TRANSFER RNA, III. RECONSTITUTION OF ALANINE ACCEPTOR ACTIVITY FROM FRAGMENTS PRODUCED BY SPECIFIC CLEAVAGE OF $tRNA_{II}^{Ala}$ AT ITS ANTICODON†*

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Abstract.—This report describes experiments showing that $tRNA_{II}^{Ala}$ can be cleaved specifically at the G residue of its anticodon to give "half" molecules in high yield. Neither of these fragments has alanine-acceptor activity, but this activity can be reconstituted by mixing the fragments in the presence of Mg^{+2}. In dilute salt, on the other hand, the active complex dissociates spontaneously at 25° into the two fragments. Thus, both "halves" of $tRNA_{II}^{Ala}$ are necessary for acceptor activity and, in the presence of Mg^{+2}, combination of these fragments to give an active duplex is thermodynamically favorable.

During our recent studies on the purification of $tRNA^{Ala}$ from yeast,[1] we discovered a new tRNA, $tRNA_{II}^{Ala}$, which differs in its physical, chemical, and biological properties from Holley's[2] $tRNA_{Iab}^{Ala}$. This paper describes the specific cleavage of $tRNA_{II}^{Ala}$ at the G residue of its anticodon, the separation of "half" molecules, and the reconstitution of alanine-acceptor activity from these fragments.

Materials and Methods.—$tRNA_{II}^{Ala}$ was prepared by a modification of our previous procedure.[1] Crude yeast tRNA (2.5 gm; Schwarz BioResearch, N.Y.) was fractionated on a 2.5 × 98-cm column of benzoylated DEAE cellulose (BD-cellulose) with a salt gradient, as described by Gillam *et al.*[3] This partially purified $tRNA^{Ala}$ was rechromatographed on a second BD-cellulose column of the same size as before, but with a salt gradient at pH 4.5 similar to that described by Gillam *et al.* (0.3–0.8 *M* NaCl, + 0.01 *M* $MgSO_4$ + 0.01 *M* sodium acetate buffer, pH 4.5, 8 liters). This procedure gave a $tRNA_{II}^{Ala}$ that was free of $tRNA_{Iab}^{Ala}$, but contaminated with other transfer RNA's. The $tRNA_{II}^{Ala}$ was purified to a specific activity of ~1700 pmoles/A_{260} by the derivative procedure described previously.[1]

"Half" molecules were produced as follows: After partial digestion of $tRNA_{II}^{Ala}$ (78 A_{260}) with RNase T-1 (4,000 Sankyo units, Sankyo Co., Ltd., Tokyo, Japan) in 6.8 ml of 0.02 *M* Tris buffer, pH 7.5, + 0.018 *M* $MgCl_2$, for 30 min at 0°, the mixture was extracted three times with 6 ml of phenol saturated with 0.02 *M* Tris, pH 8.0 + 0.02 *M* $MgCl_2$. The combined phenol extracts were back-extracted once with 5 ml of 0.02 *M* Tris, pH 7.5, + 0.02 *M* $MgCl_2$. The combined aqueous layers were extracted six times each with 12 ml of ether. The aqueous phase was aerated to remove the dissolved ether. The solution was evaporated at reduced pressure to less than 3 ml and applied to a 1 × 100-cm Sephadex G-100 column that had been equilibrated with 0.1 *M* NaCl + 0.01 *M* phosphate buffer, pH 7.5, at 56° (see Fig. 1).

Material from peak 2, Figure 1, pooled as shown, was dialyzed against three changes of distilled water and concentrated under reduced pressure almost to dryness. One milliliter of 7 *M* urea, pH 3, was added and the solution was then applied to 0.5 × 110-cm DEAE cellulose column equilibrated with 7 *M* urea adjusted to pH 3 with HCl.[4] The results are shown in Figure 2. The fractions pooled as shown in Figure 2 were adjusted to pH 7 with NaOH and dialyzed against H_2O. Exhaustive dialysis should be avoided since insoluble aggregates may form, particularly if a solution of low ionic strength is subsequently frozen and thawed.

Assay for alanine acceptor activity, complete digestion with RNase T-1, and DEAE cellulose chromatography were carried out as before.[1]

Results.—Pure $tRNA_{II}^{Ala}$ was partially digested with RNase T-1 as described in *Materials and Methods*, and the mixture was fractionated by gel filtration on Sephadex G-100 at 56°. The elution pattern is shown in Figure 1. The retention constant,[5] R, for the first peak was 0.69, which is identical with that of the starting material. R for the second peak was 0.54. This is close to the value expected for a fragment containing 35–40 nucleotides.[6] An oligonucleotide of this size would in turn correspond to "half" molecules produced by cleavage of $tRNA_{II}^{Ala}$ at the G residue in the anticodon. The yield of fragments with R values significantly smaller than 0.5 was surprisingly low. In fact, changing

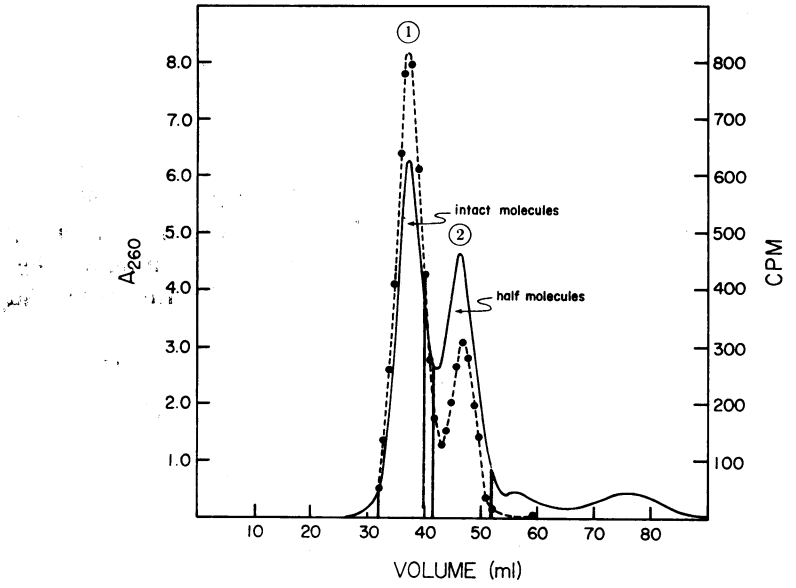

FIG. 1.—Separation of intact $tRNA_{II}^{Ala}$ from large fragments by gel filtration at 56°. See *Materials and Methods* for details. A_{260}, *solid line;* alanine-acceptor activity, *dashed line.*

the conditions slightly has given yields of peak 2 material that exceed 90 per cent of the starting material that has reacted.

When the fractions from the Sephadex column were assayed for alanine-acceptor activity, both peaks were found to contain active material (Fig. 1). However, the specific activity of peak 2 material was only half that of the intact molecules. In order to establish that the second peak really represented "half" molecules, and to see whether both fragments were necessary for acceptor activity, the material from peak 2 was fractionated further. The results of chromatography on DEAE-cellulose at pH 3 in the presence of 7 M urea are shown in Figure 2. A clean separation of two large fragments was obtained. These fragments were digested with RNase T-1, and the oligonucleotides were compared with those derived from a similar digestion of intact $tRNA_{II}^{Ala}$. The

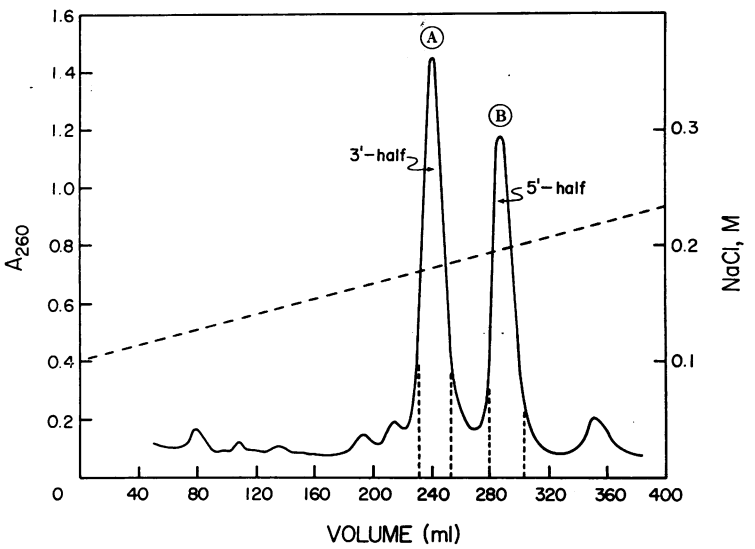

FIG. 2.—Separation of 5'-ended and 3'-ended "halves" of $tRNA_{II}^{Ala}$ by DEAE-cellulose chromatography. See *Materials and Methods* for details.

data are shown in Table 1. The presence of pGp in the digest of peak *B* material, but not in peak *A*, established peak *B* material as the 5'-phosphate-ended fragment. The oligonucleotides derived from this fragment account for 37 of 78 nucleotides in $tRNA_{II}^{Ala}$. This is consistent with a cleavage at the G residue in the anticodon and places a G residue at exactly the same position as in $tRNA_{Iab.}^{Ala}$[2] Examination of the oligonucleotides derived from peak *A*, which

TABLE 1. *Oligonucleotides*[a] *produced by complete RNase T-1 digestion of "half" molecules derived from* $tRNA_{II}^{Ala}$.

Peak number[b]	Peak *A* (3' half)	Peak *B* (5' half)
1,2	—	$C\text{-}m_2^2G > p$
3	4 Gp	4 Gp
4	C-Gp	3 C-Gp
5	A-Gp	—
6	—	H-H-Gp
7	U-U-Gp	H-A-Gp
8	—	C-A-Gp
10	A-A-Gp	pGp
11	T-Ψ-C-Gp	—
9b	C-A-A-Gp + U-C-C-A-C-C-AOH[c]	—
12	—	$C\text{-}A\text{-}C\text{-}A\text{-}U\text{-}m_1G > p$
13	C-C-A-U-C-Gp	—
14	A-U-U-C-C-Gp	—
15	—	$C(C_3U_3)U\text{-}Gp$
	(41 residues)	(37 residues)

[a] The sequences indicated here have been established. The details will be published separately. The modified nucleosides are abbreviated: H, dihydrouridine; m_2^2G, 2-dimethylguanosine; Ψ, pseudouridine; m_1G, 1-methylguanosine; T, ribothymidine; >p, cyclic phosphate.

[b] Reeves *et al.*[1]

[c] These two oligonucleotides were separated by rechromatography after being treated with alkaline phosphatase to remove the terminal phosphate from the tetranucleotide.

contains 41 nucleotide residues, showed that this material had a heptanucleotide sequence (peak 9*b*) which was identical with the sequence at the acceptor end of $tRNA^{Ala}_{Iab}$. Thus, the oligonucleotide (peak *A*) is assigned as 3′-OH-ended half. Fragments *A* and *B* account for the entire $tRNA^{Ala}_{II}$ molecule (78 nucleotide residues).

Alanine-acceptor activity assays indicated that neither fragment had significant activity by itself (Table 2). When the two fragments were mixed in 20 mM NaCl at 25° and assayed, one third of the total activity was regained. This established that *both* fragments are necessary for acceptor activity. A number of additional experiments have been conducted in an effort to explain why only one third of the initial activity was restored when the fragments were mixed in dilute salt and then assayed in the presence of Mg^{+2} at 25°.

TABLE 2. *Reconstitution of alanine-acceptor activity from "half" molecules.*

	Ala esterified (pmoles)[a]	Specific activity (pmoles/A_{260})[b]	Recovery of activity (%)
$tRNA^{Ala}_{II}$, intact	37.0	1520	—
3′ Fragment,[c] untreated[d]	0.5	13	<1
3′ Fragment,[c] treated[e]	0.6	16	1
5′ Fragment,[f] untreated[d]	2.0	56	4
5′ Fragment,[f] treated[e]	1.8	51	3
3′ + 5′ Fragments,[g] untreated[d]	17.4	512	34
3′ + 5′ Fragments,[g] treated[e]	41.9	1230	81

[a] Assayed as described by Reeves *et al.*[1]

[b] A_{260} of intact $tRNA^{Ala}$ solution was measured in water. Those of fragments were measured in 20 mM NaCl solution. When A_{260} is measured in the presence of Mg^{+2}, the specific activity is ~1700. In the calcuiation of specific activity we neglected the hypochromic effect.

[c] From peak *A*, Fig. 2.

[d] Assayed without any heating and cooling cycle.

[e] Heated to 56° for 4 min in 0.075 *M* Tris HCl (pH 7.0) + 0.01 *M* NaCl + 0.075 *M* $MgCl_2$ + 0.0015 *M* EDTA + 0.0125 *M* ATP + 0.0001 *M* (C^{14}) Ala; allowed to cool to 45° during 25 min; transferred to 25° water bath; preincubated for 10 min; and then assayed in the usual manner.

[f] From peak *B*, Fig. 2.

[g] 3′ Fragment (0.019 A_{260}) + 5′ fragment (0.015 A_{260}). This represents the peak area ratio for 3 and 5′ halves shown in Fig. 2

Time-course experiments indicated that aminoacylation reached a plateau in about 10 minutes and that no change in activity occurred during an additional 20-minute incubation. This rules out a simple equilibrium between combined and uncombined fragments under the assay conditions.

When the fragments were heated and slowly cooled in the presence of all assay components except the enzyme (see footnote *e*, Table 2), 81 per cent of the initial activity was restored (last line of Table 2). These mixing experiments suggest that *under these assay conditions* one or both of the fragments exist in conformations that are unable to combine with the opposite fragment to produce an active complex. These "incompetent" fragments can actually be separated from the active duplex by gel filtration *in the presence of* Mg^{+2} *at 25°* (Fig. 3). First, fragments were mixed under conditions where only one third of the initial activity is restored when Mg^{+2} is added (see Table 2). The solid line in Figure 3 represents gel filtration of this mixture. The peak at $R = 0.58$ corresponds to the combined fragments (intact $tRNA^{Ala}_{II}$, $R = 0.58$). This

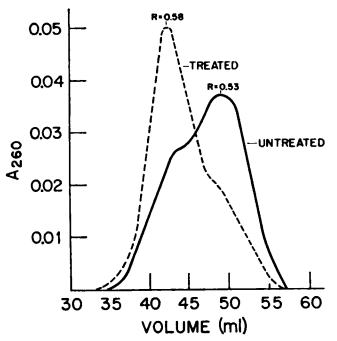

FIG. 3.—Separation of the active complex from uncombined fragments by gel filtration at 25° in the presence of Mg^{+2}. Column: 1 × 100-cm Sephadex G-100 equilibrated with 0.02 *M* $MgCl_2$ + 0.1 *M* NaCl. *Solid line*, fragments mixed as described in footnote *e* of Table 2, except that ATP and Ala were omitted. *Dashed line*, fragments mixed as above and then heated and cooled as described in footnote *e* of Table 2.

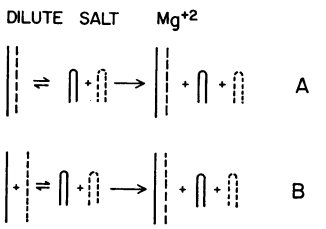

FIG. 4.—Models illustrating two possible distributions of $tRNA_{II}^{Ala}$ fragments under different ionic conditions. *Solid line*, 3′ "half"; *dashed line*, 5′ "half"; *duplex structures* (e.g., upper left), combined "halves"; *straight lines*, competent "halves"; *hairpin structures*, incompetent "halves."

peak area is approximately one-half that of the uncombined fragments (R= 0.52; 5′-phosphate-ended fragment = 0.53). After a heating and cooling cycle under conditions that restored 81 per cent of the initial activity (see Table 2), the peak areas change to those shown by the dashed line in Figure 3. The change in peak area is consistent with the change in activity. These results confirm that an equilibrium does *not* exist between incompetent molecules and the active duplex in the presence of Mg^{+2} at 25°.

At least two models are consistent with our results. They are shown schematically in Figure 4. The product distribution in both models is the same and consists of uncombined, incompetent fragments and the duplex with acceptor activity. The models differ in one important respect: In the first (A), a duplex is formed when the fragments are mixed in dilute salt, and an equilibrium exists between this duplex and the uncombined, incompetent fragments. When Mg^{+2} is added, these conformations are "locked in" to give a mixture of incompetent "halves" and a duplex with acceptor activity. In the second model (B), an equilibrium exists in dilute salt between competent and incompetent conformations. No duplex is present. When Mg^{+2} is added, competent conformations combine to form a duplex with acceptor activity. The incompetent conformations remain unchanged, but they are now "locked" in this conformation.

These models were examined by gel filtration *in dilute salt at 25°*. Model A predicts the presence of a single peak with an R value somewhere between the peak for intact $tRNA_{II}^{Ala}$ (R = 0.67) and the peak for half molecules (5′-phosphate-ended fragment, R = 0.57). Model B predicts the presence of a single with an R value identical to that of the fragments. The fragments were mixed in dilute salt and annealed *in the presence of* Mg^{+2} under conditions which restored

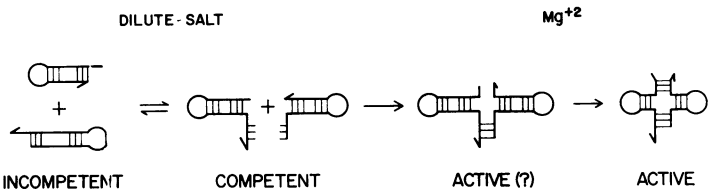

FIG. 5.—A diagrammatic view of the reconstitution of alanine-acceptor activity from "half" molecules derived from $tRNA_{II}^{Ala}$. Our data do not indicate whether the complex formed by the initial combination of fragments is active (*active ?*) or whether rearrangements of ordered structure must occur before acceptor activity is restored.

more than 80 per cent of the initial activity (footnote *e*, Table 2). The mixture was then poured onto a Sephadex G-100 column that had been equilibrated with 0.1 *M* NaCl + 0.01 *M* sodium phosphate, pH 7.5, at 25°. We have previously established that gel filtration effectively removes Mg^{+2} from tRNA under these conditions.[7] A single, sharp, symmetrical peak in the position of uncombined fragments ($R = 0.56$) was obtained. These results rule out model *A* and demonstrate that the active complex formed in the presence of Mg^{+2} dissociates at 25° in dilute salt when the Mg^{+2} is removed.

Discussion.—The above results show that the combination of fragments to form an active duplex is thermodynamically unfavorable in dilute salt. In the presence of Mg^{+2}, on the other hand, combination is thermodynamically favorable, but a kinetic barrier exists at room temperature between incompetent fragments and competent fragments. The recent work of Scheffler *et al.*[8] suggests a possible mechanism for the combination of competent fragments and the reconstitution of acceptor activity. This is shown schematically in Figure 5.

It is particularly significant that the formation of ordered structures between the two fragments of $tRNA_{II}^{Ala}$ is apparently able to overcome the unfavorable loss in entropy involved in combining two molecules. These experiments indicate that a strong driving force must exist which, given the proper conditions, ensures that the tRNA molecule will assume its biologically active conformation. In contrast to these results, it was not possible to reconstitute acceptor activity from similar fragments derived from $tRNA_{Iab}^{Ala}$.[9] The reasons for this difference between $tRNA_{Iab}^{Ala}$ and $tRNA_{II}^{Ala}$ are not clear, but it may be simply that the proper annealing conditions have not been found. In fact, Bayev *et al.*[10] have reported that valine-acceptor activity can be reconstituted from "half" molecules of $tRNA_{I}^{Val}$ isolated from yeast, and, more recently, a similar observation has been made by Oda *et al.*,[11] using $tRNA^{Val}$ from *E. coli*.

These findings open a new dimension in structure action studies on tRNA. Modification and recombination studies may provide a means to map the loci that are required for biological activity of these tRNA's. Experiments with $tRNA_{II}^{Ala}$ are in progress in this laboratory.

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