Effect of Chemical Modification of Dihydrouridine in Yeast Transfer Ribonucleic Acid on Amino Acid Acceptor Activity and Ribosomal Binding

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SUMMARY

Dihydrouridine residues in baker's yeast transfer RNA were reductively cleaved with sodium borohydride. The amino acid acceptor activity of three species of transfer RNA and the ribosomal binding of one species of transfer RNA were determined for these modified transfer RNAs. The data indicate that the reductive cleavage of the dihydrouridine residues in these transfer RNAs has no effect upon their ability to accept amino acids or to bind to ribosomes.

Although the nucleotide sequences of a number of transfer RNAs have been determined (1-6), and specific conformations have been proposed for these, the relationship of the structure to the biological functions of this class of molecules remains largely obscure. One notable exception is the identification, albeit tentative, of one triplet base sequence which is complementary and antiparallel to the specific codon to which the tRNA binds, and which is analogously located in all known tRNA sequences. This is the presumed site of interaction of tRNA and messenger RNA.

One approach to the study of the relationship between structure and function has utilized modification of a fraction of one or more of the four major nucleotides in RNA by various organic reagents. (These studies are reviewed in References 7 and 8; see also Reference 9.) Usually, only a fraction of the nucleotides in a molecule are modified, and the altered molecules produced by these manipulations represent a large class of differently and probably randomly modified molecules. Thus, assignment of function to specific sites in tRNA molecules is difficult, if not impossible (10), since one cannot determine which of the altered molecules have altered function.

Selective modification of one or more of the nucleotides present as minor components of tRNA (11, 12) promises more meaningful conclusions because the possible sites of modification are limited and sometimes restricted to a single region of the tRNA molecule.

Dihydrouridine is a pyrimidine found in small amount (as its nucleotide) in yeast tRNA; a method for its selective reduction in a tRNA molecule to ribosylureidopropanol by treatment with borohydride has been developed (13). In the light of our present ignorance about the function of dihydrouridine in tRNA and of Lindahl's recent suggestion that at least 2 dihydrouridine residues in some yeast tRNAs are necessary for amino acid acceptance, ribosomal binding, and ability to incorporate a terminal adenosine molecule (14), it seemed useful to investigate the effects of reductive cleavage of dihydrouridine on the biological activity of tRNA from yeast.

EXPERIMENTAL PROCEDURE

Bakers' yeast tRNA, prepared essentially by the method of Monier, Stephenson, and Zamecnik (15) as modified by Holley et al. (16), was treated with EDTA as described by Lebowitz et al. (17). tRNAser (more than 95% pure) was further prepared from this material as described by Lebowitz et al. (17).

Borohydride Treatment—Solid sodium borohydride (Metal Hydrides, Inc., Beverly, Massachusetts) was added to a sample of RNA at approximately 120 A250 per ml in 0.02 M MgCl2 and the mixture was incubated at room temperature (9). The amount of sodium borohydride used was chosen to produce a 200-fold molar excess relative to the total amount of dihydrouridine present in the sample. Aliquots were removed at various time intervals up to 2 hours and added to an amount of 1 N HCl (at 4°) equimolar to the amount of NaBH4 in the aliquot. The RNA thus precipitated was centrifuged, washed three times

1 A. A. Bayev, personal communication.
2 The abbreviations used are: tRNA, transfer ribonucleic acid; tRNAε2, tRNAε1, and tRNAε1, transfer ribonucleic acids specific for serine, alanine, and valine, respectively; seryl-tRNA, tRNAε2-aminoclayated with serine; poly C-A, copolymer of cytidine and adenine; poly U-G, copolymer of uridine and guanosine; poly U-C, copolymer of uridine and cytidine.
with 0.01 N HCl, and dissolved in 0.04 M Tris-HCl, pH 7.6. The A_{260} of the resulting solution was measured and appropriate amounts were used for dihydrouridine assay, acceptor activity assay, and ribosomal binding assay (below).

In the control samples the RNA was added to the borohydride solution after the borohydride had been allowed to react with HCl, but otherwise the control samples were treated exactly as the experimental samples were treated.

**Dihydrouridine Assay in RNA**—The sample of RNA, approximately 3 A_{260} per ml, was made 0.1 N in KOH and the time-dependent loss of absorbance at 235 nm and room temperature (read against an appropriate blank) was measured with a Zeiss PMQ II spectrophotometer, a Cary model 14 spectrophotometer, or a Gilford model 2000 multiple sample absorbance recorder (Fig. 1a). When the time-dependent change had reached completion, the final absorbance at 235 nm was subtracted from earlier absorbances, and the difference was plotted on semi-log paper; from this plot the value of the zero time intercept (Δ A_{235}) was obtained by extrapolation (Fig. 1b). The half-time for the loss of Δ A_{260} under these conditions was 8 min. A value of 0.2 × 10^{-4} for the molar extinction coefficient of dihydrouridine at 235 nm, pH 13, was obtained by assay of a sample of yeast tRNA from 0.1 ml of water, and used directly in the ribosomal binding assay.

This value may be compared with the molar extinction coefficient of dihydrouridine in a polynucleotide chain of greater than 95% purity (17), known to contain 3 moles of dihydrouridine per mole of tRNA (2, 6). The lower value for dihydrouridine in a polynucleotide chain is roughly compatible with the degree of hypochromicity remaining under the assay conditions.

The similarity of these values suggests that this assay may, as a first approximation, be valid for the quantitative estimation of dihydrouridine content of RNA. That it may also be qualitatively valid is supported by the fact that there was no change in absorbance at 235 nm of a similarly treated polynucleotide known not to contain dihydrouridine (a mixture of poly U-A (5:1) and poly U-G (1:1))4 (these and other copolymers were obtained from Miles Chemical Company, Clifton, New Jersey).

4 N\(^2\)-Acetylatedidine is deacylated to cytidine by treatment with alkali (29), however, this produces no significant change in A_{235} (L. B. Sheiner, unpublished results).

In our experiments the use of the calculated extinction coefficient, which may have a degree of imprecision, was bypassed by expressing the dihydrouridine content of the treated samples as a percentage of that of the control samples.

\[
\% \text{ Dihydrouridine present} = \frac{\Delta A_{235}}{A_{260}} \times 100
\]

The EP\(^{14}\) values of borohydride-treated and control samples were the same, as were thermal denaturation curves. Thus, no significant hyperchromicity was present in the borohydride-treated samples, and use of the A_{260} of the samples as a measure of the quantity of RNA seems justified.

Owing to inaccuracies in determinations of very small changes in optical density above a large background, the assay probably cannot detect the presence of dihydrouridine when it represents less than 10% of the control value.

**Acceptor Activity Assay**—Amino acid acceptor activity was measured according to the method of Malman and Cantoni (21). A highly purified serine tRNA synthetase (21) was used for assay of serine acceptor activity, and a crude yeast-activating enzyme fraction for assay of other amino acid acceptor activities.

No differences in relative activity of the samples were found in comparisons of acceptor activity for serine when highly purified enzyme (free of adenylate RNA transferase) was used or when the crude yeast-activating enzyme preparation was used. Assays for acceptor activity and for ribosomal binding (see below) were done in duplicate or triplicate, and the average values obtained were considered to be specific amino acid acceptor activity of the borohydride-treated samples is expressed as a percentage of that of the control.

**Ribosomal Binding Assay**—Ribosomes were prepared from a low nucleoside strain of *Escherichia coli* (MRE 600, kindly supplied by Dr. P. Leder) by the method of Nirenberg (22), and ribosomal binding of seryl-tRNA was determined according to the method of Nirenberg and Leder (23), with the use of copolymer U-C (1:1). Uniformly labeled \(^{14}\)C-serine (122 mC per mmole) was obtained from New England Nuclear. Radioactivity of samples was determined in a Packard model 3003 TriCarb liquid scintillation spectrometer.

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The final volume was 1.0 ml. After incubation, the reaction mixture was shaken with an equal volume of 90% phenol. The water layer was removed, diluted to 2 ml, and dialyzed overnight (at 4°C) against two changes of 3 liters of water.

The sample was then frozen, lyophilized to dryness, dissolved in 0.1 ml of water, and used directly in the ribosomal binding assay. The calculations made in order to express the ribosomal binding of the borohydride-treated samples as a percentage of that of the control sample are discussed under "Results."
RESULTS

Amino Acid Acceptance Activity—Fig. 2 shows the acceptor activities for valine, serine, and alanine of the borohydride-treated samples of unfractionated tRNA expressed as percentages of control and plotted against the percentage of dihydrouridine reductively cleaved by borohydride treatment. As can be seen, a decrease of 10 to 20% in the specific activity of these three species of amino acid-specific tRNAs lacking up to 90% of intact dihydrouridine was found (only these three species were tested). It is immediately obvious that this loss is independent of the amount of dihydrouridine destroyed; the loss is also (not shown) independent of time of reaction.

These studies were repeated with purified tRNA$^{\text{Ser}}$, rather than unfractionated tRNA, and the results were the same (approximately 80% of serine acceptor activity was retained despite reductive cleavage of 63% to 90% of the initial dihydrouridine). Under assumptions discussed below, it can readily be calculated for the last points on the curves shown in Fig. 2, where the specific amino acid acceptor activity of borohydride-treated tRNA$^{\text{Ser}}$, tRNA$^{\text{Ala}}$, or tRNA$^{\text{Val}}$ is approximately 80% of the control value, that at least 73% of tRNA molecules have no intact dihydrouridine residues whatsoever.

The reductive cleavage of dihydrouridine residues by borohydride does not affect the ability of tRNA to regain an active conformation for amino acid acceptor activity after heat denaturation. This was shown by the following experiment. A sample of borohydride-treated, unfractionated tRNA (>90% of the dihydrouridine cleaved) was dialyzed first against 0.2 M EDTA, pH 6.0, and then against water; next, it was assayed for serine acceptor activity, both before and after heating to 90° for 10 min. There were only minor and probably not significant changes in acceptor activity when this sample was compared to a similarly treated control sample (Table I).

Ribosomal Binding—Fig. 3 shows the extent of ribosomal binding of $^{14}$C-seryl-tRNA as a function of amount of $^{14}$C-seryl-tRNA used in the assay. $\odot - \odot$, control sample; $\triangle - \triangle$, a borohydride-treated sample with 78% of dihydrouridine reductively cleaved (curves plus and minus poly U-C are shown). $\cdots \cdots$, 100% binding.

As well established (23), the fraction of aminoacylated tRNA bound was found to vary non-linearly with the amount used. Curves 5 and 6 of Fig. 3 show the ribosomal binding of $^{14}$C-seryl-tRNA prepared from borohydride-treated, unfractionated tRNA. The borohydride treatment in this experiment had progressed to a 78% loss of intact dihydrouridine and a 20% loss of amino acid acceptor activity. It is obvious that the cleavage of 78% of dihydrouridine did not result in a comparable loss of polynucleotide-dependent ribosomal binding. To express the calculation more quantitatively, polynucleotide-dependent radioactivity (counts per min) bound to ribosomes, for this borohydride-treated RNA sample, was plotted as a percentage of the radioactivity of the control sample at the same seryl-tRNA concentration. This calculation is illustrated in Table II.

The same calculations were made for borohydride-treated samples with different amounts of dihydrouridine cleaved; the results are shown in Fig. 4, where the bars represent the ranges of the percentage values and the circles represent the midpoints of the ranges. These values are plotted as functions of the percentage of total dihydrouridine cleaved, and results for both

<table>
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<th>Sample</th>
<th>No heat</th>
<th>90° for 10 min</th>
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<tbody>
<tr>
<td>Control</td>
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<td>146</td>
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<td>Borohydride-treated</td>
<td>142</td>
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![Fig. 2. Amino acid acceptor activity of borohydride-treated samples as percentage of control sample as a function of the percentage of dihydrouridine cleaved by borohydride treatment.](image)

![Fig. 3. Ribosomal binding of $^{14}$C-seryl-tRNA as a function of amount of $^{14}$C-seryl-tRNA used in the assay.](image)
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"C-seryl-tRNA prepared from borohydride-treated, unfraccionated tRNA and "C-seryl-tRNA prepared from borohydride-treated, purified tRNA\textsuperscript{ser} are shown.

Correct interpretation of the data shown in Fig. 4 requires additional discussion. The data show an average loss of 10 to 20% of polynucleotide-dependent binding for all of the borohydride-treated samples. Polynucleotide-dependent binding, however, is expressed as a fraction of the seryl-tRNA used and not as a fraction of the total tRNA\textsuperscript{ser} present. In the control sample, these two amounts are presumably equal, but in the borohydride-treated samples we know there exists 10 to 20% of tRNA\textsuperscript{ser} which cannot be aminoacylated (Fig. 2). It is known that an uncharged tRNA can competitively inhibit the binding of the corresponding aminoacylated tRNA species to ribosomes (23). The extent of this inhibition under our assay conditions is shown in Fig. 5. Curve 1 shows the degree of inhibition when the unacylated tRNA used was borohydride-treated, unfraccionated tRNA (78% of the dihydrouridine cleaved), and Curve 2 shows the degree of inhibition when the unacylated tRNA used was untreated, unfraccionated tRNA. Two points should be noted. (a) The inhibition of ribosomal binding by the borohydride-treated sample is somewhat greater than that of the untreated sample. (b) When the unacylated, borohydride-treated tRNA comprises 20% of the total tRNA present, the degree of inhibition of ribosomal binding is 15%. Thus, a 15% loss of polynucleotide-dependent binding would be expected in our borohydride-treated samples from this inhibition alone. Theoretical full acceptance for the borohydride-treated samples is then 85%, and this is indicated by Curve 1 of Fig. 4.

**DISCUSSION AND CONCLUSIONS**

One of the most interesting and most baffling features of the molecular biology of tRNAs is the presence of a large variety of unusual nucleotides, such as methylated or N-acylated purines and pyrimidines and modified uridines. The function of these bases has so far been most elusive. A great variety of studies designed to reveal the unique significance of their presence in the tRNA molecules has, possibly with a few exceptions, failed to define the role that these unusual bases play in both the amino acid acceptance and the decoding functions of tRNA.

In considering the various possible relationships between reductive cleavage of dihydrouridine in an amino acid-specific tRNA and its effect on one or more of the biological functions of the tRNA, two sets of alternatives exist. (a) All the dihydrouridine residues in a given amino acid-specific tRNA are not equivalent with respect to a given biological activity. In this case, one or only one dihydrouridine cleaved by borohydride treatment would have to be intact for this biological function, while others could be intact or not. The specific case that one and only one dihydrouridine residue is important in this manner

<table>
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<th>Table II</th>
<th>Ribosomal binding of borohydride-treated samples</th>
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<tr>
<td>Borohydride-treated sample used in assay</td>
<td>Polynucleotide-dependent radioactivity bound to ribosome</td>
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<td>635</td>
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<td>Midpoint value</td>
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$^a$ Values are taken directly from experimental measurement. $^b$ Values are interpolated from curve of control sample.

**Fig. 4.** Poly U-C-dependent ribosomal binding of "C-seryl-tRNA from borohydride-treated samples as percentages of control values, plotted as a function of the percentage of dihydrouridine cleaved by borohydride treatment. $O-O \text{, } ^{14}C$-seryl-tRNA prepared from unfraccionated tRNA; $\bullet-\bullet \text{, } ^{14}C$-seryl-tRNA prepared from pure tRNA\textsuperscript{ser}. Bars represent ranges; circles are midpoints of ranges. $- - - , - - - , \text{ and } \ldots \ldots \ldots$, theoretical curves (see text).

**Fig. 5.** Poly U-C-dependent ribosomal binding of "C-seryl-tRNA prepared from unfraccionated tRNA, expressed as percentage of that of a sample with no unacylated tRNA added, plotted as a function of the percentage of total tRNA used in the assay which was unacylated. The acylated tRNA\textsuperscript{ser} and unacylated tRNA\textsuperscript{ser} were added to the assay mixture at the same time. $O$, the unacylated tRNA used was unfraccionated tRNA; $\bullet$, the unacylated, unfraccionated tRNA used was treated with borohydride (78% of the dihydrouridine was cleaved).

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would give rise to a loss of amino acid acceptor activity, as shown by Curve 1 in Fig. 2, or to the loss of ribosomal binding activity as shown in Curve 2 of Fig. 4, or to the loss of both these activities. (b) All the dihydrouridine residues in a given amino acid-specific tRNA are equivalent with respect to a given biological activity. Two possibilities then follow. One is that all the dihydrouridine residues must be intact for this biological activity and destruction of any one would lead to inactivation of the molecule. This alternative would give rise to a loss of amino acid acceptance activity, as shown in Curve 2 of Fig. 2, to a loss of ribosomal binding capability, as in Curve 3 of Fig. 4, or to loss of both activities, for a tRNA species which has three dihydrouridine residues in its structure, such as bakers' yeast tRNA\textsubscript{Ser}. The second possibility is that none of the dihydrouridine residues need be intact for the activity. Clearly, this would predict 100% biological activity for all samples without respect to their fraction of intact dihydrouridine.

The theoretical curves in Figs. 2 and 4 were calculated on the assumption that all dihydrouridine residues are reductively cleaved by borohydride at the same rate no matter where they are located in the polynucleotide chain, or whether they are in polynucleotide chains of differing sequences. Although this assumption is not easily verifiable at the present state of our technology, it is used because, in the absence of more specific knowledge, random action by borohydride seems most likely and because it is the only nonarbitrary assumption that allows quantitative interpretation of the data.

Comparison of the experimental curves shows that the last discussed hypothesis (that none of the dihydrouridine residues need be intact for activity) is the most consistent one and permits the conclusion that dihydrouridine residues as such do not perform a unique or critical role, since their reductive cleavage does not impair the ability of tRNAs to function as amino acid acceptors or to undergo polynucleotide-directed ribosomal binding. While this conclusion is based on examination of only three amino acid acceptor species in tRNA from bakers' yeast, and only one species in the ribosomal binding assay, it seems logical at this time to extend our conclusions tentatively to all species of tRNA that contain dihydrouridine. Although the loss of 10 to 20% of specific acceptor activity in all borohydride-treated samples remains unexplained, it is of sufficiently small magnitude not to detract from our conclusions.

Cerutti and Miller (13) have shown, under the conditions used in our experiments, that of the minor and major nucleotides present in yeast tRNA, borohydride produces a modification only of dihydrouridine and N\textsuperscript{4}-acyctethylcytidine,\textsuperscript{6} which is partially deacylated to cytidine and partially reduced to N\textsuperscript{4}-acyctetyl-3',4,5',6-tetrahydrocytidine. Since (a) N\textsuperscript{4}-acyctethylcytidine is destroyed more rapidly than dihydrouridine by borohydride, (b) yeast tRNA\textsubscript{Ser} contains one N\textsuperscript{4}-acyctethylcytidine residue (2, 6), while both tRNA\textsubscript{Ala} and tRNA\textsubscript{Val} do not (1, 5), and (c) our results show no difference in the biological activities of these borohydride-treated tRNAs as far as these have been examined, we conclude, also, that intact N\textsuperscript{4}-acyctethylcytidine in yeast tRNA\textsubscript{Ser} is not critically important to the activities measured.

We have not established experimentally that borohydride treatment in our experiments leads solely to modification of dihydrouridine (and of N\textsuperscript{4}-acyctethylcytidine). This question, however, is tangential to the main conclusion from the studies reported above, since no important effect on amino acid acceptor or ribosome-binding activities is seen as a result of borohydride treatment.

By extrapolation from the known primary structures of yeast tRNA, it may tentatively be concluded that most of the dihydrouridine residues in tRNA are to be found in the same general region, namely between nucleotides 16 and 22 from the 3' terminus of the molecule, although one or more dihydrouridine residues may be found in other parts of the molecule as well (e.g. nucleotides 47 to 49 in yeast valine- (5), tyrosine- (3), and, perhaps, alanine-tRNA (1)). The clustering of dihydrouridine residues in the area between nucleotides 16 and 22 may impart some special properties to this area; whether these are of significance to the over-all secondary or tertiary structure of the tRNA is not known with certainty. Be that as it may, we must then conclude that either these properties and this region (or both) are of no importance for two major biological functions of tRNA, amino acid acceptor activity and polynucleotide-dependent ribosomal binding, or that the ribosylureidopropanol residues formed as the result of reductive cleavage of dihydrouridine residues by borohydride are capable of fulfilling the same role.

Our results and the conclusions that we draw from them are at variance with the conclusions drawn by Lindahl (14) from irreversible heat inactivation studies of tRNA. He concluded from his studies that "destruction of dihydrouridine residues appears to be a qualitatively significant factor" in the loss of amino acid acceptance and binding activities of several amino acid-specific species of tRNA caused by heat treatment at 90° for 10 to 30 hours in the absence of Mg\textsuperscript{2+}. It should be pointed out, however, that Lindahl did not relate experimentally the effect of heat treatment to the dihydrouridine content of tRNA; rather, his conclusions are indirect and are based on the rate of destruction of commercial 5,6-dihydrouridine as judged by the loss of absorption at 220 mp.

Our conclusion that dihydrouridine residues, as such, are not strictly essential to the amino acid acceptor and ribosomal binding functions of tRNA receives indirect support from the observation that tyrosine-tRNA from Escherichia coli does not contain any dihydrouridine residues. The role of dihydrouridine and its origin thus remain an interesting and open question.

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\textsuperscript{6} Numbering, International System.
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