*TRANSFER RNA, II. A STRUCTURAL BASIS FOR ALANINE ACCEPTOR ACTIVITY**

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In an effort to elucidate the chemical basis for recognition of a transfer RNA (tRNA) by its aminoacyl synthetase, we have been studying the inactivation of purified $tRNA^{ala\ Iab}$ (ref. 1) by ultraviolet light. There are four known photoreactions that might cause inactivation of tRNA:[3] (1) photohydration of C and U residues; (2) dimerization of adjacent C and U residues; (3) chain cleavage at ψ residues;[4] (4) modification of ψ residues *without* chain cleavage.[5] We investigated the pseudouridine cleavage reaction in detail and found that inactivation of alanine acceptor activity can occur without chain cleavage.[4, 5] In this paper, we shall confine ourselves to inactivation of intact molecules.

The potential targets for these photoreactions are shown in Figure 1. These targets are not equally sensitive, however. The reaction cross sections for the four above reactions are different at the nucleotide level, and ordered structure alters the reaction cross sections still further in nucleic acids.[6–8] It is not surprising, then, that the irradiation conditions play an important role on the photochemistry of $tRNA^{ala\ Iab}$.

In dilute salt, inactivation of alanine acceptor activity is rapid and complex.[9] The inactivation shows a D_2O isotope effect of 1.8, an indication that photohydration is an inactivating event.[9] Although the photoproducts formed under these conditions have not been examined further, it is probable that most of the targets shown in Figure 1 react.

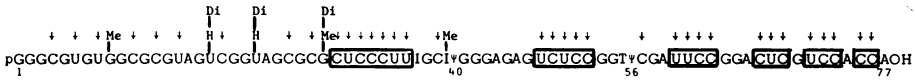

FIG. 1.—Primary sequence of $tRNA^{ala\ Iab}$.[2] *Arrows:* possible sites of photohydration; *boxes:* possible sites of photodimerization. Pseudouridine residues occur at positions 40 and 56 from the Gp end of the molecule.

In the presence of Mg^{2+}, inactivation is much slower and the kinetics are first-order.[9] This indicates that a single photoreaction is sufficient to inactivate the acceptor activity. This does not mean, of course, that a single target is necessarily involved, but if more than one target exists, a hit in any one of them is an inactivating event.

In the presence of Mg^{2+}, inactivation does *not* show any D_2O isotope effect.[9] Thus, photohydration is not a necessary event for the occurrence of inactivation. This finding plays an important role in the location of the inactivation target in that it rules out 11 isolated pyrimidines (hydration targets, Fig. 1) as inactivation sites and leaves only the dimer targets and the ψ residues for further consideration. With one exception, these targets occur in separate

oligonucleotides when $tRNA^{ala}$ is digested with T1 RNase. These oligonucleotides give a characteristic profile when they are fractionated on DEAE-cellulose in the presence of 7 *M* urea.[10] Since all the known photoreactions lead to a loss in A_{260}, changes in the targets can be followed by measuring absorbance changes in the oligonucleotide profile after irradiation of $tRNA^{ala}$ and digestion with T1 RNase.

In order to correlate structural changes with loss in alanine acceptor activity, it is necessary to start with material having full *biological activity* and to separate active and inactive molecules after irradiation. Under these conditions, one can distinguish modifications which have no effect on acceptor activity from those which cause inactivation. The work reported in this paper was carried out with this experimental design in mind. The results suggest that $tRNA^{ala\ Iab}$ is inactivated by formation of a single photoproduct located in the "stem" nucleotides near the acceptor end of the molecule.

Materials and Methods.—The preparation of yeast aminoacyl-tRNA synthetase, assay of $tRNA^{ala}$, preparation of pure $tRNA^{ala\ Iab}$, T1 RNase digestion, and DEAE-cellulose chromatography are described in paper I.[11]

Irradiation of tRNA and separation of active and inactive molecules: Pure $tRNA^{ala\ Iab}$ (specific activity 1700 pmoles/A_{260})[11] was irradiated with 5 $\mu E/cm^2$ of 254 nm light in the presence of 0.01 *M* Mg^{2+}. Loss of alanine acceptor activity (60%) followed first-order kinetics and was accompanied by a 4.4% loss of A_{260}. Although $tRNA^{ala\ Iab}$ is quite resistant to *inactivation* under these conditions, it does undergo extensive photochemical modification. In fact, examination of the oligonucleotide pattern (not shown) after T1 RNase digestion of the irradiated $tRNA^{ala}$ indicated that most of the targets have been altered to some degree.

In order to correlate the photochemical changes with loss of acceptor activity, the active and inactive molecules were separated. This was accomplished by Tener's method: First the active molecules were esterified enzymatically with alanine and then the ala-$tRNA^{ala}$ was derivatized with the *N*-hydroxysuccinimide ester of phenoxyacetic acid.[12] The derivatized, active molecules were separated from the inactive molecules by chromatography on BD-cellulose,[13] as shown in Figure 2. The *inactive* material was eluted in

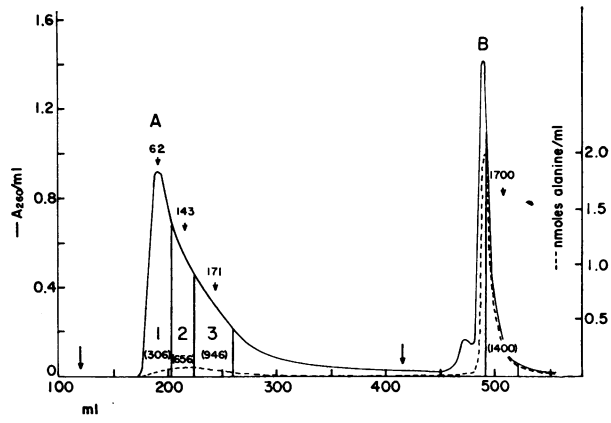

FIG. 2.—Separation of active and inactive alanine tRNA after irradiation. Stepwise elution on a 2 × 19-cm column of BD-cellulose: 100 ml of solution I (0.01 *M* $MgSO_4$ + 0.01 *M* NaOAc + 0.3 *M* NaCl, pH 4.5) followed by 300 ml of solution II (0.01 *M* $MgSO_4$ + 0.01 *M* NaOAc + 0.8 *M* NaCl, pH 4.5) to elute peak A, and then 130 ml of solution III (0.01 *M* $MgSO_4$ + 0.01 *M* NaOAc + 1 *M* NaCl + 10% ethanol, pH 4.5) to elute peak B. ———, Absorbance at 260 nm. — — —, Radioactivity. Numbers above the arrows indicate specific activities after irradiation; numbers in parentheses indicate specific activities after isolation of each fraction (see text).

peak A. The small amount of radioactivity seen in this peak is due to a small fraction of ala-tRNA$^{ala\ Iab}$ (active molecules) that failed to react with the phenoxyacetyl ester. The derivatized ala-tRNA$^{ala\ Iab}$ was eluted in peak B. The peak B material, pooled as shown in Figure 2, had a specific activity of 1700 pmoles/A_{260}. After removal of the phenoxyacetylalanyl group from this material, the resulting tRNA had a specific activity of 1400 pmoles/A_{260}. The reason for this small drop in specific activity (20%) of the modified, active tRNA compared to the starting material (1700 pmoles/A_{260}) is not clear. It is important to note, however, that four out of five molecules in the active fraction accepted alanine.

Results and Discussion.—Active, modified tRNA^{ala} from peak B (Fig. 2) was digested with T1 RNase, and the oligonucleotides were fractionated on *O*-(diethylaminoethyl) cellulose (DEAE-cellulose) in the presence of 7 *M* urea. The oligonucleotide pattern is shown in Figure 3*A*. When the pattern is compared with that of the control, it is clear that many of the targets have undergone extensive photochemistry. Peak 13ab, for example, has disappeared completely. This means that *every* molecule in this population has undergone photomodification in this region. Therefore, this region clearly cannot be involved in the inactivation process. Peaks 14 and 15 have decreased by more than 50 per cent. Thus, a large proportion of the active molecules have also been hit in these regions, and these targets may be ruled out as inactivation sites. Several photohydration targets also undergo extensive change without inactivating the acceptor activity (peaks 1, 2, 8, and 11). This supports our conclusion from kinetic studies that photohydration is not an inactivating event when the tRNA is irradiated in the presence of Mg^{2+}.

These results demonstrate that the primary structure of tRNA^{ala} can be modified considerably without destroying its acceptor activity. Studies of heat denaturation and circular dichroism spectra indicate that large changes in conformation accompany photochemical alteration of the primary structure. These results, combined with the kinetic data showing that ultraviolet (UV) inactivation of alanine acceptor activity is sensitive to changes in ordered structure, form the basis for our conclusion that a *localized ordered structure is necessary for acceptor activity, but a unique conformation of the entire molecule is not required to maintain the stereochemical integrity of the recognition site.*

The data in Figure 3*A* rule out as inactivation sites the dimer targets in peaks 13, 14, and 15 as well as the ψ target in peak 11, since these peaks undergo dramatic changes without loss of acceptor activity. This leaves the ψ target in peak 10 and the adjacent pyrimidines (potential dimers) in peaks 9b and 12 as possible inactivation targets.

The *photoinactivated* tRNA (peak *A*, Fig. 2) was examined next. This peak contained a small amount of radioactivity due to the failure of some of the active molecules to react with the phenoxyacetyl ester. These active molecules were not equally distributed throughout the peak, and the specific activities of different sections are indicated above the arrows in Figure 2. Fragments were removed from each of the pooled fractions by gel filtration on Sephadex G-100 at 56°.[4] Reassay of the intact molecules obtained showed that partial recovery of acceptor activity had occurred. The final specific activities are shown in parentheses in Figure 2. Approximately 18 per cent of the molecules in fraction 1 and 39 per cent in fraction 2 were now active.

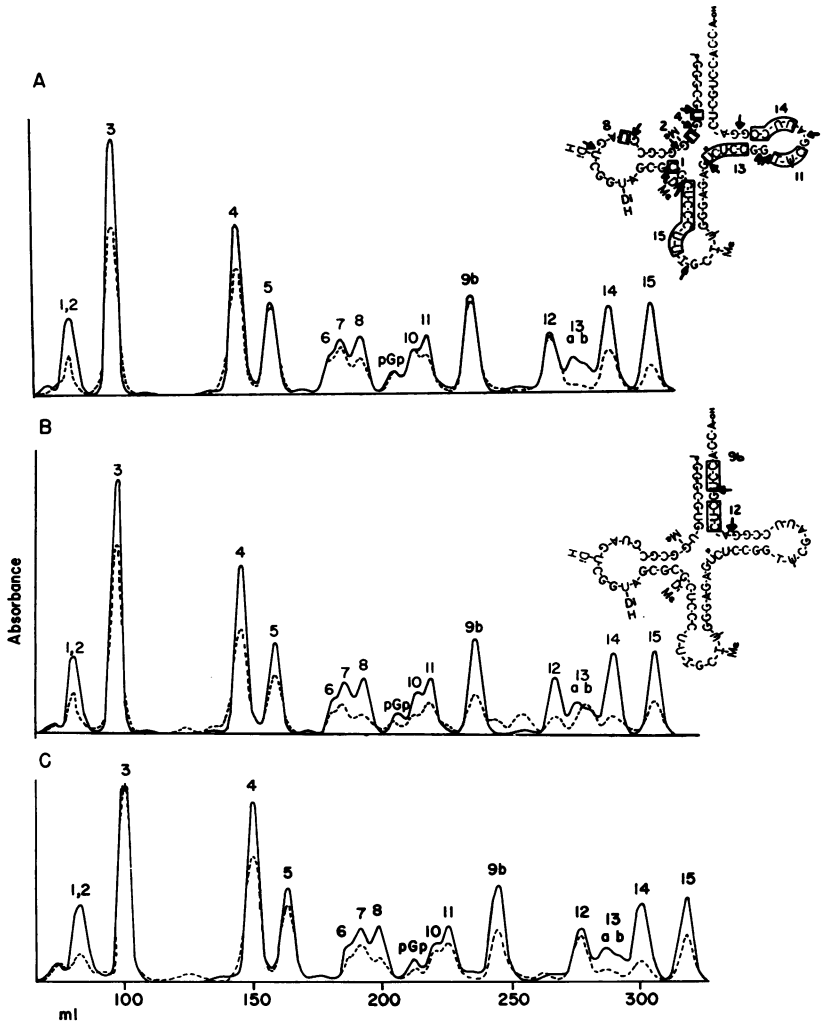

FIG. 3.—Fractionation of T1 RNase digests on DEAE-cellulose. Gradient elution from 0.5 × 120-cm columns as described previously.[11] ———, Control (acceptor activity 1700 pmoles/A_{260}).

(*A*) — — —, Irradiated $tRNA^{ala\ I}$ from peak B of Fig. 2 (acceptor activity 1400 pmoles/A_{260}). Boxes in the structure indicate the pyrimidine residues in the regions undergoing photoreactions.

(*B*) — — —, Irradiated $tRNA^{ala\ I}$ from peak A, section 1, of Fig. 2 (acceptor activity 306 pmoles/A_{260}). Boxes in the structure indicate possible sites of UV inactivation.

(*C*) — — —, Irradiated $tRNA^{ala\ I}$ from peak A, section 2, of Fig. 2 (acceptor activity 656 pmoles/A_{260}). This fraction has recovered 21% more activity than the irradiated $tRNA^{ala\ I}$ shown in Fig. 3*B*.

Fraction 1 (82% of the molecules inactive) was digested with T1 RNase and examined by DEAE chromatography. The results are shown in Figure 3*B*. Peaks 10, 9b, and 12 are now dramatically reduced, compared to the control; this indicates that any or all of these targets may be involved in inactivation.

The ψ residue in the oligonucleotide of peak 10 can be eliminated as an *essential* inactivation target on the following grounds: The reaction cross section for the conversion of ψ to the unknown photoproduct, X, is too small[5] to account for the 60 per cent inactivation observed in this experiment. Furthermore, studies on cyanoethylation of $tRNA^{ala}$ have shown that this particular ψ residue can be modified in 40 per cent of the molecules with only an 8 per cent loss in alanine acceptor activity.[14]

We conclude, therefore, that the UV-inactivation site lies in the oligonucleotide sequences corresponding to peak 9b or 12, or both. This target is located in the 12 terminal nucleotides at the acceptor end. Furthermore, the kinetic data indicate that formation of one of the five potential dimers in this area is sufficient to inactivate the molecule.

Studies on poly dI·dC[7] and CpC[15] have shown that the yield of CC dimers is only 10–15 per cent of the theoretical maximum when the irradiation is carried out at 280 nm. The yield decreases to 7–4 per cent at 265 nm and 2 per cent at 240 nm.[7, 15] Therefore, under the conditions we are using (254 nm), the yield is probably <5 per cent. Thus, formation of a dimer at the CCA end is insufficient to explain the observed loss of activity. Furthermore, this potential target cannot explain the effect of ordered structure on the inactivation kinetics. Therefore, the inactivation target lies in the double-stranded region near the acceptor end as shown in Figure 3*B*.

The oligonucleotide pattern in Figure 3*C* was obtained from material isolated from peak A, fraction 2 of Figure 2. After work-up, this material had 21 per cent more acceptor activity than the material shown in Figure 3*B*. Both peaks 9b and 12 increased at the same time that the activ ty returned. Peak 12, however, returned almost to normal, whereas peak 9b recovered to only a small extent. In addition, it was found by examination of several such patterns that the maximum recovery of 9b is equal to the maximum recovery of activity. This, combined with the first-order inactivation kinetics, suggests that *formation of a single dimer between residues 5 and 6 or 6 and 7 (counting from the acceptor end) is sufficient to inactivate* $tRNA^{ala\ Iab}$.

It must be emphasized that a single *inactivation target* is not *required* by our data. We cannot say that a particular dimer is the *sole* cause of inactivation, nor can we be sure that *all* the inactivation is confined to hits in residues 5, 6, and 7. It is clear, however, that these nucleotides constitute a major inactivation target. Therefore, contrary to the conclusion reached by other workers,[16, 17] this region of the tRNA molecule must play an important role in esterification of alanine to $tRNA^{ala}$ by the alanyl-$tRNA^{ala}$ synthetase.

The change in the oligonucleotide pattern and the partial recovery of activity in an inactive fraction *without further irradiation* requires an explanation because our data show that dimer formation must be the inactivating event, and dimers require UV irradiation for reversal.

The mechanism of the photoreactions in the regions of peaks 9b and 12 will be discussed in detail in a subsequent publication. Briefly, dimer formation occurs first in this region of ordered structure and is followed by rapid photohydration of adjacent pyrimidine residues. As the irradiation is continued,

the dimers undergo slow photochemical reversal, but the molecules remain inactive due to the presence of the hydrate. Those inactive molecules that contain only C hydrates when the light is turned off are able to undergo thermal reversal during the work-up procedure.[18] This leads to restoration of a normal stem region in a certain fraction of the molecules and partial recovery of alanine acceptor activity.

Relationship of the UV-inactivation site to aminoacyl acceptor activity: Our data show that the UV-inactivation site must lie in an ordered structure region and that photomodification of residues 5, 6, and 7 (counting from the acceptor end) destroys the alanine acceptor activity. It follows that these residues must play some role in esterification of alanine to $tRNA^{ala}$ by alanine-$tRNA^{ala}$ synthetase. Formally, a number of possibilities for this role exist. These residues might (1) be the specific recognition site[19] (or part of it) for the synthetase; (2) exert some essential effect on the recognition site through ordered structure; (3) play some critical role in the esterification reaction without having any effect on the recognition site. Modification experiments on tRNA, by themselves, cannot distinguish between these possibilities. The possibilities can be examined, however, with the aid of structural data from the literature.

In order to maintain absolute specificity in the recognition process, the recognition sites must be *different* (1) in tRNA's with different acceptor activities within a pure strain of a species and (2) in tRNA's which are *not* aminoacylated by heterologous enzymes. As shown in Figure 4, residues 5, 6, and 7 from the acceptor end of several tRNA's, whose structures are known, fulfill these requirements. This sequence is unique in $tRNA^{ala,\ ser,\ val,\ tyr,\ phe}_{yeast}$ and in $tRNA^{ala,\ tyr,\ phe,\ F\text{-}met}_{coli}$, satisfying condition (1), as well as in $tRNA_{coli\ and\ yeast}^{tyr}$ and $tRNA_{coli\ and\ yeast}^{phe}$, satisfying condition (2). In addition, this sequence is the same for pairs of tRNA with the same acceptor activity which are aminoacylated by heterologous enzymes ($tRNA_{yeast\ and\ coli}^{ala}$, $tRNA^{ser\ I\ and\ II}_{yeast}$ and $tRNA_{yeast\ and\ wheat\ germ}^{phe}$). However, structural *identity* of tRNA's that are substrates for heterologous enzymes is not an *absolute* requirement. It is possible that structural degeneracy exists in the recognition site so that tRNA's with the *same* acceptor activity may have *either* the same or different recognition sites.

Possible involvement of the peak-12 region in the inactivation of $tRNA^{ala\ Iab}$ plus the fact that three base pairs are probably insufficient to maintain the structural integrity of the first three base pairs suggests that the entire stem region in the cloverleaf model may be necessary for activity. However, the pairs corresponding to residues 8, 9, 10, and 11 cannot be a *specific* recognition site by themselves because the corresponding sequences in $tRNA^{tyr}_{coli}$ and $tRNA^{F\text{-}met}_{coli}$ are identical.

On this basis, we wish to propose the *hypothesis* that *the specific recognition site for the aminoacyl-tRNA synthetase involves the first three base pairs of tRNA as shown in Figure 4, but the entire stem region plays a role in maintaining the stereochemical integrity of the recognition site.* This hypothesis makes some definite predictions and suggests a number of experiments that can be done to test the fundamental ideas contained in it. For example, the 5, 6, 7 sequence from the acceptor end must be unique for each tRNA which accepts a different

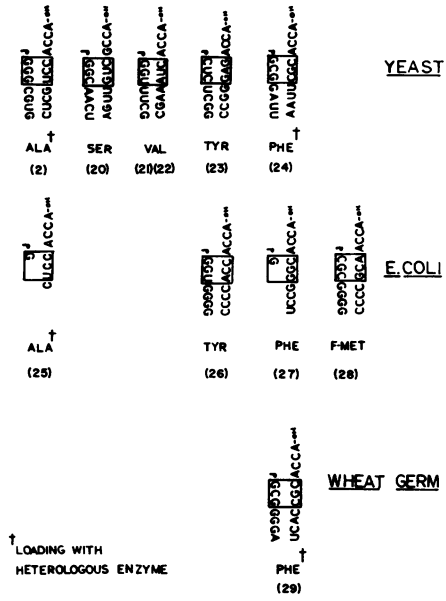

FIG. 4.—Postulated specific recognition sites for aminoacyl synthetases in tRNA. Numbers in parentheses indicate literature references.

amino acid within a species. In addition, if *only* the "stem" nucleotides (Fig. 4) are necessary for aminoacylation, then the 5, 6, 7 sequence must be unique for tRNA's that are not aminoacylated by heterologous enzymes, and the correctly ordered complex derived from *fragments* corresponding to this area should be a substrate for the corresponding synthetase. Rate studies of active, modified tRNA or of tRNA's aminoacylated by heterologous enzymes may indicate whether any additional structural elements are required. Finally, chemical alteration of a single base in the recognition region (Fig. 4) might be lethal (if ordered structure is disrupted) or it might change the aminoacyl specificity. A mutation of this kind occurring in *DNA*, *within the species*, could give rise to the long-sought suppressor tRNA with a "normal" anticodon, but altered aminoacyl specificity.

Summary and Conclusions.—The experiments described in this paper lead us to the following conclusions: (1) $tRNA^{ala\ Iab}$ undergoes extensive photochemical modification without loss of its acceptor activity; (2) $tRNA^{ala\ Iab}$ is inactivated by formation of a single photoproduct located in the "stem" nucleotides near the acceptor end of the molecule; (3) *localized* ordered structure is necessary for acceptor activity, but a *unique* conformation of the entire molecule is not necessary.

Based on our UV-inactivation studies and the structural data in the literature for various tRNA's, we suggest that the specific recognition site for the aminoacyl-tRNA synthetase involves the first three base pairs of tRNA as shown in Figure 4. However, the ordered structure of the entire stem region near the acceptor end, and possibly other portions of the molecule, may be necessary for the correct stereochemical alignment of the recognition nucleotides.

* For paper I in this series, see ref. 11. An abbreviated account of this work was presented at an international symposium (in New York) on "Transfer of Genetic Information in Protein Synthesis," sponsored by Miles Laboratories, June 7, 1968. The work was supported by grants from the Health Research Council of the City of New York (U-1530), USPHS (5 RO1 GM07262), and the American Cancer Society (P-476).

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[1] Abbreviations: $tRNA^{ala\ Iab}$, a mixture of alanine tRNA's differing in a single base;[2] pmoles, 10^{-12} moles; μE, 10^{-6} einsteins; C, cytidine; U, uridine; ψ, pseudouridine (5-β-D-ribofuranosyluracil).

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[19] For the purposes of this discussion, the recognition site is defined as the sum total of all structural elements involved in physical or chemical contact with the enzyme (i.e., H-bonds, van der Waal contacts, ionic linkages, etc.).

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