Isoaccepting Lysine Transfer Ribonucleic Acid Species of *Pseudomonas Aeruginosa*

By BAYAR THIMMAPPAYA and JOSEPH D. CHERAYIL

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

(Received 12 May 1975)

Pseudomonas aeruginosa tRNA was treated with iodine, CNBr and *N*-ethylmaleimide, three thionucleotide-specific reagents. Reaction with iodine resulted in extensive loss of acceptor activity by lysine tRNA, glutamic acid tRNA, glutamine tRNA, serine tRNA and tyrosine tRNA. CNBr treatment resulted in high loss of acceptor ability by lysine tRNA, glutamic acid tRNA and glutamine tRNA. Only the acceptor ability of tyrosine tRNA was inhibited up to 66% by *N*-ethylmaleimide treatment, a reagent specific for 4-thiouridine. By the combined use of benzoylated DEAE-cellulose and DEAE-Sephadex columns, lysine tRNA of *Ps. aeruginosa* was resolved into two isoaccepting species, a major, tRNA₁^{Lys} and a minor, tRNA₂^{Lys}. Co-chromatography of ¹⁴C-labelled tRNA₁^{Lys} and ³H-labelled tRNA₂^{Lys} on benzoylated DEAE-cellulose at pH 4.5 gave two distinct, non-superimposable profiles for the two activity peaks, suggesting that they were separate species. The acceptor activity of these two species was inhibited by about 95% by iodine and CNBr. Both the species showed equal response to codons AAA and AAG and also for poly(A) and poly(A₁G₁) suggesting that the anticodon of these species was UUU. Chemical modification of these two species by iodine did not inhibit the coding response. The two species of lysine of *Ps. aeruginosa* are truly redundant in that they are indistinguishable either by chemical modification or by their coding response.

Lysine tRNA from various sources has been of special interest in the last few years because of the existence of multiple species of this tRNA in a number of systems, the high susceptibility of this tRNA to thionucleotide-specific reagents and the presence of 2-thiouridine derivatives in certain tRNA species which recognize codons of the type NNA and NNG. According to the wobble hypothesis (Crick, 1966), only one lysine tRNA species is required to recognize the codons of lysine, AAA and AAG. However, in microbial (Soll & RajBhandary, 1967; Chuang & Doi, 1972a; Sen & Ghosh, 1973; Smith *et al.*, 1973) and a number of animal systems (Carbon *et al.*, 1965; Caskey *et al.*, 1968; Rudloff & Hilse, 1971; Ortwerth & Liu, 1973; Ortwerth *et al.*, 1973; Juarez *et al.*, 1974) the presence of multiple species of lysine tRNA has been demonstrated and their codon responses have been found to be different. In general three types of lysine tRNA species have been observed: (1) species that recognize both codons, AAA and AAG, their presumed anticodon being UUU; (2) those which recognize only AAG, with anticodon CUU; and (3) those which have preference for the AAA codon. The latter type is presumed to have a 2-thiouridine derivative as the first letter of the anticodon. Lysine tRNA specific for the codon AAA has been isolated from rat liver (Liu & Ortwerth, 1972), rabbit reticulocytes (Rudloff & Hilse, 1971; Wood-

ward & Herbert, 1972) and yeast (Sen & Ghosh, 1973). The presence of 5-methyl-2-thiouridine in rat liver lysine tRNA (Harada *et al.*, 1971) and 2-thio-5-carboxymethyluridine methyl ester in yeast lysine tRNA (Madison *et al.*, 1972; Smith *et al.*, 1973) has been demonstrated. Two lysine tRNA species isolated from *Escherichia coli* coded for both AAA and AAG (Soll & RajBhandary, 1967); however, one of them is presumed to have a 5-methylaminomethyl-2-thiouridine residue in the first position of the anticodon (Ohashi *et al.*, 1970). *Bacillus subtilis* also has been shown to contain two lysine tRNA species. One of them was bound equally well to AAA and AAG, whereas the other showed some preference for the AAG codon, and both the species were shown to have UUU as their anticodon (Chuang & Doi, 1972a).

Pseudomonas is a very versatile genus and is academically important for several reasons (Stanier *et al.*, 1966; Holloway, 1969). Important among them are its innate resistance to antibiotics, high sensitivity to ionizing radiation, existence as saprophytes and as both animal and plant pathogens, high biosynthetic and biodegradative abilities, increasing importance at the industrial level as an agent of spoilage, and non-clustered and scattered arrangement of genes for biosynthetic pathways. We have reported the presence and identification of

thionucleotides in *Pseudomonas aeruginosa* tRNA (Thimmappaya & Cherayil, 1972). The relative proportion of thionucleotides present in this organism is very much different from that of *E. coli* (Rao & Cherayil, 1973; Thimmappaya, 1974). Further, this bacterium is unique in that the common 2-methylthioisopentenyladenosine found in other bacterial tRNA species is absent, but in its place, 2-methylthioribosylzeatin, a plant cytokinin, is present in its tRNA (Thimmappaya & Cherayil, 1974). In the present paper we describe some properties of two isoaccepting lysine tRNA species of *Ps. aeruginosa*.

Materials and Methods

Poly(A) was from Sigma Chemical Co., St. Louis, Mo., U.S.A. Poly(A₁,G₁) of Miles Laboratories was a gift from Dr. R. Mayasundari, Albert Einstein College of Medicine, New York, N.Y., U.S.A. Codons AAA and AAG were purchased from Miles Laboratories, Kankakee, Ill., U.S.A. Millipore filters (0.45 µm pore size, 25 mm diameter) were from Millipore Filter Corp., Bedford, Mass., U.S.A. [¹⁴C]- and [³H]-lysine used in these experiments were from Bhabha Atomic Research Centre, Bombay, India, and had a specific radioactivity of 110 and 250 µCi/µmol respectively. ¹⁴C-labelled algal protein hydrolysate of specific radioactivity 13 mCi/mg-atom was supplied by Bhabha Atomic Research Centre, Bombay, India. All the other materials used in these studies, including the details of radioactive amino acids, were described in two papers from this laboratory (Anandraj & Cherayil, 1974b; Rao & Cherayil, 1974).

Growth of *Ps. aeruginosa* and *E. coli* B

Ps. aeruginosa, strain no. 155, from the Microbiology and Pharmacology Laboratory of this Institute was grown in a nutrient medium containing (per litre); Bacto-peptone (V.P. Chest Institute, Delhi, India), 5.0 g; NaCl, 3.5 g; yeast extract (V.P. Chest Institute), 1.5 g; bovine extract (V.P. Chest Institute), 1.5 g; the pH of the medium was adjusted with NaOH to 7.2. The cells were grown at room temperature (25–28°C) in 500 ml culture flasks with constant shaking. At the late exponential phase (20 h) the cells were harvested, washed twice with a buffer containing 0.01 M-Tris-HCl, pH 7.2, 0.01 M-MgCl₂ and 0.001 M-EDTA, and stored at –15°C. The culture was grown in 20-litre batches, and the cells obtained were pooled for the extraction of tRNA. The yield of cells in this medium was 5 g/litre.

E. coli B was grown in M9 medium (Adams, 1959) containing casamino acids (Difco Laboratories, Detroit, Michigan, U.S.A.). The composition of the medium (per litre) was Na₂HPO₄, 5.8 g; KH₂PO₄,

3.0 g; NaCl, 0.5 g; NH₄Cl, 1.0 g; glucose, 10.0 g; MgSO₄, 246.5 mg; FeCl₃, 23.0 mg; and 0.25% casamino acids. The culture was grown for 5 h at 37°C with constant shaking, harvested, washed and used. Yield of the cells was 4 g/litre.

Extraction of tRNA

tRNA was extracted as described by Anandraj & Cherayil (1974a). Briefly, the procedure was as follows. Cells were suspended in 7–8 vol. of buffer containing 0.05 M-sodium acetate, 0.001 M-EDTA, 0.5% sodium dodecyl sulphate; the final pH was 4.5. An equal volume of water-saturated phenol was added, the mixture was stirred for 30 min, centrifuged at 3000 g for 30 min and the aqueous layer was once again treated with phenol. The aqueous layer was made 2% in sodium acetate, the RNA was precipitated by adding 2.5 vol. of ice-cold ethanol, and kept at –15°C overnight. The RNA was collected, dissolved in 0.1 M-NaCl and the tRNA was separated from the total RNA by using a DEAE-cellulose column (Anandraj & Cherayil, 1974a).

Preparation of aminoacyl-tRNA synthetase and the assay for acceptor activity

The procedure for preparation of aminoacyl-tRNA synthetases and the conditions for the amino acid-acceptor assay were already described (Anandraj & Cherayil, 1974b; Rao & Cherayil, 1974). The frozen synthetase preparation of *Ps. aeruginosa* was active for several weeks when stored at –15°C.

Chromatography of ribonuclease T₁ digest

Ribonuclease T₁ digestion of acylated tRNA species was carried out by the method of Ishida & Miura (1965) and DEAE-cellulose column chromatography was done as described by Walker & RajBhandary (1970).

Preparation of ribosomes

Ribosomes were prepared as described by Nishimura *et al.* (1965). Cells (*Ps. aeruginosa* or *E. coli* B) were ground at 0°C with twice their weight of alumina and in 2 vol. of extraction buffer containing 0.01 M-Tris-HCl, pH 7.8, 0.01 M-magnesium acetate, 0.06 M-KCl and 0.006 M-β-mercaptoethanol. Alumina and intact cells were removed by centrifugation at 30000 g for 30 min. This step was repeated once more and the resulting supernatant was again centrifuged at 105000 g for 90 min. The pellet was suspended in 2.0 M-KCl containing 0.03 M-magnesium acetate, 0.006 M-β-mercaptoethanol and 0.01 M-Tris-HCl, pH 7.8, for 8 h at 0°C. The suspension was again centrifuged at 105000 g

for 90 min and the pellet thus obtained was suspended in extraction buffer and stored as small batches at -15°C .

Ribosome-binding assay

The procedure of Nirenberg & Leder (1964) was used, with a 0.05 ml reaction mixture containing 0.1 M-Tris-HCl, pH 7.2, 0.05 M- NH_4Cl , 0.01 M- MgCl_2 , various concentrations of poly(A) or poly(A_1G_1) and [^{14}C]lysyl-tRNA. Incubation was carried out at 23°C for 20 min.

Chemical modifications of tRNA

Iodine oxidation was performed as described by Carbon *et al.* (1965). *N*-Ethylmaleimide treatment was done by the method of Carbon (1971). CNBr treatment of the tRNA was done as described by Saneyoshi & Nishimura (1970). Spectra of the tRNA samples were measured in a Beckman DU-2 spectrophotometer manually.

Fractionation of lysine tRNA species

Two types of columns were used for the fractionation of lysine tRNA. BD-cellulose (benzoylated DEAE-cellulose) column chromatography was carried out essentially by the method of Gillam

et al. (1967). DEAE-Sephadex column chromatography was done by the method of Bock & Cherayil (1967).

Results

Unfractionated *Ps. aeruginosa* tRNA was treated with three reagents, iodine, CNBr and *N*-ethylmaleimide, which specifically react with thiopyrimidines, and the absorption spectra of the treated samples were scanned from 300 to 390 nm. In all three cases, the shoulder in the absorption spectrum corresponding to 4-thiouridine (Lipsett, 1965) disappeared after the treatment, indicating that the reagents reacted completely with the 4-thiouridine (Figs. 1a, 1b and 1c).

The samples were assayed for their acceptor activity after chemical modification. Table 1 gives the percentage activity retained after treating the sample with the reagents. Iodine treatment resulted in remarkable loss of acceptor activity for lysine, serine, glutamic acid, glutamine and tyrosine and a partial loss of activity for alanine, asparagine, isoleucine and valine. CNBr treatment resulted in high loss of acceptor activity for glutamic acid, glutamine and lysine, along with partial loss of activity for arginine, methionine, proline and serine. Iodine and CNBr have been shown to react with all the thiopyrimidines present in tRNA

Fig. 1. Absorption spectra of chemically modified total tRNA of *Ps. aeruginosa*

tRNA samples at a concentration of 40–50 E_{260} units/ml were taken in 0.02 M-Tris-HCl buffer, pH 7.2, containing 0.01 M- MgCl_2 . In each case the spectrum was taken manually and the readings were calculated for 40 E_{260} units/ml and plotted. ●, Control; ○, chemically treated; (a) iodine-treated; (b) CNBr-treated; (c) *N*-ethylmaleimide-treated.

Table 1. Percentage acceptor ability of *Ps. aeruginosa* tRNA for various amino acids after different chemical modifications

Experiments were carried out in duplicate and the values agreed within 5–10%. In a few cases several independent experiments were carried out and the values corresponding to maximum inhibition are presented.

Amino acid	Iodine treatment	CNBr treatment	N-Ethylmaleimide treatment
Alanine	61	83	62
Arginine	95	42	—*
Aspartic acid	93	82	89
Asparagine	61	—*	—
Glutamic acid	16	5	76
Glutamine	15	1	71
Glycine	86	78	—
Histidine	92	85	—
Isoleucine	70	102	—
Leucine	60	99	—
Lysine	15	12.5	83
Methionine	94	70	—
Phenylalanine	80	85	—
Proline	113	73	—
Serine	24	70	78
Threonine	117	100	—
Tyrosine	21	84	34
Valine	70	84	—
Algal protein hydrolysate	55	65	68

* Not checked.

(Carbon *et al.*, 1965; Saneyoshi & Nishimura, 1971; Agris *et al.*, 1973; Rao & Cherayil, 1974). N-Ethylmaleimide, however, has been shown to react only with 4-thiouridines present in tRNA (Carbon & David, 1968). The N-ethylmaleimide-treated sample was assayed for acceptor activity towards a few amino acids, which was extensively inactivated by iodine and CNBr. N-Ethylmaleimide treatment decreased the acceptor activity for tyrosine by 66% and that for lysine by about 20% (Table 1). Comparative data on the effect of three thiopyrimidine-specific reagents on three bacterial systems studied so far, namely *E. coli* (Carbon *et al.*, 1965; Carbon & David, 1968; Saneyoshi & Nishimura, 1971), *B. subtilis* (Gohler & Doi, 1968; Chuang & Doi, 1972a) and *Ps. aeruginosa* (the present paper) are presented in a tabular form for convenience (Table 2). The acceptor activity of lysine tRNA, glutamic acid tRNA and serine tRNA is inhibited by iodine treatment to more or less the same extent in all the three bacterial systems. On the other hand, glutamine tRNA, phenylalanine tRNA and tyrosine tRNA do not seem to follow a general pattern with regard to the effect of chemical modification on acceptor activity. Only *Ps. aeruginosa* tyrosine tRNA showed a high degree of inhibition

Table 2. Comparative data on the effect of iodine, CNBr and N-ethylmaleimide treatment on the acceptor activity of different tRNA species of *E. coli*, *B. subtilis* and *Ps. aeruginosa*

Results are given wherever available and only maximum values are presented.

Amino acid	Organism	Percentage inhibition of acceptor activity after chemical modification with		
		I ₂	CNBr	N-Ethylmaleimide
Lysine	<i>E. coli</i> *	83	85	8
	<i>B. subtilis</i> †	80	—	12
	<i>Ps. aeruginosa</i> ‡	85	88	17
Glutamic acid	<i>E. coli</i>	92	86	0
	<i>B. subtilis</i>	90	—	18
	<i>Ps. aeruginosa</i>	84	95	14
Glutamine	<i>E. coli</i>	86§	72	0§
	<i>B. subtilis</i>	3	—	—
	<i>Ps. aeruginosa</i>	85	99	19
Serine	<i>E. coli</i>	77	34	39
	<i>B. subtilis</i>	50	—	15
	<i>Ps. aeruginosa</i>	76	30	12
Phenylalanine	<i>E. coli</i>	76	69	38
	<i>B. subtilis</i>	15	—	—
	<i>Ps. aeruginosa</i>	20	15	—
Tyrosine	<i>E. coli</i>	6§	2	22§
	<i>B. subtilis</i>	70	—	2
	<i>Ps. aeruginosa</i>	79	42	66

* For iodine treatment see Carbon *et al.* (1965); for N-ethylmaleimide treatment see Carbon & David (1968) and for CNBr treatment see Saneyoshi & Nishimura (1971).

† Gohler & Doi (1968); Chuang & Doi (1972a).

‡ Table 1 of the present paper.

§ B. Thimmappaya & J. D. Cherayil, unpublished work.

by N-ethylmaleimide treatment. It is possible that lysine tRNA, glutamic acid tRNA and serine tRNA may have common structural features between these three bacterial systems.

Fractionation of lysine tRNA

As lysine tRNA was one of the species most affected by iodine and CNBr it was decided to fractionate the tRNA and to study the properties of isoaccepting species. When a sample of total tRNA was fractionated on a BD-cellulose column at pH 5.0, the lysine-acceptor activity was partially separated into two peaks, a major peak, tRNA₁^{Lys}, and a minor peak, tRNA₂^{Lys} (Fig. 2a). Separation on this column for other tRNA species in general was very good. For example, tyrosine tRNA was well resolved from the lysine tRNA species. We could not achieve complete separation of the two isoaccepting

Fig. 2. Fractionation of lysine tRNA of *Ps. aeruginosa*

(a) Elution profile of *Ps. aeruginosa* lysine tRNA and tyrosine tRNA from BD-cellulose column (2.2cm×110cm); 5100 E_{260} units of bulk tRNA isolated from the cells grown in a nutrient medium as described in the Materials and Methods section was applied to the column. Elution was performed with a linear NaCl gradient from 0.4 to 1.0M-NaCl (900ml each) containing 0.05M-sodium acetate, pH 5.0, 0.01M-MgCl₂ and 0.05% NaN₃ at a flow rate of 38 ml/h. Fractions (8.2ml) were collected and 0.1 ml of the fractions was taken for acceptor assay. Recovery of E_{260} units was 100%. ———, E_{260} ; ●, [¹⁴C]lysine; △, [³H]tyrosine; ———, NaCl gradient. (b) Rechromatography of tRNA₁^{lys} on DEAE-Sephadex. Fractions 100–111 (615 E_{260} units) from the BD-cellulose column containing tRNA₁^{lys} were concentrated and applied to a DEAE-Sephadex column (1cm×90cm). Elution was performed with a linear NaCl gradient from 0.44 to 0.59M containing 0.02M-sodium acetate, pH 4.5, 0.01M-MgCl₂ and 7.0M-urea. The flow rate was 6.6 ml/h; 1.8 ml fractions were collected and 0.1 ml of the fractions was used for acceptor assay. ———, E_{260} ; ●, [¹⁴C]lysine; ———, NaCl gradient. Fractions from the hatched area in this Figure were taken as tRNA₁^{lys}. (c) Rechromatography of tRNA₂^{lys} from the BD-cellulose column (a) on a DEAE-Sephadex column. Fractions 112–130 (1400 E_{260} units) from the BD-cellulose column containing tRNA₂^{lys} were concentrated and chromatographed on a DEAE-Sephadex column as described for (b). The gradient in this run was 0.46–0.61M-NaCl. ———, E_{260} ; ●, [¹⁴C]lysine; ———, NaCl gradient. Fractions from the hatched area in this Figure were taken as tRNA₂^{lys}. (d) Co-chromatography of [¹⁴C]lysyl-tRNA₁^{lys} and [³H]lysyl-tRNA₂^{lys} on a BD-cellulose column (0.5cm 80cm); 56000 c.p.m. of [¹⁴C]lysyl-tRNA₁^{lys} was mixed with 52800 c.p.m. of [³H]lysyl-tRNA₂^{lys} and chromatographed. The column was developed with a linear 0.4–0.8M-NaCl gradient (70 ml each), containing 0.02M-sodium acetate, pH 4.5, and 0.01M-MgCl₂. Flow rate was 3.5 ml/h; 1.1 ml fractions were collected and 0.9 ml of each fraction was applied to Whatman no. 3 filter paper strips (2cm×6cm), washed and counted for radioactivity as described earlier (Anandraj & Cherayil, 1974b). □, [¹⁴C]lysyl-tRNA₁^{lys}; ■, [³H]lysyl-tRNA₂^{lys}; ———, NaCl gradient.

lysine tRNA species in any single column, such as the DEAE-cellulose column at both neutral and acid pH or with DEAE-Sephadex at pH 4.5 and 7.0 or with reversed-phase chromatographic system 5 (RPC-5, supplied by Miles Laboratories) at both neutral and acidic pH. Therefore a second column was used for further purification of the two lysine tRNA species. The two lysine tRNA peaks from the first column were pooled separately and each of the peaks was rechromatographed on a DEAE-Sephadex column in the presence of 7.0M-urea at pH 4.5. tRNA₁^{Lys} emerged from the column as a fairly pure species (Fig. 2b). The earlier fractions (cross-hatched area in Fig. 2b) in the peak were pooled and used as tRNA₁^{Lys}; peak 2 on rechromatography on a DEAE-Sephadex column was partially resolved into two peaks (Fig. 2c). The second peak (cross-hatched area in Fig. 2c) in this column was taken as tRNA₂^{Lys} and used for studies mentioned below. The first peak in this column was probably due to tRNA₁^{Lys}, present as contamination in the fractions from BD-cellulose (Fig. 2a) chosen for rechromatography.

To confirm that the two lysine-acceptor-activity peaks represented two distinct species, they were separately labelled with [¹⁴C]lysine and [³H]lysine, mixed and co-chromatographed at pH 4.5 on a BD-cellulose column. The ¹⁴C and ³H radioactivity was eluted separately and the profiles were not superimposable (Fig. 2d). Such non-superimposable profiles for two species is the best criterion to show that the two activity peaks are two distinct species (Soll *et al.*, 1967). Therefore the results suggested that the two activity peaks of lysine tRNA observed on a BD-cellulose column (Fig. 2a) were two separate lysine tRNA species and not artifacts. The 3' ends of the two species were identical, as shown by co-chromatography of a T₁-ribonuclease digest of the ¹⁴C-labelled and ³H-labelled species; on chromatography of the digest on a DEAE-cellulose column at pH 4.5 in the presence of 7.0M-urea, the radioactivity due to ¹⁴C and ³H coincided (profile not shown).

Effect of chemical modification on the acceptor activity of the lysine tRNA species

The two lysine tRNA species were subjected to mild iodine oxidation and CNBr treatment. Both the isoaccepting lysine tRNA species were tested for their acceptor activity after modification. As shown in Table 3, both the isoaccepting species were inactivated almost completely (90–95%) after iodine and CNBr treatment. These results suggested that thionucleotides were present in both the lysine tRNA species. The spectrum at 330nm corresponding to 4-thiouridine of partially purified tRNA₁^{Lys}, and paper chromatography of thio-

Table 3. *Amino acid acceptor activity of isoaccepting tRNA species of Ps. aeruginosa before and after chemical modifications*

Experiments were carried out in triplicate, and the results agreed within 5%.

Chemical modification	tRNA	Acceptor activity (c.p.m./E ₂₆₀ unit)		Percentage acceptor activity retained after modification
		Untreated	Treated	
I ₂ treatment	Lys ₁	15750	732	4.6
	Lys ₂	13850	960	7.6
CNBr treatment	Lys ₁	14340	814	5.6
	Lys ₂	12970	871	6.8

nucleosides prepared from partially purified ³⁵S-labelled tRNA₁^{Lys} (B. Thimmappaya & J. D. Cherayil, unpublished work) suggested that there was a 4-thiouridine residue present in tRNA₁^{Lys}. But we could not ascertain the presence or absence of 2-thiopyrimidine in it. At present we do not know the nature of the thionucleoside present in the minor lysine tRNA species.

Chuang & Doi (1972b) have shown that the lysyl-tRNA of *B. subtilis*, after treatment with iodine, was eluted from a BD-cellulose column at a lower salt concentration when compared with the untreated lysyl-tRNA, and this was believed to be due to the conformational change induced by iodine treatment. They could purify lysine tRNA to 60% by this method (Chuang & Doi, 1972b). We have carried out similar studies, by subjecting the pre-labelled [¹⁴C]lysyl-tRNA of *Ps. aeruginosa* to iodine treatment, with subsequent co-chromatography of the sample with [³H]lysine-labelled control tRNA on a BD-cellulose column at pH 4.5. There was no significant change in the elution profiles of the iodine-treated lysyl-tRNA species compared with the control (profiles not shown). If a shift in the elution profile was any indication, one could conclude that there was no significant change in the conformation of the two lysine tRNA species after iodine treatment.

Coding responses of tRNA₁^{Lys} and tRNA₂^{Lys}

The coding responses of the two lysine tRNA species were determined by the membrane-binding technique (Nirenberg & Leder, 1964) by using codons AAA and AAG at 20mM-Mg²⁺ and also poly(A) and poly(A₁G₁) at 10mM-Mg²⁺. Both tRNA species were bound to poly(A) equally well (Fig. 3a). As the response of the two species to poly(A) was equal, the anticodon of these species should be UUU. Both the lysine tRNA species were bound equally well to poly(A₁G₁) (Fig. 3b). The base ratio of the poly(A₁G₁) used in these studies

Fig. 3. Coding responses of isoaccepting lysine tRNA species of *Ps. aeruginosa*

(a) and (b) Stimulation of binding of isoaccepting lysine tRNA species to *Ps. aeruginosa* ribosomes with poly(A) and poly(A₁G₁); 4000 c.p.m. of ¹⁴C-labelled lysyl-tRNA species were used for the experiments and the details are described in the Materials and Methods section. Radioactivity bound to ribosomes in the absence of template (250–300 c.p.m.) was subtracted from each reading and Δc.p.m. are shown in the Figures. (a) Binding with poly(A): ○, lysyl-tRNA₁^{ys}; ▲, lysyl-tRNA₂^{ys}. (b) Binding with poly(A₁G₁): ○, lysyl-tRNA₁^{ys}; ▲, lysyl-tRNA₂^{ys}. (c) and (d) Effect of iodine oxidation on the binding efficiency of [¹⁴C]lysyl-tRNA₁^{ys} with poly(A) and poly(A₁G₁) on *Ps. aeruginosa* ribosomes; 2500 c.p.m. each of iodine-treated and control lysyl-tRNA₁^{ys} were used. Δc.p.m. was plotted. (e) and (f) Effect of iodine oxidation on the binding efficiency of [¹⁴C]lysyl-tRNA₂^{ys} with poly(A) and poly(A₁G₁) with *Ps. aeruginosa* ribosomes; 2500 c.p.m. each of iodine-treated and control lysyl-tRNA₂^{ys} were used and Δc.p.m. was plotted. (e) Iodine-treated lysyl-tRNA₂^{ys} (▲) and control (○) against poly(A). (f) Iodine-treated lysyl-tRNA₂^{ys} (▲) and control (○) against poly(A₁G₁).

was 1:1. Hence, the codons AAA and AAG would be present in equal proportions in the random co-polymer (A₁G₁). The amounts of synthetic polymer were sufficient to saturate the ribosomes being used (Figs. 3a and 3b). As the two species showed equal response to poly(A₁G₁), both the lysine tRNA species read codons AAA and AAG without discrimination. To rule out the possibility that the two species read only the AAA codons present in poly(A₁G₁), triplet-binding experi-

ments using AAA and AAG were carried out separately. *Ps. aeruginosa* ribosomes did not give good stimulation when triplets were used. Therefore *E. coli* ribosomes were used in these studies. These results are presented in Table 4. Both species exhibited almost equal response to codons AAA and AAG. From this it can be inferred that both the messages AAA and AAG present in the random copolymer (A₁G₁) were read by the two lysine tRNA species with almost equal efficiency.

Table 4. Triplet-stimulated ribosomal binding of lysine tRNA species of *Ps. aeruginosa*

The reaction mixture contained approx. 2500 c.p.m. of either [^{14}C]lysyl-tRNA $_{1}^{\text{Lys}}$ or [^{14}C]lysyl-tRNA $_{2}^{\text{Lys}}$; 0.1 M-Tris-HCl, pH 7.2; 0.05 M-KCl; 0.02 M-magnesium acetate; 0.15 E_{260} unit of trinucleotide; and 1.4 E_{260} units of *E. coli* B ribosomes in a volume of 0.05 ml. Incubation was carried out for 15 min at 25°C. The values presented below are after deducting the values obtained for the assay without template (none). The values in parentheses are the c.p.m. converted into pmol. The [^{14}C]lysine used in these experiments had a specific radioactivity of 180 $\mu\text{Ci}/\mu\text{mol}$. Efficiency of counting was taken as 55%.

Template	Ac.p.m. of [^{14}C]lysyl-tRNA bound to ribosomes	
	tRNA $_{1}^{\text{Lys}}$	tRNA $_{2}^{\text{Lys}}$
AAA	148 (0.67)	153 (0.7)
AAG	148 (0.67)	147 (0.67)
None	195 (0.89)	200 (0.90)

Studies from different laboratories have shown that the modification of a 2-thiouridine derivative, if present, in the first position of the anticodon, either by iodine or CNBr, inhibits the coding response of a particular tRNA (Soll & RajBhandary, 1967; Sane-yoshi & Nishimura, 1971; Liu & Ortwerth, 1972; Sen & Ghosh, 1973). Conformational change in tRNA due to iodine oxidation has also been shown to inhibit the coding response (Goehler & Doi, 1966; Chuang & Doi, 1972a). Therefore it was decided to study the coding response of the two lysine tRNA species, after modification with iodine. The two lysine tRNA species were acylated and then subjected to iodine oxidation along with parallel control samples and the coding responses of these species were studied by using poly(A) and poly(A_1G_1). These results are shown in Figs. 3(c)–3(f). There was no significant difference in the coding response of the iodine-treated tRNA species compared with the control. The maximum inhibition observed for lysyl-tRNA $_{1}^{\text{Lys}}$ with poly(A_1G_1) (Fig. 3d) or lysyl-tRNA $_{2}^{\text{Lys}}$ with poly(A) (Fig. 3e) did not exceed more than 22% of that for the corresponding control lysyl-tRNA. These results therefore show that the chemical modification of lysyl-tRNA species by iodine did not affect their coding responses.

Discussion

The high loss of acceptor activity by lysine tRNA, glutamic acid tRNA, glutamine tRNA, serine tRNA and tyrosine tRNA due to iodine or CNBr treatment suggests the presence of thionucleotides in these tRNA species. Considerable loss of acceptor activity of tyrosine tRNA caused by *N*-ethylmale-

imide treatment suggests the presence of 4-thiouridine residues in this tRNA. Tyrosine tRNA of *Ps. aeruginosa* appears to be the only case in the three bacterial systems so far reported (see Table 2) where *N*-ethylmaleimide treatment results in high loss of activity. *E. coli* tRNA $^{\text{Trp}}$ has been shown to contain two 4-thiouridine residues in the eighth and ninth positions from the 5' end, and a methylthioisopentenyl adenosine at the 3' end, next to the anticodon (Nishimura, 1972). In spite of this, none of the thiopyrimidine-specific reagents affected the acceptor activity (Table 2). This observation, along with the results presented for glutamine tRNA and phenylalanine tRNA (Table 2), suggests that the pattern of modification or role of thionucleotides in the structure and function of these tRNA species in the three bacterial systems studied so far, i.e. *E. coli*, *B. subtilis* and *Ps. aeruginosa*, may not follow a general pattern.

The two isoaccepting lysine tRNA species could be fractionated from *Ps. aeruginosa* tRNA by the combined use of BD-cellulose and DEAE-Sephadex. Co-chromatographic experiments using ^{14}C -labelled tRNA $_{1}^{\text{Lys}}$ and ^3H -labelled tRNA $_{2}^{\text{Lys}}$ suggest that they are two distinct species. This is a reliable criterion to show that the two activity peaks observed in a BD-cellulose column (Fig. 2a) represent two lysine tRNA species (Soll *et al.*, 1967). However, they were inactivated almost to the same extent by iodine and CNBr treatment. Since both tRNA $_{1}^{\text{Lys}}$ and tRNA $_{2}^{\text{Lys}}$ can bind codons AAA and AAG as well as poly(A) and poly(A_1G_1) with an equal efficiency, the anticodon of these two tRNA species, in theory, should be the same. The wobble hypothesis (Crick, 1966) predicts that the first base on the anticodon wobbles and can translate both A and G (i.e. AAA and AAG), and therefore the sequence in the anticodon must be UUU and not UUC. Both the lysine tRNA species bind to the same extent to the codon AAG (Table 4) and also to poly(A_1G_1) (Fig. 3b), reading both the messages AAA and AAG present in the random copolymer without discrimination. Ohashi *et al.* (1970) have observed that the *E. coli* tRNA $^{\text{Glu}}$ could bind to codons GAA and GAG equally well at 20 mM-Mg $^{2+}$. However, at 10 mM-Mg $^{2+}$ the stimulation of binding for only GAA was observed; GAG was not read. This was shown to be due to the presence of 5-methylamino-methyl-2-thiouridine in the first position of the anticodon of glutamic acid tRNA (Ohashi *et al.*, 1970). When we carried out the triplet-binding assay of lysine tRNA species with codons AAA and AAG, at 10 mM-Mg $^{2+}$, no detectable stimulation was observed even for triplet AAA. At the same time, our ribosome-binding studies with poly(A) at 10 mM-Mg $^{2+}$ (Fig. 3a) show that both the lysine tRNA species recognize the AAA codon equally well.

It is therefore clear that for the triplets AAA and AAG, the minimum Mg^{2+} concentration required for detectable stimulation of binding is 20mM. Iodine oxidation of lysyl-tRNA species does not cause any serious inhibition in reading either poly(A) or poly(A_1G_1). The inhibition observed with tRNA $_{Lys}^1$ with poly(A_1G_1) (Fig. 3d) or tRNA $_{Lys}^2$ with poly(A) (Fig. 3e) is less than 22%. The results discussed above give a good indication that 5-methylaminomethyl-2-thiouridine may not be present in the first position of the anticodon of either of the lysine tRNA species. It is noteworthy that iodine treatment results in almost total inhibition of acceptor activity, but does not influence the coding ability of these tRNA species to a great extent. Therefore the structural change in the molecule as a consequence of iodine treatment may not be in the anticodon or anticodon loop, but elsewhere, which probably is crucial for the interaction with cognate synthetase. We suggest (B. Thimmappaya & J. D. Cherayil, unpublished work) that there is a 4-thiouridine in tRNA $_{Lys}^1$, but we do not know the nature of the thionucleotide present in tRNA $_{Lys}^2$. The effects of *N*-ethylmaleimide treatment on the acceptor activity of total lysine tRNA (Table 1) suggest that 4-thiouridine residues might not play an important role in aminoacylation. Obviously, explanation for inactivation of lysine tRNA by iodine or CNBr treatment should be sought elsewhere. If 2-thiopyrimidine residues are responsible for the inactivation, they may be present in the molecule, in a region other than the anticodon loop. Alternatively, one could also imagine the presence of any other new modified bases, other than the thionucleotides, which may be sensitive to iodine and CNBr treatment and which would also be crucial for the aminoacylation of lysine tRNA. Knowledge of complete nucleotide sequence or base composition analysis of purified lysine tRNA species is essential to clarify this point. In *B. subtilis* and *E. coli*, the two lysine tRNA species were inactivated by iodine to different extents, but not completely (Chuang & Doi, 1972a; Soll & RajBhandary, 1967). One of the *B. subtilis* lysine tRNA species was shown to bind to poly(A_1G_1) preferentially. However, the anticodon of these species was shown to be UUU and the preference for poly(A_1G_1) observed was believed to be due to the inherent property of this tRNA (Chuang & Doi, 1972a). A small difference in the coding response observed for *Ps. aeruginosa* lysine tRNA species with either poly(A) or poly(A_1G_1) (Fig. 3a and 3b), may be due to such an inherent property of the molecules. Either in *E. coli* or in *B. subtilis*, treatment of lysine tRNA species either by iodine or by CNBr resulted in complete or high degree of inhibition of coding response (Soll & RajBhandary, 1967; Saneyoshi & Nishimura, 1971;

Chuang & Doi, 1972a). *Ps. aeruginosa* lysine tRNA species appear to be very different from those of the other two species.

The two lysine tRNA species of *Ps. aeruginosa* were eluted very close together in any column-chromatographic system, irrespective of the type of column we used. However, the pattern is highly reproducible, and in several independent experiments that we have carried out an identical pattern was obtained. Such close and overlapping elution profiles suggest that the two species may have many resemblances in their structures. Alternatively, a single lysine tRNA species may exist in two conformations and therefore appear as two species. These possibilities can be confirmed only when the nucleotide sequence of these species is worked out. The two lysine tRNA species of *Ps. aeruginosa* are redundant in that both the species code for AAA and AAG to the same extent and are indistinguishable with regard to the effect of chemical modification on acceptor activity. It has been postulated (Carbon *et al.*, 1965) that the reversible oxidative inactivation of tRNA species of the type mentioned above may be a basis for the control of translation by adaptor modification (Sueoka & Kano-Sueoka, 1964). *Ps. aeruginosa* appears to be a suitable system for the study of such an enzyme if it exists.

We are indebted to Mrs. A. Gunakreeta for excellent technical assistance and to Dr. R. Maya Sundari for poly(A_1G_1). This research project is supported by the National Institutes of Health, Washington from PL-480 funds.

References

- Adams, M. H. (1959) *Bacteriophages*, Interscience, New York
- Agris, P. F., Soll, D. & Seno, T. (1973) *Biochemistry* **12**, 4331-4337
- Anandraj, M. P. J. S. & Cherayil, J. D. (1974a) *Anal. Biochem.* **58**, 190-194
- Anandraj, M. P. J. S. & Cherayil, J. D. (1974b) *Biochem. J.* **141**, 647-653
- Bock, R. M. & Cherayil, J. D. (1967) *Methods Enzymol.* **12**, 638-644
- Carbon, J. A. (1971) *Methods Enzymol.* **20**, 129-135
- Carbon, J. A. & David, H. (1968) *Biochemistry* **7**, 3851-3858
- Carbon, J. A., Hung, L. & Jones, D. S. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **53**, 979-986
- Caskey, T., Beaudet, A. & Nirenberg, M. (1968) *J. Mol. Biol.* **37**, 99-118
- Chuang, R. Y. & Doi, R. H. (1972a) *J. Biol. Chem.* **247**, 3476-3489
- Chuang, R. Y. & Doi, R. H. (1972b) *Biochim. Biophys. Acta* **272**, 53-55
- Crick, F. (1966) *J. Mol. Biol.* **19**, 548-555

- Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmor, E. & Tener, G. M. (1967) *Biochemistry* **6**, 3043-3056
- Goehler, B. & Doi, R. H. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1047-1051
- Goehler, B. & Doi, R. H. (1968) *J. Bacteriol.* **95**, 793-800
- Harada, F., Saneyoshi, M. & Nishimura, S. (1971) *FEBS Lett.* **13**, 335-338
- Holloway, B. W. (1969) *Bacteriol. Rev.* **33**, 419-443
- Ishida, T. & Miura, K. (1965) *J. Mol. Biol.* **11**, 341-357
- Juarez, H., Juarez, D. & Hedgoth, C. (1974) *Biochem. Biophys. Res. Commun.* **61**, 110-116
- Lipsett, M. N. (1965) *J. Biol. Chem.* **240**, 3975-3978
- Liu, L. P. & Ortwerth, B. J. (1972) *Biochemistry* **11**, 12-17
- Madison, J. T., Boguslawski, S. J. & Tector, G. H. (1972) *Science* **176**, 687-689
- Nirenberg, M. & Leder, P. (1964) *Science* **145**, 1399-1407
- Nishimura, S. (1972) *Prog. Nucleic Acid Res. Mol. Biol.* **12**, 49-85
- Nishimura, S., Jones, D. S., Ohtsuka, E., Hayatsu, J., Jacob, T. M. & Khorana, H. G. (1965) *J. Mol. Biol.* **13**, 283-301
- Ohashi, Z., Saneyoshi, M., Harada, F., Hara, H. & Nishimura, S. (1970) *Biochem. Biophys. Res. Commun.* **40**, 866-872
- Ortwerth, B. J. & Liu, L. P. (1973) *Biochemistry* **12**, 3978-3984
- Ortwerth, B. J., Yonuschot, G. R. & Carlson, J. V. (1973) *Biochemistry* **12**, 3985-3991
- Rao, Y. S. P. & Cherayil, J. D. (1973) *Biochim. Biophys. Acta* **299**, 1-7
- Rao, Y. S. P. & Cherayil, J. D. (1974) *Biochem. J.* **143**, 285-294
- Rudloff, E. & Hilse, K. (1971) *Eur. J. Biochem.* **24**, 313-320
- Saneyoshi, M. & Nishimura, S. (1970) *Biochim. Biophys. Acta* **204**, 389-399
- Saneyoshi, M. & Nishimura, S. (1971) *Biochim. Biophys. Acta* **246**, 123-131
- Sen, G. C. & Ghosh, H. P. (1973) *Biochim. Biophys. Acta* **308**, 106-116
- Smith, J. C., Teh, H., Ley, A. N. & D'Obrenan, P. (1973) *J. Biol. Chem.* **248**, 4475-4485
- Soll, D. & RajBhandary, U. L. (1967) *J. Mol. Biol.* **29**, 113-124
- Soll, D., Cherayil, J. D. & Bock, R. M. (1967) *J. Mol. Biol.* **29**, 97-112
- Stanier, R. Y., Palleroni, N. J. & Doudroff, M. (1966) *J. Gen. Microbiol.* **43**, 159-271
- Sueoka, N. & Kano-Sueoka, T. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **52**, 1535-1540
- Thimmappaya, B. (1974) Ph.D. Thesis, Indian Institute of Science, Bangalore
- Thimmappaya, B. & Cherayil, J. D. (1972) *Life Sci.* **11**, 211-219
- Thimmappaya, B. & Cherayil, J. D. (1974) *Biochem. Biophys. Res. Commun.* **60**, 665-672
- Walker, R. T. & RajBhandary, U. L. (1970) *Biochem. Biophys. Res. Commun.* **38**, 907-914
- Woodward, W. R. & Herbert, E. (1972) *Science* **177**, 1197-1199