THE AMINO ACID RECOGNITION AND RIBOSOME COMBINING SITES OF E. COLI TRANSFER RNA

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A possible approach to the study of the mechanism of action of transfer RNA (s-RNA) involves the modification of the RNA by a chemical treatment known to cause specific changes in certain nucleotides, followed by observation of the effect of the modification on the biological functions of the RNA. This approach was recently employed by Yu and Zamecnik (1963), who studied the effect of bromination of s-RNA from yeast and E. coli on the amino acid acceptor ability of the RNA. These workers obtained evidence that the amino acid recognition sites differed in the s-RNA from the two species. Takanami et al. (1963) presented evidence, based on modifications of s-RNA with hydroxylamine, that indicates the integrity of both the terminal - CCA sequence and the internal polynucleotide chain to be necessary for binding of s-RNA to the ribosomes. Zillig et al. (1960) showed that the leucine, tyrosine, and alanine acceptor abilities of E. coli s-RNA were destroyed rapidly by treatment with nitrous acid.

We have been engaged in a study of the effect of nitrous acid treatment on the ability of E. coli s-RNA to (1) accept labeled amino acids onto the terminal nucleotide (amino acid activation), and (2) transfer the activated amino acids into polypeptides using a polynucleotide message (Nirenberg et al., 1962). Nitrous acid is a particularly interesting reagent for studies of this type since the base changes induced in viral RNA by this reagent frequently result in mutations (Schuster et al., 1958). Similar changes in s-RNA could

conceivably produce changes in the biological specificity of protein synthesis, and shed some light on the coding problem. In addition to bringing about the deamination of cytosine, adenine, and guanine, nitrous acid has been shown to be capable of cross-linking DNA strands (Geiduschek, 1961).

Experimental and Results. E. coli s-RNA was purchased from General Biochemicals, Chagrin Falls, Ohio or was prepared as previously described (Zubay, 1962). The nitrous acid treatment was carried out by a modification of the method used by Schuster et al. (1958). A solution of the s-RNA (5 mg./ml.) in 0.25 M acetate buffer (pH 4.3) was treated at room temperature with one-half volume of 3.0 M sodium nitrite (dissolved in the pH 4.3 buffer). Aliquots were withdrawn at 0, 2, 4, 6, 10, and 24 hours, and precipitated in the cold with two volumes of ethanol. The RNA was purified by three precipitations from 1 M sodium acetate (pH 5) with ethanol; washed with ethanol, ether, and finally dried in vacuo. Control tubes, in which sodium acetate was substituted for the sodium nitrite, were also employed. The acceptor ability of the control RNA remained unchanged for at least 10 hours under these conditions. The treated RNA samples were taken up in sufficient water to make a final concentration of approximately 10 mg./ml., and the exact concentration determined from the absorbance at 260 mμ.

The amino acid activating system (aminoacyl-RNA synthetase) was prepared from frozen E. coli paste. A 100,000 x g supernatent, prepared by the method of Nirenberg et al. (1961), fraction S-100, was treated with one gram of streptomycin sulfate per 50 ml. of supernatent, and centrifuged at 12,000 x g for 30 minutes. The resulting supernatent was brought to 60% saturation with solid ammonium sulfate, the precipitate isolated by centrifugation at 30,000 x g for 2 hours, dissolved in "standard buffer" (Nirenberg et al., 1961), and dialyzed against the same buffer overnight in the cold.

The cell-free protein synthesizing system (S-30), dependent upon exogenous polynucleotides for activity, was prepared from E. coli as described previously (Nirenberg et al., 1961). Maximal activity was obtained with preparations ground with alumina for no longer than 5-10 minutes. The S-30 preparation was

incubated at 37° for 45 minutes and then dialyzed overnight against "standard buffer" before using.

Various samples of s-RNA, previously treated with nitrous acid for different time intervals, were tested for their ability to accept labeled leucine, lysine, phenylalanine, and valine, using an assay system similar to that described previously (Starr, 1963). The results are summarized in Fig. 1. It should be emphasized that the RNA was allowed to become saturated with labeled amino acid under the conditions of the assay, and the results thus represent the maximum uptake. Separate kinetic studies indicated that the rate of amino acid uptake was not changed appreciably by the nitrous acid treatment, and all of the reactions were complete in 10 minutes at 37°.

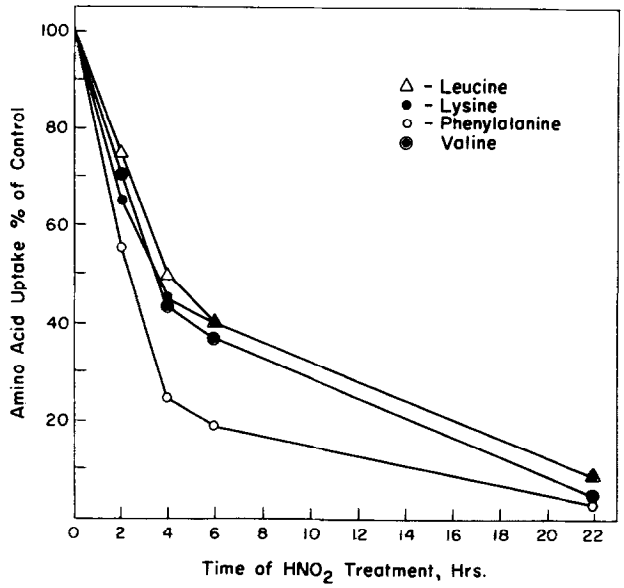

Fig. 1. Amino acid uptake by E. coli s-RNA after treatment with nitrous acid for various times. For conditions of the HNO_2 treatment, see the text.

Reaction mixtures for measurement of amino acid uptake contained in 250 µl: 12.5 µmoles Tris (pH 7.4); 1.25 µmoles $MgCl_2$; 2.5 µmoles ATP; 2.5 µmoles phosphoenolpyruvate; 10 µg. pyruvate kinase; 0.25 µcurie of C^{14} amino acid (10 µc/µmole); 0.5 mg. of s-RNA; and 1 mg. of enzyme protein. After incubation at 37° for 15 min., 50 µl aliquots were withdrawn, placed onto Whatman #3 mm paper discs, washed with 5% TCA (five times), with ethanol (twice), dried, and counted by the scintillation method.

It was repeatedly observed that the phenylalanine-specific s-RNA was inactivated at a much faster rate than the leucine, lysine, or valine acceptors.

Thus after 4 hours of nitrous acid treatment, approximately 75% of the phenylalanine acceptor had been inactivated, whereas only 55% of the RNA molecules specific for the other measured amino acids had been changed sufficiently for inactivation to occur.*

C^{14}-L-Phenylalanyl-s-RNA and C^{14}-L-lysyl-s-RNA were prepared from both normal s-RNA and from nitrous acid treated material (reaction conditions of Fig. 1 expanded 20-fold), and purified as previously described (von Ehrenstein et al., 1961). The ability of the labeled aminoacyl s-RNA to transfer the amino acids into polypeptides was measured by the method of Nirenberg et al., (1962), using poly U as the message for C^{14}-phenylalanine incorporation and poly A as message for C^{14}-lysine incorporation. Since the original uptake of labeled amino acid by the various s-RNA samples differed, reaction mixtures were set up to contain nearly identical amounts of C^{14}-aminoacyl s-RNA, but different amounts of unlabeled material.

The results (Table 1) indicate the C^{14}-aminoacyl s-RNA prepared from nitrous acid treated s-RNA to be markedly less efficient in transferring the amino acid into polypeptides. This reduction in polypeptide synthesizing ability cannot be attributed to an inhibition by the altered unlabeled s-RNA in the preparations, since the addition of large amounts of unlabeled nitrous acid-treated s-RNA did not affect the ability of normal C^{14}-phenylalanyl s-RNA to function in this system (see Table 1). These results indicate that after treatment with nitrous acid for 2-6 hours, a population of s-RNA molecules exists that can still accept amino acids, but can no longer successfully transfer the amino acids into polypeptides.

Discussion. The faster rate of inactivation of the phenylalanine-specific s-RNA, as shown in Fig. 1, is in marked contrast to the results of Yu et al.

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* In separate experiments to be reported elsewhere, we have determined that under the conditions of our nitrous acid reactions, approximately one C to U change occurs per 900 nucleotides per hour; one A to I change occurs per 650 nucleotides per hour; and one G to X change occurs per 400 nucleotides per hour. Thus the inactivation curves (Fig. 1) are due to the change of only a very few nucleotides per s-RNA molecule (assuming 75-100 nucleotides per chain).

Table 1

Polypeptide Synthesis by C^{14}-Aminoacyl-s-RNA Prepared from Nitrous Acid Treated s-RNA

Additions	1		2	
	Cpm in Reaction	Cpm Incorporated	Cpm in Reaction	Cpm Incorporated
C^{14}-Phenylalanyl-s-RNA (normal), without poly U	420	24	2,730	115
C^{14}-Phenylalanyl-s-RNA (normal)	420	160	2,730	1,040
C^{14}-Phenylalanyl-s-RNA (from 2 hr. HNO_2 treated RNA)	390	120	2,560	830
C^{14}-Phenylalanyl-s-RNA (from 4 hr. HNO_2 treated RNA)	390	90	2,820	750
C^{14}-Phenylalanyl-s-RNA (normal), without poly U	2,300	110		
C^{14}-Phenylalanyl-s-RNA (normal)	2,300	580		
C^{14}-Phenylalanyl-s-RNA (normal) + 250 µg. s-RNA (4 hr. HNO_2 treated)	2,300	600		
C^{14}-Phenylalanyl-s-RNA (normal) + 500 µg. s-RNA (10 hr. HNO_2 treated)	2,300	570		
C^{14}-Lysyl-s-RNA (normal), without poly A	22,000	60	9,500	55
C^{14}-Lysyl-s-RNA (normal)	22,000	225	9,500	180
C^{14}-Lysyl-s-RNA (from 2 hr. HNO_2 treated RNA)	21,500	75		
C^{14}-Lysyl-s-RNA (from 4 hr. HNO_2 treated RNA)	24,000	70	10,500	70
C^{14}-Lysyl-s-RNA (from 6 hr. HNO_2 treated RNA)			11,000	70

Reaction mixtures contained the following in µmoles/ml.: 100 Tris (pH 7.8); 10 magnesium acetate; 50 KCl; 6 mercaptoethanol; 10 GTP; 10 phosphoenolpyruvate; 8 µg. pyruvate kinase; 40 µg. poly U or poly A; C^{14}-aminoacyl-s-RNA as indicated; and 300 µl. of incubated S-30 containing 20 mg. protein/ml. After incubation at 37° for 30 min., the phenylalanine reactions were worked up as directed by Nirenberg et al. (1961) except that scintillation counting was used. The lysine reactions were worked up using tungstic acid in TCA as directed by Wahba et al. (1962).

(1963), who found that bromination of E. coli s-RNA inactivated the lysine acceptor much faster than the phenylalanine or valine acceptors. The two sets

of data are not in conflict, however, since bromine is known to react preferentially with pyrimidines, while nitrous acid reacts fastest with the purines (Schuster et al., 1958). This provides suggestive evidence that the amino acid recognition site of the E. coli phenylalanine acceptor s-RNA is rich in purine nucleotides. In this case, it is tempting to equate the amino acid recognition site with the triplet coding site (probably AAA in the phenylalanine acceptor). However the fairly rapid destruction of the lysine acceptor by nitrous acid suggests that the observed inactivations (Fig. 1) are not due entirely to changes at the triplet coding site, since the lysine acceptor coding site is probably rich in unreactive uridine nucleotides (complementary to the AAA coding unit on the messenger).

It is apparent that, in a small percentage of the molecules, the ability of the s-RNA to combine with the ribosomes and transfer the amino acid into polypeptides can be destroyed by nitrous acid, without affecting the ability to accept an amino acid onto the terminal nucleotide. Thus, after a 4 hour nitrous acid treatment, 25% of the phenylalanine-specific molecules will still function properly as an acceptor (see Fig. 1), however these active molecules are only 60-70% as efficient as the normal ones in transferring their amino acid into polypeptides (Table 1). Similarly, of the 45% that will still accept lysine after 4 hours exposure to nitrous acid, only 30-40% can transfer the lysine into polylysine.

These findings are in agreement with the concept of separate sites on the s-RNA molecule for amino acid recognition and for ribosome interaction and amino acid transfer. Treatment of s-RNA with nitrous acid would be expected to cause some changes in secondary structure, due to a change of some of the normal A-U or G-C pairs. The possibility therefore exists that s-RNA-ribosome interactions are more sensitive to small changes in secondary structure than are the amino acid activation reactions. This interpretation would not require a separate ribosome "combining site", but only that the secondary structure of the entire molecule be unchanged for successful binding to occur.

Since the code for lysine incorporation is probably AAA (Gardner et al., 1962), the lysine acceptor would be expected to contain a UUU sequence at the coding site. It is therefore evident that the low efficiency for polylysine synthesis demonstrated by C^{14}-lysyl-s-RNA (from nitrous acid treated s-RNA) cannot be due to destruction of the coding site, since nitrous acid does not affect uracil residues. Successful ribosome binding and amino acid transfer apparently requires the integrity of most if not all of the s-RNA molecule.

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