) ; (d) (^{3}H) Trp : tRNA (Su^+) and (^{14}C) Su^+ tRNA (CCA*) ; (e) sample as in (c) but denatured before application ; (f) sample as in (e) but renatured before application. 3H is shown —●— , ^{14}C is shown —o— and the ratio ^{3}H/^{14}C is shown —Δ—. The column buffer was in all cases 20 mM sodium cacodylate-HCl, pH 7.4, 10 mM $MgCl_2$ and in addition (a) and (b), 0.7 M NaCl, 20°C ; (c), 0.7 M NaCl, 10°C ; (d), 0.1 M NaCl, 15°C ; (e) and (f) 0.4 M NaCl, 20°C.

the quantity of immobilised tRNA in the column freely available. Assuming, however, that all tRNA initially bound is available, and that the retardation in elution in units of column void volume is proportional to the ratio of bound to free $tRNA^{Trp}$, one obtains a value of 7×10^5 M^{-1} at 20°C in 0.7 M NaCl, 10 mM-$MgCl_2$, 20 mM sodium cacodylate pH 7.4. Similar experiments have been performed at temperatures between 10°C and 25°C and concentrations of NaCl between 0.1 M and 1.0 M. Retardation was favoured by low temperatures and high ionic strength, but in all cases the retarded peaks of wild type and suppressor tRNAs were found to be co-eluted from the column.

Co-chromatography of (^{3}H) $Su^-tRNA^{Trp}(CC^*)$ and (^{14}C)Su^-tRNA^{Trp} CCA^* shows (fig. 1b) that readdition of the terminal A increases the association constant by only 5 %. A similar result was found with the mutant tRNA (results not shown) and a direct comparison been the wild type and mutant tRNAs, both labelled in the terminal A, reveals no difference between them (Fig. 1 C). It was possible also to examine the effects of charging the tRNA. Although considerable discharge occurs during the chromatography at pH 7.4, this was of no consequence provided that only TCA-insoluble radioactivity eluted from the column was measured. The presence of the tryptophanyl group was found to increase the association constant by about 2 % in both suppressor (Fig. 1 d) and wild-type tRNA (not shown). Preliminary experiments at pH 5 showed that the association was greatly reduced with respect to that at pH 7.4 ; whether this was a direct effect on the base pairing between anticodons or resulted from a change in tRNA conformation was not investigated further.

It is known that the wild type tRNA can be denatured under conditions which do not affect the suppressor species (1, 8) and that the rate of renaturation is negligible under the conditions of operation of the affinity column (7, 19). Treatment under denaturing conditions prior to chromatography of the two tRNAs labelled in the terminal A did not affect the behaviour of the suppressor species but eliminated the interaction of the wild type tRNA with $tRNA^{Pro}$ (Fig. 1 e). Thus the ^{3}H -labelled wild-type tRNA was eluted from the column in the void volume, even when chromatography was conducted at 10°C in 0.7 M NaCl,

10 mM Mg^{2+}, conditions such that the retardation of the suppressor species was increased to 29 column volumes (not shown). Renaturation of a denatured sample restored the behaviour to that normally observed (Fig. 1 f).

D I S C U S S I O N

Binding of tryptophanyl-$tRNA^{Trp}$ to the UGA triplet on the ribosome is very difficult to demonstrate *in vitro* even in the case of the suppressor species, which is a "strong" suppressor (20) and translates UGA nonsense codons considerably more efficiently than the wild-type tRNA (21). We have attempted to study the codon binding properties of $tRNA^{Trp}$ in a ribosome-free model system consisting of two tRNA-species with complementary anticodons. The important characteristic of this system is that the entropy change on association is small and positive in contrast to tRNA triplet binding, where the change is large and negative (3). This leads to binding constants about three orders of magnitude higher for the complementary anticodon system. It is thus a much closer model to codon recognition on the ribosome than tRNA : triplet interactions in solution. The most direct analogy to UGA recognition in such a model system would be the interaction between $tRNA^{Trp}$ and $tRNA^{Ser}_1$ (*E. coli*) which carries the anticodon VGA (22) where V is 5-oxyacetic acid uridine. This system has not, in our hands, yielded satisfactory results, possibly because the method of fixing $tRNA^{Pro}$ to the support chemically modifies the base 2-methylthio-N^6-isopentenyl adenosine adjacent to the anticodon, which may be important to codon : anticodon interaction (23). Therefore we have studied tRNA binding to $tRNA^{Pro}$, the analogue of UGG translation, in anticipation that a change in anticodon conformation that significantly increases the binding to UGA nonsense codons would also affect binding to UGG. The system of complementary anticodon association with $tRNA^{Pro}$ has been found to function well with $tRNA^{Pro}$ immobilised on a solid support and to permit a precise comparison of the association constants with the wild type and mutant $tRNA^{Trp}$. Our finding that there is no detectable difference between the two tRNAs does not support the hypothesis that the

anticodon structure is changed by the mutation in such a way as to affect directly the codon : anticodon interaction. It is conceivable, though it seems to us unlikely, that binding to a complementary anticodon is unaffected by a change in anticodon conformation that nevertheless enhances binding to UGA nonsense codons. Alternative hypotheses for the mechanism of suppression are that the base change is important in modifying the interaction between the tRNA and the ribosome, or between the tRNA and elongation factor EFT_u.GTP. It is clear that interactions other than the codon : anticodon pairing are essential to account for the stability of the complex on the ribosome. These ancillary interactions may be directly modified by an altered conformation of the Su^+ tRNA so as to increase the "leakiness" in translation by this tRNA. It may also be that the discriminatory process in codon recognition entails a conformational change in the tRNA before tight binding is achieved. It is probable that the conformational stability of the tRNA may be altered by the mutation in the dihydrouridine stem (1) and this may facilitate a change in conformation required on the ribosome to unmask a common binding site such as the TψC loop (24). This has been fully discussed recently (5). Evidence for a conformational difference between the wild type and mutant tRNAs has come from the kinetics of photochemical cross-linking (25) and the susceptibility to degradation by polynucleotide phosphorylase (Buckingham, R.H., Dondon, L. and Thang, M.N. unpublished results).

The small enhancement in binding to $tRNA^{Pro}$ when the terminal adenosine is restored to $tRNA^{Trp}$ and the weak binding by other tRNA species which nevertheless carry the CCA terminal sequence is in line with the expectation that this part of the molecule has considerable configurational freedom. Association of this sequence with a complementary anticodon is thus more analagous to triplet : anticodon binding. Our finding that the acylated molecule behaves almost identically to the uncharged species does not support the suggestion that charging induces a conformational change in the anticodon that augments codon : anticodon binding (26, 27).

We can detect no binding of the denatured wild-type $tRNA^{Trp}$ to immobilised $tRNA^{Pro}$. This would indicate that there

is an important change in the anticodon region of the molecule on denaturation. It is clear that the secondary structure of the denatured form is altered (1, 8), and the most straightforward explanation for the lack of binding of the anticodon sequence is that it is involved in base pairing. It is also possible that the sequence is rendered unavailable by being buried in tertiary structure, or that the conformation of the anticodon is no longer favourable. The change in association constant is at least by a factor of thirty which would argue against any model for the denatured form in which the terminal base pairs of the anticodon stem and the anticodon loop were the same as in the native structure. The models that have been proposed so far are of this type and offer no explanation for the lack of anticodon accessibility that we observe (8, 10).

ACKNOWLEDGMENTS

Thanks are due to Dr M. Grunberg-Manago for her constant advice and encouragement. This work was supported by grants to Dr M. Grunberg-Manago from the Centre National de la Recherche Scientific (Groupe de Recherche N° 18) and the Délégation Générale à la Recherche Scientifique et Technique (Convention N° 74.7.0356) and by a fellowship to R.H. Buckingham from E.M.B.O.

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