Bisulfite-induced C → U Transitions in Yeast Valine tRNA*

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The reaction of yeast tRNA\textsubscript{\textsuperscript{\textdagger}} with NaHSO\textsubscript{3} at 25° and pH 5.8 has been studied. Six reactive residues have been located. C-17 in loop I is the most reactive (31% conversion) and C-73 in the first base pair of the acceptor stem the least reactive (8%). Three of the remaining reactive residues (C-39 in loop II, C-75 and C-76 near the acceptor stem) react to the same extent (36 to 38%) under the conditions of the experiment. C-37 in the anticodon reacted to a lesser extent (28%) than C-39 (36%), located just 2 residues away in the anticodon loop. No other changes were detected, but kinetic data suggest one or more additional residues may react very slowly. The C → U change in the anticodon (iac → iau) is a missense change (Val → Ile). Both mechanistic considerations and experimental data from the literature show that HSO\textsubscript{3}\textsuperscript{-}-induced deamination of cytosine residues occurs only at unstacked residues. We interpret the quantitative changes in tRNA\textsuperscript{\textdagger} to indicate that C-17 spends a large portion of its lifetime in an unstacked conformation. The stacking lifetimes of C-37, C-39, C-75, and C-76 seem to be similar but not identical. All other cytidine residues are much more tightly stacked. These results are consistent with the folded cloverleaf models that have been proposed from x-ray diffraction studies of yeast tRNA\textsuperscript{\textdagger}. Residues C-46, C-49, C-57, and C-61, which are present in the single-stranded regions of the unfolded cloverleaf structure, do not react, suggesting that they are tightly stacked in solution under the conditions of this experiment. The data also suggest that anticodon loop conformations other than the extremes with five bases stacked on either the 3' or 5' portion of the anticodon stem exist in solution and that the anticodon loop is flexible.

Because of the central position of tRNA in protein synthesis, it has become important to elucidate the relationship of the structure of tRNA to the function of the molecule. One of the approaches we have used involves introduction of C → U transitions by treating the tRNA with bisulfite ions under controllable conditions (1-7). This produces sequence changes that are of particular biological interest because they represent, in principle, the expression of specific mutations in a tRNA gene. By studying the effect that these changes have on the tRNA gene, we hope to gain insight concerning the fundamental chemical rules that underlie the biologically active activity of this macromolecule. Ultimately, the goal is to predict how specific mutations will affect the function of the altered tRNA in vivo.

Although the bisulfite-catalyzed deamination reaction proceeds smoothly (8-11), more than 1 residue always reacts, and 2 different molecular species are possible (n = number of reactive residues). This paper deals with the location of the reactive cytosine residues in baker's yeast tRNA\textsuperscript{\textdagger}. We will describe the position of these reactive sites and the extent to which each reacts under one set of reaction conditions (2.5 × 10\textsuperscript{-3} M tRNA, 2.8 × 10\textsuperscript{-3} M MgCl\textsubscript{2}, 3.2 M NaHSO\textsubscript{3}, pH 5.8, 25°, 3 h). These experiments provide information concerning the complexity of the mixture that must be dealt with in order to determine the effect that each of these changes has on the biological activity of the tRNA. The results also provide some insight concerning the overall conformation of the tRNA and the localized ordered structure that controls the reaction rate at each cytosine residue.

**Experimental Procedures**

Materials

Yeast tRNA\textsubscript{\textdagger} was purified as described previously (12) and had a specific activity of 1,825 pmol/A\textsubscript{260} unit. The material is a mixture of two isoaccepters differing by uridine/dihydrouridine (59-41) modification at position 48 (12). All attempts to separate these isoacceptors have failed. Highly purified, nucleoside-free Val:tRNA ligase is purified from Saccharomyces cerevisiae AS288C (13). Siliconized glassware was employed in handling and using the purified enzyme. Unless specified, the tRNA solution was always 10\textsuperscript{-3} M in Mg\textsuperscript{2+}. All other materials were from sources described previously (1).

Aminocyclization

The assay was carried out as described previously (14) at the following final concentrations: Tris-HCl, pH 7.0, 60 mM; MgCl\textsubscript{2}, 30 mM; EDTA, pH 7.0, 1.2 mM; ATP, 5 mM; l-\textsuperscript{14}CValine, 0.2 mM; tRNA, 6.5 × 10\textsuperscript{-3} M; purified enzyme, 6.5 millionunits/ml; final volume, 0.125 ml. Incubation was carried out at 25° for 120 min.

**Reaction with Sodium Bisulfite**

The kinetic data were measured by carrying out the reaction for different times in sealed capillary tubes. Each reaction mixture was made 10\textsuperscript{-3} M in Mg\textsuperscript{2+}. The solution was concentrated in \textit{vacuo} to 0.1 ml. The absorbance of this solution at 260 nm was measured on a Cary model 14 spectrophotometer by diluting a 10-µl aliquot with 500 µl of 10 mM MgCl\textsubscript{2}. One portion of this solution (~1.0 A\textsubscript{260} unit) was analyzed for its nucleoside composition as described previously (12).

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A second portion (~0.4 A_260 unit) was assayed in duplicate for valine acceptor activity. The inactivation kinetics will be discussed in detail in the next paper. The half-time for inactivation was found to be 3 h under these assay conditions.

For structural analysis, a sample (155 nmol) of tRNA_{Val}^{2567} (specific activity = 1,825 pmol/A_260 units) was treated with freshly prepared 4 M NaHSO_3 for 3 h at 25°C exactly as described for the kinetic measurements. The reaction mixture was worked up as described above. The tRNA fractions were pooled, concentrated, and filtered through a sterile, Millipore membrane (average pore size 0.45 μm). The filtrate was made 0.05 M in MgCl_2. The recovery of the tRNA was 93% based on absorbance. This modified tRNA was used to locate the C → U transition sites by sequence analysis.

Steps 1 and 2: Digestion with Bacterial Alkaline Phosphatase - The oligonucleotide in 100 μl of 0.01 M NaH_2HCO_3, pH 8.0, was digested with 5 μl of Escherichia coli alkaline phosphatase (50 units/ml). The solution was evaporated to dryness under reduced pressure. In order to inactivate the phosphatase activity, the residue containing the nucleotide material was dissolved in 100 μl of 0.005 M EDTA, pH 7.0. After keeping the mixture at room temperature for 20 min, it was heated in a boiling water bath for 1 min and immediately cooled in ice. The heat treatment was repeated, and the solution was evaporated to dryness.

Steps 3 and 4: Degradative Oxidation with Periodate - The residue from Step 1 was dissolved in 40 μl of water, and 20 μl of 0.1 M NaIO_4 was added. After 20 min at 25°C, 20 μl of 1 M cyclohexylamine adjusted to pH 8.0 with HCl was added. The mixture was incubated at 45°C for 2 h. The reaction mixture was cooled to 25°C, and 30 μl of 0.4 M rhamnose and 40 μl of 1 M NH_4HCO_3, pH 8.4, were added. The reaction mixture was kept at 25°C for 30 min, diluted with 1.5 ml of water and applied to a column (0.4 × 3 cm) of DEAE-cellulose (HCO_3 form). The column was eluted with 4 ml of water; the total eluent was concentrated to dryness under reduced pressure. The residue was dissolved in 140 μl of 0.4 M ammonium formate, pH 4.65, and 20-μl aliquots were used for analysis of guanine. The nucleotide material was eluted from the column with 4 ml of 1.5 M NH_4HCO_3, and desalted as described previously (1).

Steps 5 and 6: Digestion with Bacterial Alkaline Phosphatase - The desalted nucleotide material obtained from Step 4 was dissolved in 100 μl of 0.01 M NaH_2HCO_3, pH 8.0. E. coli alkaline phosphatase (5 units/ml, 50 units/ml) was added. The digestion and inactivation of phosphatase were carried out as described in Steps 1 and 2.

Steps 7 and 8: Digestion with RNase U_2 - The residue from Step 6 was dissolved in 80 μl of the 0.05 M sodium acetate, pH 4.5, containing 0.002 M EDTA. RNase U_2 and bovine serum albumin were added (final concentration in 100 μl = 1 unit/ml and 0.1 mg/ml, respectively). The mixture was incubated at 37°C for 2 h and diluted to 2.4 ml with water. The diluted solution was applied to a DEAE-cellulose (HCO_3 form) column (0.4 × 3 cm), and the column was eluted with 4 ml of water. The effluent was evaporated to dryness in vacuo and dissolved in 100 μl of 0.4 M ammonium formate, pH 4.65. Nucleoside analysis of the residue was carried out with 20-μl aliquots. The mononucleotide and dinucleotides that remained on the column were eluted with 4 ml of 1.5 M ammonium bicarbonate, and the effluent was desalted as described earlier.

Step 9: Separation of Mono- and Dinucleotides - The mono- and dinucleotide residues from the previous step were dissolved in 0.02 M Tris HCl buffer, pH 8.0, + 7 μM urea and chromatographed on DEAE-cellulose column (0.5 × 90 cm). The elution was carried out with a linear gradient, 0 to 0.35 M NaCl in 7 μM urea containing 0.02 M Tris HCl, pH 8.0, total volume 500 ml. The absorbance was monitored at 254 nm with a single channel continuous flow absorbance detector at a full-scale of 0.04 absorbance. The dinucleotide peak was desalted and digested with alkaline phosphatase and venom phosphodiesterase in the usual manner. The digestion mixture was evaporated to dryness, dissolved in 60 μl of 0.4 M ammonium formate, pH 4.65, and 20-μl aliquots were analyzed for nucleotide content to give the C → U change at residue 37. Nucleoside analysis of the mononucleotide peak was carried out by the usual procedure. In addition to the mono- and dinucleotide peaks, two additional peaks were observed. Further analysis indicated they did not contain any nucleotide material, and they represent unknown impurities that invariably accumulate during this procedure.

Degradation of 3'-Terminal Oligonucleotide from Peak 15

The degradation was carried out according to Scheme 2 on 2.3 nmol of oligonucleotide 15 isolated from the initial chromatographic fractionation of RNase T₁ digest of HSO₃⁻-modified tRNA. The procedure used was similar to that described above for degradation of oligonucleotide 13 from peak 15.

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RESULTS

Bakers’ yeast tRNA<sub>Val</sub> has 20 cytosine residues (15). The total C → U transitions occurring as a function of the reaction time with HSO<sub>3</sub><sup>-</sup> was measured as described under "Experimental Procedures." The reaction was carried out at 25°C and pH 5.8 in the presence of 2.5 × 10<sup>-5</sup> M tRNA, 2.8 × 10<sup>-3</sup> M MgCl<sub>2</sub> and 3.2 M NaHSO<sub>3</sub>. The results are shown in Fig. 1.

The data do not show a true plateau, and there is a suggestion of a break in the curve at 5 residues. This phenomenon has been observed with several tRNAs<sup>5</sup> (1) although it does not always occur at 5 reactive residues. The increase in uridine residues occurring after 16 h is slow.

Location of Reactive Residues and Measurements of Extent of Reaction—A reaction time of 3 h was selected. This corresponds to the half-time for inactivation of valine acceptor activity measured under standard assay conditions described under "Experimental Procedures." A preparative scale reaction was carried out, worked up, and fractionated as described under "Experimental Procedures." Separation of the oligonucleotides derived from a RNase T<sub>1</sub> digest is shown in Fig. 2.

Fig. 2A shows the control profile obtained with unmodified starting material. The profile obtained with HSO<sub>3</sub>-modified tRNA is shown in Fig. 2B. Each of the oligonucleotides containing a cytidine residue was analyzed for their nucleoside composition. The results are shown in Table I.

Transitions were detected in oligonucleotides 4, 13, and 15. All oligonucleotides, except 1 (U-m'G), 3 (C-G), 5 (C-A-G), and 15 (A-A-A-U-C-A-C-A) were rechromatographed at pH 3.7. An oligonucleotide containing a C → U change usually separates from the unchanged parent at this pH. Each oligonucleotide isolated by rechromatography was analyzed for its nucleoside composition. The only changes that could be detected were found in oligonucleotide 4* (Fig. 2C), 13* and 13** (Fig. 2D), and unfractionated oligonucleotide 15 (Fig. 2F).<sup>6</sup>

The change in oligonucleotide 4 was identified as the sequence change, D-C-G → D-U-G, at position 17 of the tRNA. The data are shown in Table II. The extent of the change was measured in three ways: direct analysis of unfractionated oligonucleotide 4 (Fig. 2B), nucleoside analysis of oligonucleotide 4 and 4* after separation (Fig. 2C) and by corrected peak area measurements of oligonucleotide 4 and 4* (Fig. 2C). As shown in Table II, the agreement between these different methods was excellent. The mean change was 51%, ε = 0.8.

Two changes were identified in oligonucleotide 13. Isolation of oligonucleotide 13** (Fig. 2D) by rechromatographing the mixture of oligonucleotides 13 and 14 (Fig. 2B) followed by nucleoside analysis indicated it had the composition, A-G-U. This corresponds to the sequence change, A-C-A-C-G → A-U-A-U-G, at positions 37 and 39. The change at position 37 represents a missense change in the anticodon. Since an oligonucleotide containing both changes was isolated, both single-hit oligonucleotides must be present in oligonucleotide 13<sup>5</sup>. The ratio of the peak areas corrected for the change in extinction coefficient for C → U was 41:50:9 for oligonucleotides 13<sup>13</sup>, 13, and 13** (Fig. 2D). This shows that the reactivity of C-37 and C-39 is not equal (theory for equal reactivity is 25:50:25).

The actual changes occurring at positions 37 and 39 were quantitatively measured as follows: oligonucleotides 13, 13<sup>5</sup>, and 13** (Fig. 2D) were recombined and degraded as described under "Experimental Procedures." The entire degradation was carried out on 1.65 nmol of material. The results are shown in Table III.

<sup>5</sup>E. Sabban, and R. W. Chambers, unpublished observations.

<sup>6</sup>The peak shown as 15 in Fig. 2F represents a mixture of oligonucleotides derived from C → U transitions in the parent oligonucleotide, A-A-A-U-C-A-C-A. The analysis of these changes is discussed later in the text.
Determination of the percentage change at position 39 is not affected by incomplete oxidation, \( \beta \) elimination, or dephosphorylation since the degradation scheme precludes cross contamination of 37 and 39. The mol fraction, \( \text{Urd}/(\text{Urd + Cyd}) \) for each residue was measured so that physical losses do not influence the quantitative measurements. The results (Table III) indicate 28% conversion at C-37 (anticodon) and 36% at C-39.

The remaining C → U transitions were identified in oligonucleotide 15 derived from the 3' terminus of the tRNA. Since the sequence contains 3 cytidine residues (C-73, C-75, C-76), the change occurring at these positions was determined by degradation as shown in Scheme 2. The degradation was carried out on 2.3 nmol of material from unfractionated peak 15 (Fig. 2B). The details are described under "Experimental Procedures"; the results are shown in Table IV.

The change at residue 75 was obtained by isolating the dinucleotide mixture, A-C- and A-U-, under conditions where no separation of these two could occur. Physical losses were not a factor since the ratio Urd/(Urd + Cyd) was measured, as in all our analyses. The C → U change at C-75 was 38%.

In this degradation, residue 73 can contaminate 76 if the removal of phosphatase activity is incomplete. In that case, A-A-A-U-, A-A-A-U-, and A-\( \overline{C} \) (expected A-\( \overline{C} \)), will be among the products after Step 5. Under the conditions used in Step 6, A-\( \overline{C} \) should appear in the water wash and be detected by nucleoside analysis.

**FIG. 1.** Reaction kinetics for deamination of cytosine residues in yeast tRNA\( _{\text{Val}} \) induced by 3.2 M NaHSO\(_3\) at pH 5.8 and 25°C. For details see "Experimental Procedures." The + mark indicates the half-time for C → U-dependent inactivation of the aminoaclation activity of tRNA\( _{\text{Val}} \). Dashed line represents possible break in kinetic data.

**FIG. 2.** Fractionation of oligonucleotides produced by RNase T\(_1\) digestion of yeast tRNA\( _{\text{Val}} \) and HSO\(_3\)-modified tRNA\( _{\text{Val}} \). For details see "Experimental Procedures." Shaded areas indicate oligonucleotides where C → U transitions were detected by nucleoside analysis. A, oligonucleotide from control tRNA\( _{\text{Val}} \), chromatographed at pH 8. B, oligonucleotides from HSO\(_3\)-modified tRNA\( _{\text{Val}} \), chromatographed at pH 8. C, rechromatography at pH 3.7 of oligonucleotides 13, 13*, 13**, and 14 from HSO\(_3\)-modified tRNA\( _{\text{Val}} \). D, rechromatography at pH 3.7 of oligonucleotides 13, 13*, 13**, and 14 from HSO\(_3\)-modified tRNA\( _{\text{Val}} \); 13 = A-C-A-C-G-; 13* = A-A-A-U-, and 14 = C-A-A-U-G-; 14* = U-U-A-U-G.; 14 = C-A-A-U-G-.
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TABLE I
Nucleoside composition of oligonucleotides produced by RNase T, digestion of bisulfite-modified yeast tRNA<sub>Val</sub>

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Nucleoside content&lt;sup&gt;a&lt;/sup&gt; (molar ratios)</th>
<th>Theory</th>
<th>Found</th>
<th>Residue θ</th>
<th>C → U transitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T-C-G</td>
<td>U&lt;sup&gt;1.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>U-C-G</td>
<td>G&lt;sup&gt;0.99&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>D-C-G</td>
<td>G&lt;sup&gt;1.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>G-C-G</td>
<td>U&lt;sup&gt;0.52&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>0.47</td>
<td>17</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>C-A-G</td>
<td>G&lt;sup&gt;1.00&lt;/sup&gt;A&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>0.97</td>
<td>41</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>C-C-G</td>
<td>G&lt;sup&gt;1.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>C-G</td>
<td>U&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>T-G-C-G</td>
<td>U&lt;sup&gt;2.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>2.00</td>
<td>32, 57</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>A-U-C-G</td>
<td>U&lt;sup&gt;2.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>2.00</td>
<td>61, 62</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>D-D-A-U-G</td>
<td>U&lt;sup&gt;2.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>2.00</td>
<td>6</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>U-G-U-C-D</td>
<td>U&lt;sup&gt;2.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>2.00</td>
<td>12</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>U-C-D-A-G</td>
<td>U&lt;sup&gt;2.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>2.00</td>
<td>26, 29, 37, 39</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>A-C-C-A-G</td>
<td>U&lt;sup&gt;2.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>2.00</td>
<td>73, 75, 76</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>C-A-T-U-G-C-A-G</td>
<td>U&lt;sup&gt;2.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>2.00</td>
<td>46, 49, 51, 52</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>A-A-A-A-C-A-</td>
<td>U&lt;sup&gt;2.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>2.00</td>
<td>46, 49, 51, 52</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>A-A-U-C-D-U-A</td>
<td>U&lt;sup&gt;2.00&lt;/sup&gt;C&lt;sup&gt;1.00&lt;/sup&gt;</td>
<td>2.00</td>
<td>46, 49, 51, 52</td>
<td>No</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Given in order of emergence from the nucleoside analyzer. Data from oligonucleotide 15 are normalized to A; all others are normalized to G

<sup>b</sup> Not analyzed

<sup>c</sup> Equivalent per tRNA molecule

<sup>d</sup> The low value of C obtained in this analysis is not due to C → U transitions in the oligonucleotide 16. Similar low value of C was obtained for this oligonucleotide derived from unmodified tRNA<sub>Val</sub> (12). In order to confirm further that no C → U transition has occurred in oligonucleotide 16 obtained from bisulfite-modified yeast tRNA<sub>Val</sub>, it was rechromatographed at pH 3.7. Only one peak corresponding to the parent oligonucleotide 16 was obtained (Fig. 2E). The reason for the low C value is still unknown.

TABLE II
Cytidine → uridine transitions at residue 17 or yeast tRNA<sub>Val</sub>

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Residue</th>
<th>C → U (fraction/residue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>D-C-G</td>
<td>0.52&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>4*</td>
<td>D-U-G</td>
<td>0.51&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>e</sup> Nucleoside analysis of mixtures.

<sup>f</sup> Nucleoside analysis after separation of parent and progeny oligonucleotides.

<sup>g</sup> Peak area analysis.

<sup>h</sup> Nucleoside analysis of oligonucleotide 4, 4* (Fig. 2B). C → U = (pmol Urd)/(pmol Cyd + pmol Urd).

<sup>i</sup> Nucleoside analysis of oligonucleotides 4 and 4* after separation (Fig. 2C). C → U = (pmol Urd)/(pmol Cyd + pmol Urd).

<sup>j</sup> Corrected peak areas of 4 and 4*. The area of the peak containing the product oligonucleotide was corrected for change in extinction coefficient: Peak area × (sum of extinction coefficients of the nucleotides present in product oligonucleotide)/(sum of extinction coefficients of the nucleotides present in parent oligonucleotide) at the pH of the chromatography. C → U = (peak area 4*)/(peak areas of 4 + 4*).

TABLE III
Cytidine → uridine transitions at residues 37 and 39 of yeast tRNA<sub>Val</sub>

<table>
<thead>
<tr>
<th>Step</th>
<th>Base or nucleotide</th>
<th>Obtained</th>
<th>Recovery</th>
<th>C → U (fraction/residue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Gua</td>
<td>1.45</td>
<td>88</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>U</td>
<td>0.54</td>
<td>91</td>
<td>0.36</td>
</tr>
<tr>
<td>9</td>
<td>C-U</td>
<td>0.35</td>
<td>75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28</td>
</tr>
<tr>
<td>10</td>
<td>C-U</td>
<td>0.89</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Analysis dimonucleotide peak.

<sup>b</sup> Overall recovery from Step 8 is 82%.

A-U by nucleoside analysis as above. No adenosine was detected. We conclude that inactivation of phosphatase was complete, and residue 17 does not containmate residue 76 under the conditions used.

Similarly, residue 76 may containo 73 if oxidation, β elimination, or dephosphorylation are incomplete. Since the recovery of adenine in Step 2 was low (84%), we investigated the oxidation step by analyzing the water wash from Step 6 directly. If oxidation had been incomplete, adenosine would have been released by pancreatic RNase and would appear in the water wash. None was found under the conditions where 2% would have been detected. We conclude that the oxidation was complete.

separated from A-<sup>U</sup><sub>73</sub>. To test this, the water wash was digested with snake venom phosphodiesterase and examined for adenosine; none was found. The fraction eluted with 1.5 M NH₄HCO₃ was also checked for the presence of A-<sup>U</sup><sub>73</sub> and A-A-
Quantitation of the β elimination step and the dephosphorylation of the resulting product is more difficult. We have no independent data to show that the 8% change observed at residue 73 is actually due to a slow HSO₃⁻-induced deamination reaction and not to an artifact of incomplete degradation during structural analysis. However, the following calculation is relevant. The recovery of residue 76 after six steps was 88%. This includes not only any incomplete reactions that might have occurred, but also physical losses during manipulation of the reaction mixtures. If we assume that all losses (12%) are due to incomplete reactions, then a maximum of 0.12 x 2.3 x 0.37 = 0.10 nmol of uridine from residue 76 might contaminate residue 73. This would account for the 0.13 nmol observed. It is unreasonable, however, to assume that the recovery through six steps including two DEAE-cellulose columns was quantitative. Some of the 15% loss must have been due to physical losses. A recovery of 90% represents a reasonable estimate. This would allow a contamination of 0.02 x 2.3 x 0.37 = 0.10 nmol of uridine from residue 76 to contaminate residue 73. This could account for the 0.13 nmol observed.

The results are summarized in Fig. 3. The numbers represent the mole fraction changed at each of the reactive residues. Six reactive residues have been detected under the conditions given above. Sites and that ordered structure markedly reduces the reactivity of the other 15 cytosine residues in this tRNA.

**DISCUSSION**

The experiments described in this paper were designed to produce a C → U missense change at the third position of the anticodon in tRNA_I, and to provide part of the data necessary to determine what effect this specific change has on aminoacylation. The results shown in Fig. 3 show that this change has been produced and that the missense tRNA, tRNA₁ (AUU, AUC, AUA)₅₃ is present in the reaction mixture.

The data in Fig. 3 also show that five other sites (residues 17, 39, 73, 75, and 76) react to varying degrees. Since partial reactions at six different sites have occurred under the conditions used (2.5 x 10⁻⁵ M tRNA, 2.8 x 10⁻⁵ M MgCl₂, 2.5 x 10⁻³ M; pH 5.8; 25°, 3 h), 2⁶ is 64 different molecular species are possible in the reaction mixture. If the reactions at these different sites are independent events, then one can calculate the amount of each molecular species that is actually present from the data given in Fig. 3. We have no independent kinetic data to indicate whether or not each of the reactions is first order, but some of our chromatographic data suggests that the reactions are indeed independent.

For example, the fractionation of oligonucleotide 13 at low pH (Fig. 2D) gives peaks corresponding to no hits (A₅₃C₅₃G), one hit (A₅₃C₅₃G, peak 13⁺) and two hits (A₅₃U₅₃G, peak 13++) for independent events at residues C₃₇ and C₃₉. The distribution should be: no hit, 0.46; one hit, 0.44; two hits, 0.10. We found: no hits, 0.41; one hit, 0.50; two hits, 0.09. The agreement for these 2 cytosine residues with the theoretical values is good, and it seems highly unlikely that cooperative reactivity is involved.
bution for three independent events at C-73, C-74, and C-75 are: no hits, 0.36; one hit, 0.46; two hits, 0.17; three hits, 0.01. We found: no hits, 0.42; one hit, 0.44; two hits, 0.14; three hits, 0. Again, the agreement between the theoretical and experimental values is good. There seems to be no indication in any of our data for cooperative reactivity involving residues 37, 39, 73, 75, and 76. We cannot make any statements regarding residue 17.

If all the reactions are independent, as the data available suggest, then the amount of any given molecular species present can be calculated. For example, the fraction of the total molecular population (ignoring the isosceles problem) that is changed only at the anticodon is (1 - 0.61)(0.28)(1 - 0.36)(1 - 0.09)(1 - 0.38)(1 - 0.37) = 0.032. Isolation at this particular molecular species from the complex mixture presents a serious experimental problem. For this reason, we have chosen an alternative approach for determining the effect that each of the changes, by itself, has on the aminoacylation reaction under standard assay conditions. Our approach has been to separate active from inactive molecules using amino acid acceptor activity as a basis for fractionation (16, 17) and searching for inactivation targets.

The changes shown in Fig. 3 are accompanied by a loss of 45% of the valine acceptor activity. In a preliminary report (3), we have shown that the 3rd position of the anticodon (residue 37) is an inactivation target. The data in Fig. 3 show that only 28% of the molecules have a change in the anticodon, so only 62% of the loss in activity can be due to this change. This result is similar to that found with E. coli tRNA\textsubscript{V}\textsuperscript{w} (18) and is in sharp contrast to the results obtained with yeast tRNA\textsubscript{V} (1), where a similar change in the anticodon does not prevent aminoacylation.

From our preliminary data (3), we suggested that the additional loss of activity might be due to another inactivation target at residue 73 in the acceptor stem. The data in Fig. 3 show that the 8% change at this position is not sufficient to account for 38% inactivation that must come from a change outside the anticodon. It is also clear from our data on the active fraction\textsuperscript{4} that residue 73 is not an inactivation target. From these observations, it became clear that the approach we were using to study structure-action effects is capable of detecting not only all-or-none effects (inactivation targets) but also modulation effects in which a change at a given position produces only a partial loss of activity under standard assay conditions. Further discussion of these activity effects will be deferred to a separate paper dealing specifically with this subject.

In addition to providing data for analyzing activity effects, the changes shown in Fig. 3 give some information concerning the ordered structure of this tRNA. Of the 20 cytosine residues in yeast tRNA\textsubscript{V} (5 residues in the variable loop and 15 in the other secondary structure elements), five (C-17, C-37, C-39, C-75, C-76) react readily under the conditions used. One other (C-73) reacts much more slowly. No reaction was detected at any other residue at a reaction time of 3 h. Any model proposed for the three-dimensional structure of this tRNA must account for these observations.

In a similar paper on tRNA\textsuperscript{A} (1), we have discussed the sensitivity of the HSO\textsubscript{3}--induced C → U reaction to stacking in terms of the electronic and steric requirement of the reaction. From these considerations, it follows that the stacking lifetime of a given residue, according to the simple scheme shown below, will play a critical role in determining the relative rate at which that residue reacts.

\[ \text{C (stacked) } \rightleftharpoons \text{C (unstacked)} \rightleftharpoons \text{U} \]

Our data (Fig. 3) indicate that all of the cytosine residues, except 17, 37, 39, 75, and 76, are tightly stacked since they do not react at a significant rate. Residue 73 in the acceptor stem is not stacked, but it spends a small portion of its lifetime in an unstacked conformation since it reacts slowly under the reaction conditions employed.

The data do not fit the cloverleaf model. This predicts that residues 17, 37, 39, 46, 49, 57, 61, 75, and 76 should react at similar rates since they are in single-stranded regions. Residues 17, 37, 39, 75, and 76 do react, but residues 46, 49, 57, and 61 do not. Thus, the cloverleaf is not an adequate description of the tRNA in solution under the conditions of this experiment, although it may be part of a more ordered structure such as that proposed for yeast tRNA\textsuperscript{w} (19-22).

Fig. 4A shows the sequence of yeast tRNA\textsuperscript{V} folded into the stacking pattern of the MIT-Duke model for tRNA\textsuperscript{w} (21). The residues that actually react are shown by the solid arrows. Additional residues that are in single-stranded regions in the cloverleaf model, but do not react, are shown by broken arrows. This stacking pattern agrees with our results reasonably well. For comparison, the results with yeast tRNA\textsuperscript{A} are shown in Fig. 4B. Except for slightly different reaction times (3 h for tRNA\textsuperscript{V} and 3.25 h for tRNA\textsuperscript{A}), the conditions used to modify these tRNAs were essentially identical.

Residues 49 and 57 in tRNA\textsuperscript{V} occupy positions similar to 48 and 56 in tRNA\textsuperscript{A}. Both are hydrogen-bonded; 49 is stacked on one face. The data suggest both are tightly stacked since they do not react in either tRNA. This is in agreement with the important role that C-49 plays in this tertiary structure (the C-49-G-15 hydrogen-bonded couple).

Residues 46 and 61 in tRNA\textsuperscript{V} correspond to G-45 and U-60 in tRNA\textsuperscript{A}. The failure of these cytidine residues to react in either tRNA suggests that they are also tightly stacked. This provides further evidence for the generalization that the residue in the variable loop and the TVC loop are protected by the tertiary structure of the tRNA and held in a relatively rigid, stacked conformation.

The reactivity of C-17 in the D-loop is different in these two tRNAs. In tRNA\textsuperscript{A}, its reactivity is the same as the 2 cytidines at theCCA end. In tRNA\textsuperscript{V}, C-17 reacts significantly faster than C-75 and C-76 at the CCA end. The nearest neighbor sequence around C-17 is the same in both tRNAs, D-C-G. The tertiary environment (C-49, G-19, U-60 in tRNA\textsuperscript{A}; C-48, G-19, U-50 in tRNA\textsuperscript{V}) is also the same. The D-loop contains 1 more residue (an extra dihydrouracil) in tRNA\textsuperscript{V}. The data suggest that the larger loop leads to a decrease in stacking lifetime at C-17 in tRNA\textsuperscript{V} compared to tRNA\textsuperscript{A}.

Interpretation of the data for the changes in the anticodon loop are complicated by the presence of U-34 which may add HSO\textsubscript{3}--. We have not measured this, but a reaction at this position of tRNA occurs (23). The difference between the reactivity of C-39 and the anticodon, C-37, is small, but it seems to be real. The reason for this difference is not clear. It may be a reflection of different nearest neighbor sequences (24). We can conclude that conformations other than the two extremes (25) shown in Fig. 4A must be possible because C-39 cannot react in either of these. Yet one of these stacked conformations almost certainly exists during codon-anticodon interactions (25, 26). Whether a conformational change in this loop occurs normally in solution or whether it is induced by addition of HSO\textsubscript{3}-- to C-37 or perhaps U-34 is uncertain. Nevertheless, the results indicate the anticodon loop is flexible in solution (1, 26-29).
Bisulfite-induced C $\rightarrow$ U Transitions in Yeast Valine tRNA

FIG. 4. Diagrammatic representation of stacking interactions in A, yeast tRNA$^{Val}$, and B, yeast tRNA$^{Ala}$ based on the MIT-Duke model of tRNA$^{Ala}$ (21). The solid arrows indicate the reactive cytosine residues and dashed arrows point to the unreactive cytosine residues that are present in loop regions of the cloverleaf model.

There is another interesting difference between these two tRNAs. The actual C $\rightarrow$ U kinetics for tRNA$^{Ala}$ does not agree well with the theoretical curve derived from the sequence data by assuming that each reaction is independent. The difference in the curve suggests 2 additional residues react at longer times (1). This could involve unfolding of the tRNA to expose residue 49 (variable loop) and 57 (TΨC loop), although we have no direct, experimental support (sequence data) for this. The data in Fig. 5 show the theoretical curve for

using pseudo-first order rate constants derived from the data in Fig. 3.

The difference between the experimental points and the theoretical curve is very small at the early reaction times. Therefore, it is difficult to decide whether the difference is real or not. At much later reaction times, the difference becomes significant, but even then, the difference is smaller than with tRNA$^{Val}$ (1). The explanation offered for the tRNA$^{Val}$ data seems unlikely for tRNA$^{Val}$ since an unfolding of tertiary structure should expose 4 residues (46, 49, 57, and 61) instead of only two as in the case of tRNA$^{Val}$.

Obviously, additional data are needed to find out which cytosine residues react with increased reaction time, but it seems clear that the tertiary structure of tRNA$^{Val}$ is held together quite firmly under the conditions of this experiment.

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Bisulfite-induced C → U Transitions in Yeast Valine tRNA

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