Selective Inactivation of E. coli tRNA by Ethylenimine

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Studies in this laboratory on the S-aminoethylation of cysteine by ethylenimine while attached to transfer RNA (tRNA) led to the observation that the ability of E. coli B tRNA to accept certain amino acids was abolished by exposure to this reagent. The demonstration of limiting amounts of thiopyrimidines, predominantly 4-thiouridine, in E. coli tRNA by Lipsett (1965) led us to consider the S-aminoethylation of thionucleotides as a possible explanation for the selective inactivation of amino acid acceptance by ethylenimine. The present communication demonstrates that aminoethylation by ethylenimine of 4-thiouridine residues in E. coli tRNA does occur under conditions mild enough to avoid covalent rupture of tRNA molecules. The kinetics of alkylation of 4-thioU residues as well as the kinetics of inactivation of acceptance for four different amino acids are reported.

Materials and Methods: E. coli B stripped tRNA was obtained from General Biochemicals, Chagrin Falls, Ohio. Reaction with ethylenimine was carried out as follows: tRNA was dissolved in 0.2 M KH_2PO_4 - 1 mM dithiothreitol - 1 mM $Mg^{++}OAc_2$ buffer pH 6.3 to a concentration of 10 mg/ml. After 30 minutes at 25°C, ethylenimine (Matheson, Coleman, Bell) was titrated into the experimental vessel to a pH of 8.0 (10 µl per ml). The control was titrated to pH 8.0 with ethanolamine. After various periods of reaction at pH 8.0 and 25°C, aliquots containing 20 or 30 mg tRNA were placed on

a rapidly flowing Sephadex G-25 (coarse) column (10 cm x 2 cm) equilibrated with 0.1 M KOAc pH 5.0 to stop the reaction. The tRNA was eluted with 0.1 M KOAc pH 5.0, located by absorbance at 260 mμ and pooled. The tRNA was then precipitated at 0°C by the addition of 2 volumes of precooled (-18°C) ethanol, centrifuged and redissolved in standard TMSH buffer (50 mM TrisCl, 10 mM $MgOAc_2$, 10 mM mercaptoethanol pH 7.4) for assay of amino acid acceptance. The ethanolamine-treated controls were treated in the same way. The tRNA samples were then adjusted to an A 260 mμ equivalent to 100 in a 10 mm light path with TMSH buffer and assayed for acceptance in a reaction containing 0.28 ml tRNA solution, 20-50 μl E. coli B supernatant protein (approximately 11 mg per ml) and 0.20 ml of a mixture containing 50 μl 0.1 M ATP, 15 μl 0.3 M $Mg^{++}OAc_2$, 25 μl 0.1 M creatine phosphate, 10 μl 0.05 M CTP, 25 μl 0.8 M TrisCl pH 7.5, 25 μl creatine kinase (1 mg/ml), 5 μl 0.1 M dithiothreitol, 15 μl C-14 amino acid (20 μC/ml and 1.65 mM) and 30 μl of water. The E. coli supernatant protein was prepared by centrifuging an extract of E. coli B, homogenized with glass beads in a Bronwill MSK homogenizer, for 90 minutes at 150,000 x g then dialyzing the supernatant protein for 12 hours against two changes of 1:10 diluted TMSH buffer. The complete mixture was incubated at 30°C in a shaker bath and aliquots (usually 90 μl) were removed at various intervals to determine the kinetics of amino acid charging. The aliquots were pipetted onto filter paper discs, dropped into cold 10% TCA, washed twice in cold 10% TCA then in ethanol-ether and finally ether before drying and counting the discs in a scintillation counter in 0.4% scintillator solution in toluene (2,5-bis-[2-(5-tert-Butylbenzoxazolyl)]-thiophene).

Spectral analyses of the reaction of tRNA with ethylenimine were performed under conditions identical to those used in acceptor inactivation as was the reaction of ethylenimine with 4-thiouridine (purchased from Cyclo Chemical Corp. as the disulfide and reduced in 2 mM dithiothreitol prior to use). Spectra were measured in a Cary 15 recording spectrophotometer.

Results: Figure 1 shows the progressive destruction of absorbance at 340 mμ

(due to 4-thioU) in E. coli B tRNA exposed to 0.19 M ethylenimine at pH 8.0 and 25°C. The exponential plot of direct O.D. reading with time was found to deviate from linearity after 1 - 2 hours had elapsed, making the calcul-

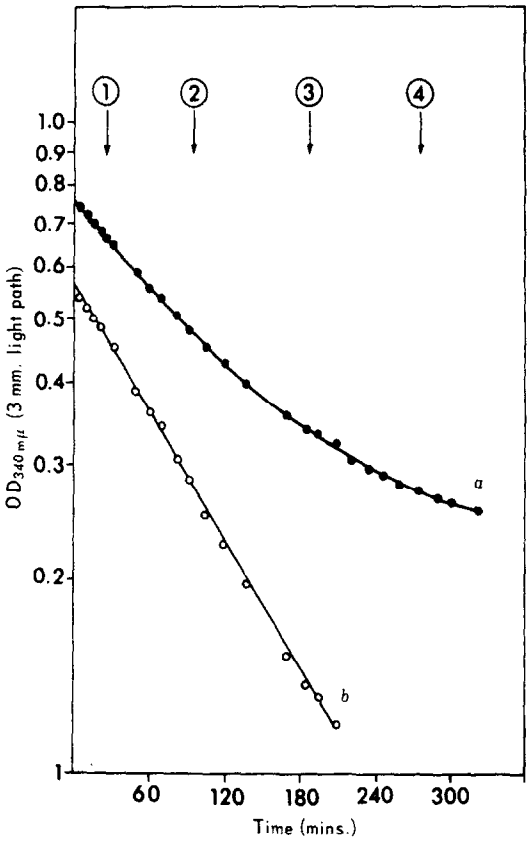

Figure 1. Kinetics of destruction of 340 mμ absorbance of E. coli B tRNA by ethylenimine (0.19 M, pH 8.0, 25°C). An aliquot of the same reaction mixture as in Fig. 3 was read at 340 mμ in a 3 mm. light path. Curve a: actual O.D. readings. Curve b: curve a after subtraction of asymptotic residual absorbance value at each point.

ation of a pseudo first-order kinetic constant impossible. Under a variety of conditions tested the direct O.D. 340 mμ reading was found to asymptotically approach a residual O.D. equivalent to approximately 30% of the initial optical density. The reasons for this are not clear at present although the curvature is not due to destruction of ethylenimine during the course of the reaction since the addition of further reagent did not cause any further decrease in absorbance. Furthermore, by analogy with the aminoethylation of free 4-thioU (see later), this residual absorbance is probably not due to the aminoethylated product (although the effect of neighboring

base "stacking" in tRNA on the spectrum of the product is not yet known). Another possible explanation is the presence of a group of "inaccessible" 4-thioU residues which alkylate at an immeasurably slow rate - however this seems unlikely in view of the fact that even at elevated temperatures this same residual absorbance is observed. Even if this is caused by a very slowly reacting class of residues the reaction rate for the "accessible" class of 4-thioU residues can be determined by subtracting out the residual absorbance as shown in curve b of Figure 1. This yields an exponential process with a pseudo first-order kinetic constant for *in situ* aminoethylation of 4-thioU of 0.00736 $min.^{-1}$ (half-life = 94 minutes under these conditions).

Further investigation of the *in situ* aminoethylation of 4-thioU was carried out by following the complete spectrum of tRNA from 300 to 380 mμ at various time intervals after the addition of ethylenimine to 0.19 M at pH 8.0 and 25°C. As shown in Figure 2a there is a progressive decrease in the 4-thioU peak at 335 mμ with a discrete isosbestic point at 322 mμ and the

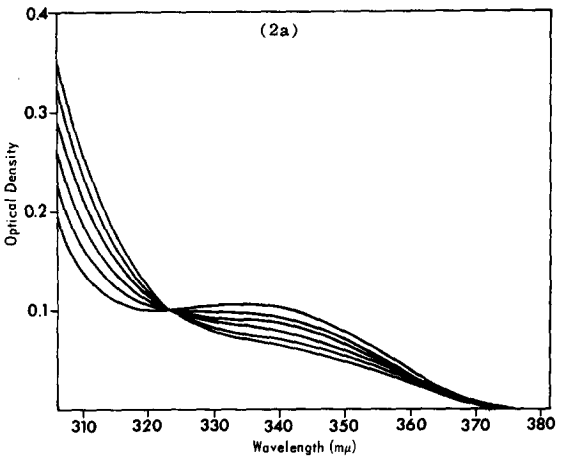

Figure 2a. Spectral analysis of the reaction of *E. coli* B tRNA with ethylenimine (0.19 M, pH 8.0, 25°C). Spectra were taken at 15 mins. (upper curve at 340 mμ), 35 mins., 60 mins, 80 mins, 120 mins and 150 mins (lower curve at 340 mμ).

generation of a new species absorbing below 310 mμ (presumably S-aminoethyl 4-thiouridine). This reaction shows marked similarities to the aminoethylation of free 4-thioU under identical conditions as shown in Figure 2b (the different λ_{max} values for free 4-thioU and 4-thioU in tRNA have been pointed out by Lipsett).

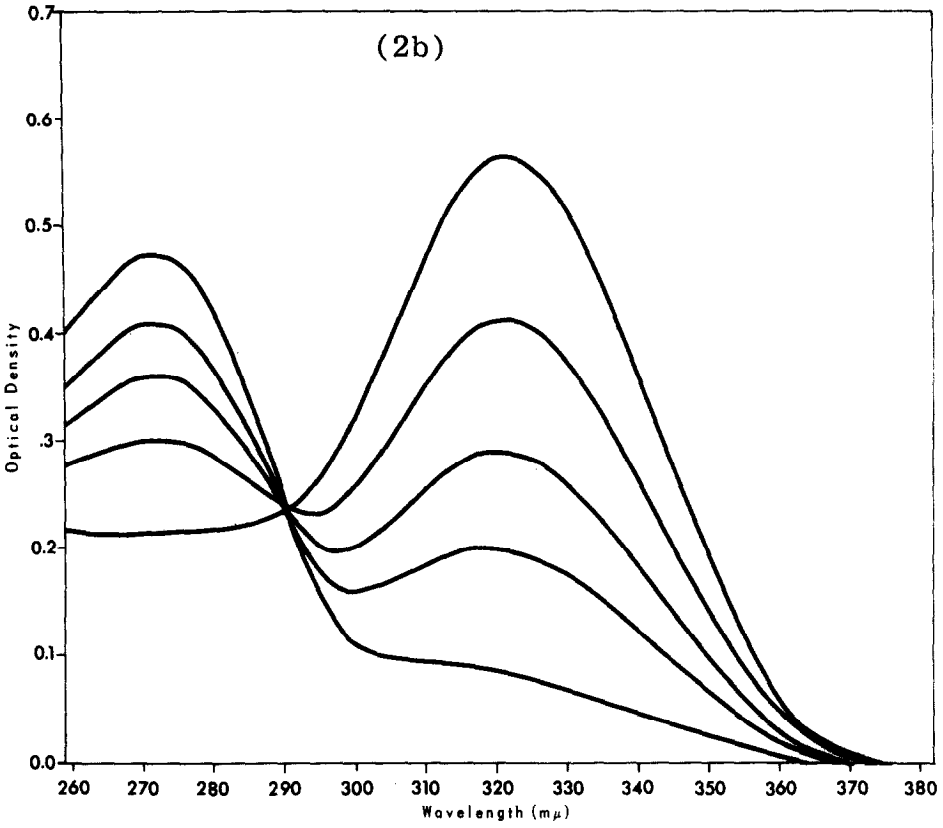

Figure 2b. Spectral analysis of the reaction of 4-thioU with ethylenimine (0.19M, pH 8.0, 25°C). Spectra were taken at 15 mins (upper curve at 320 mμ), 30 mins, 50 mins, 80 mins and 150 mins (lower curve at 320 mμ).

The effect of aminoethylation on amino acid acceptor activity is shown in Figure 3. Samples 1 - 4 were removed from the same reaction mixture shown in Figure 1 at the indicated times and prepared for assay of amino acid acceptance with E. coli supernatant enzymes as described in Methods. Samples 5 and 6 were controls titrated to pH 8.0 with ethanolamine. As can be seen from Figure 3 exposure to ethylenimine caused a progressive rapid decrease of lysine acceptance whereas isoleucine acceptance was unaffected (a small and variable inactivation at pH 8.0 over a 5 hour period was observed

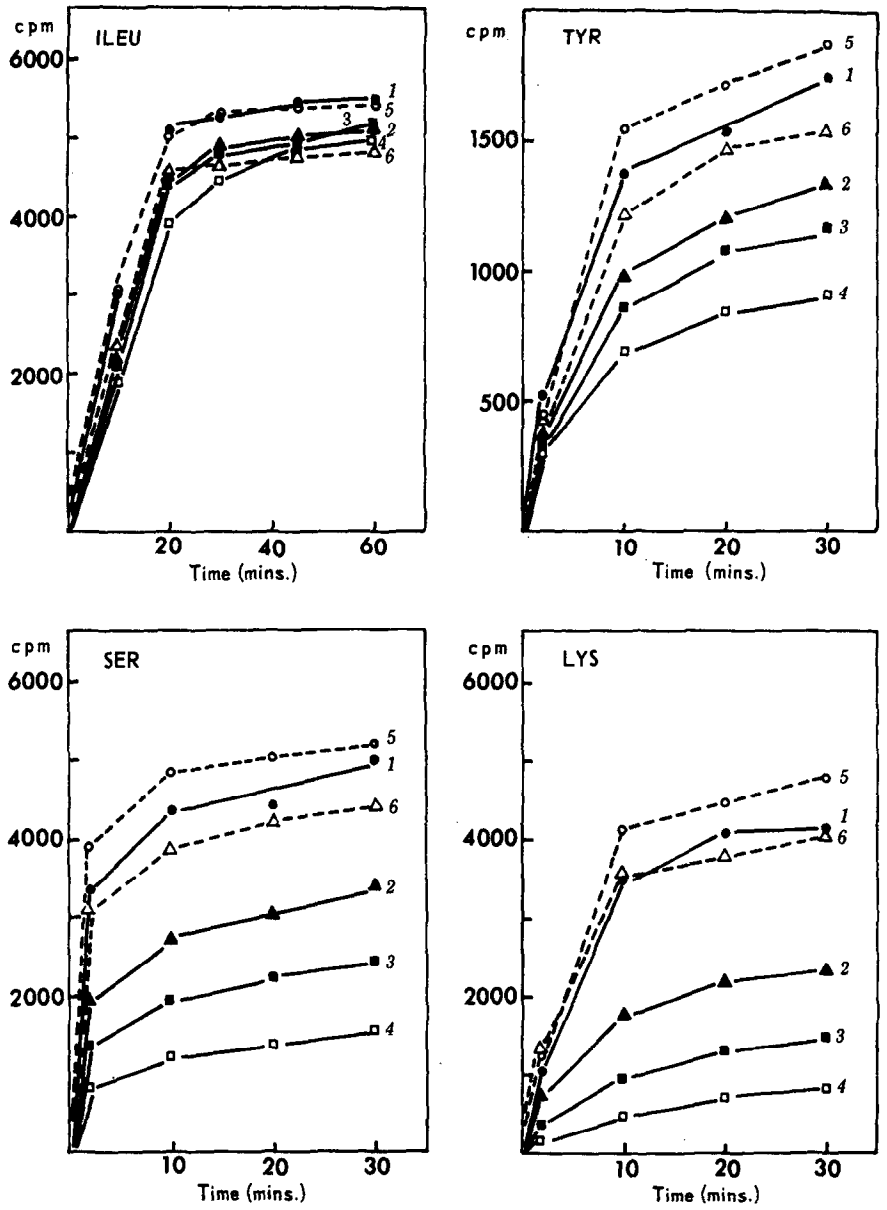

Figure 3. Amino acid acceptance of E. coli tRNA after varying lengths of exposure to ethylenimine (0.19 M, pH 8.0, 25°C). Samples of tRNA were withdrawn from the ethylenimine reaction mixture at various times and prepared for acceptor assay as described in Methods. Samples 1,2,3 and 4 are the same samples indicated in Figure 1 and represent 26, 96, 185 and 274 minutes of reaction with ethylenimine respectively. Control samples 5 and 6 represent 60 and 210 minutes of exposure to ethanolamine under identical conditions. Each assay (0.5 ml) contained 1.47 mg tRNA (0.059 μmoles) and 0.3 μC C-14 amino acid containing 0.0248 μmoles. Each point (0.09 ml.) corresponds to 0.0106 μmoles tRNA and represents maximal charging for Ileu, Tyr, Ser and Lys of 3.9%, 1.4%, 3.8% and 3.5% respectively.

in the ethanolamine-treated controls and was in the range of 5-20% for the amino acids shown). Inactivation of serine and tyrosine acceptance was intermediate between these two extremes. By plotting the maximum plateau acceptance values from Figure 3 as a function of length of exposure to ethylenimine the

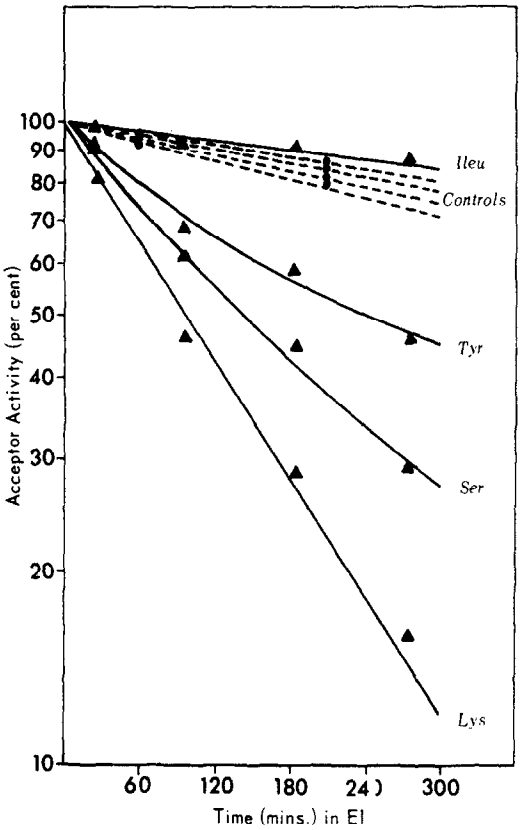

Figure 4. Semi-log plot of inactivation of amino acid acceptance by ethylenimine (0.19 M, pH 8.0, 25°C). The maximum acceptor capacities were taken from the plateau values in Fig. 3. The 100% value for each amino acid was obtained by back extrapolation of the 60 minute and 210 minute ethanolamine samples to zero time.

kinetics of inactivation can be obtained as shown in Figure 4. From this plot, lysine acceptance (which is the most susceptible to ethylenimine) is seen to decay with a first-order constant of 0.00722 min^{-1} (corresponding to a half-life of 96 minutes under these conditions) which is in fairly good agreement with the rate of aminoethylation of 4-thioU residues.

Discussion: The above data demonstrate the selective inactivation of E. coli B tRNA amino acid acceptance by ethylenimine and the in situ aminoethylation of 4-thiouridine residues by this reagent. The kinetic data on lysine acceptor inactivation and 4-thiouridine alkylation show quite good correlation. Furthermore, experiments at 35°C in the absence of added Mg^{++} again gave good correlation with a half-life of 19 - 20 minutes for 4-thioU aminoethylation (340 mμ) and a half-life of 17 - 18 minutes for lysine acceptance and further indicate that under the conditions used in this paper the aminoethylation of 4-thioU is probably rate limited by the secondary and tertiary conformation of the tRNA. The intermediate inactivation of serine and tyrosine acceptance is open to a variety of interpretations. The presence of many iso-accepting tRNA molecules for the same amino acid coupled with the observation that there is less than one 4-thioU residue per tRNA molecule in E. coli (Lipsett, 1965) might explain the appearance of a refractory fraction among the redundant tRNAs for a given amino acid. Furthermore the recent demonstration in E. coli tRNA of 2-thio-substituted cytidine and uridine derivatives (which presumably aminoethylate more slowly than 4-thio pyrimidines) by Carbon et al. (1968) might also explain intermediate rates of inactivation of amino acid acceptance. These possibilities are presently under investigation.

The simplest interpretation of the lack of susceptibility of isoleucine acceptance would be the absence of thio-bases in the tRNAs for this amino acid - however this is by no means conclusive from the above data. The lack of inactivation of isoleucine acceptance is, however, indirect evidence that pseudouridine is not being aminoethylated under these conditions since cyanoethylation of pseudouridine by acrylonitrile in E. coli tRNA (Ofengand, 1968) or in yeast tRNA (Rake and Tener, 1966) leads to inactivation of acceptor activity. Direct evidence for the lack of aminoethylation of pseudouridine and inosine by ethylenimine will be presented at a later date.

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