Chemical Modification as a Probe of Conformational Changes in Transfer Ribonucleic Acid on Aminoacylation

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Treatment of *Escherichia coli* CA265 phenylalanyl-tRNA with 3M-NaHSO₃, pH 6.0, at 25°C resulted in modification of four bases and in the deacylation of the charged tRNA^{Phe}. The similarity of the rates of base modification and of the deacylation of the phenylalanyl-tRNA permitted the isolation of partially modified phenylalanyl-tRNA^{Phe} and partially modified deacylated tRNA^{Phe}. The sites and extents of base modification in these fractions were determined and found to be the same as those in uncharged tRNA^{Phe} modified under identical conditions. These findings are discussed in relation to previous evidence for and against a conformational change in tRNA on its aminoacylation. The methods described should prove adaptable to study of other aminoacyl-tRNA species.

Aminoacyl-tRNA has been reported to interact differently from tRNA with *Escherichia coli* elongation factor EF-Tu (Ono *et al.*, 1968), aminoacyl-tRNA ligases (Lagerkvist *et al.*, 1966), the histidine-operon repressor (Lewis & Ames, 1972) and ribosomal binding sites (Grajevskaja *et al.*, 1972). To account for these differences, it has been suggested that tRNA molecules undergo a conformational change on aminoacylation (Sarin & Zamecnik, 1965; Schofield, 1970; Woese, 1970). C.d. and u.v. studies of *E. coli* tRNA^{Val} and tRNA^{Met} (Adler & Fasman, 1970), partial-nuclease-digestion studies of *E. coli* tRNA^{Phe}, yeast tRNA^{Phe} and yeast tRNA^{Ser} (Hanngi & Zachau, 1971), small-angle X-ray-scattering studies of *E. coli* tRNA^{Val} (Ninio *et al.*, 1972), ³H-exchange studies of *E. coli* tRNA^{Met} (Englander *et al.*, 1972), n.m.r. studies of yeast tRNA^{Phe} (Wong *et al.*, 1973) and Raman-spectroscopy studies of yeast tRNA^{Phe} (Thomas *et al.*, 1973) did not show any detectable change in the conformation of the tRNA on aminoacylation.

However, small conformational changes were detectable on aminoacylation by other c.d. and u.v. studies of *E. coli* tRNA^{Met} (Watanabe & Imahori, 1971), changed kinetics of binding of ethidium bromide to tRNA (Tritton & Mohr, 1973), increased binding of Mn²⁺ and oligo(C) to aminoacylated tRNA (Cohn *et al.*, 1969; Danchin & Grunberg-Manago, 1970) and change in spin-label (Caron *et al.*,

1976) and nuclear-magnetic (Kan *et al.*, 1976) resonance peaks. Most of the available evidence implies that, if any changes do occur on aminoacylation, they are small, being restricted to slight changes in tertiary structure.

Forget & Weissman (1967) suggested that binding of aminoacyl-tRNA to the ribosome might involve interactions between the sequence G-T-Ψ-C-R that occurs in the T-Ψ-C loop of nearly all tRNA species, and a C-G-A-A-C sequence contained in 5S RNA of the large ribosomal subunit. There is considerable evidence supporting such a hypothesis (Sprinzl *et al.*, 1976, and references therein), although detailed models of tRNA structure based on X-ray crystallography of yeast tRNA^{Phe} (Jack *et al.*, 1976; Quigley & Rich, 1976) indicate that the G-T-Ψ-C-R sequence is heavily involved in tertiary-structure hydrogen-bonding in the uncharged tRNA. It is therefore probable that a conformational change exposing the G-T-Ψ-C-R sequence occurs either on aminoacylation of the tRNA or on EF-Tu-dependent binding of the aminoacyl-tRNA to its site on the ribosome (i.e. the ribosome A-site).

Determination of the sites and extents of chemical modification of residues in tRNA species has been extensively used to study the conformation of tRNA molecules in solution, and the results of these solution studies are in remarkably good agreement with those of X-ray-crystallographic studies, suggesting that tRNA conformation is the same in both states. One of the chemical reagents most widely used in such studies is NaHSO₃ (Lowdon & Goddard, 1976, and references therein). This reacts reversibly with pyrimidines to form 5,6-dihydropyrimidine-6-sulphonate (Hayatsu *et al.*, 1970; Shapiro *et al.*, 1970).

Abbreviations used: ms²i⁶A, 2-methylthio-*N*⁶-isopentyladenosine; D, 5,6-dihydrouridine; m⁷G, *N*⁷-methylguanosine; s⁴U, 4-thiouridine; in this paper X is 3-(3-amino-3-carbodiethyl)uridine (i.e. not xanthosine) and N is an alkali-stable nucleoside produced by reaction of m²i⁶A with NaHSO₃ (i.e. not 'unspecified nucleoside').

This 'bisulphite-adduct' formation facilitates the deamination to uracil of exposed cytosine bases that are not involved in hydrogen-bonding, whereas hydrogen-bonded cytosine molecules are not deaminated (Goddard & Schulman, 1972). If a conformational change were to occur on aminoacylation of tRNA^{Phe} that altered the environment of a cytidine residue, it would be readily detected by a difference in the reactivity to bisulphite of that residue in tRNA^{Phe} and phenylalanyl-tRNA^{Phe}. For example, the sequence G-T-Ψ-C-R, which involves residues occurring at positions 53–57 from the 5'-end, may be exposed on aminoacylation. If this were so, residue C₍₅₆₎, which in tRNA^{Phe} is hydrogen-bonded to G₍₁₉₎ (Jack *et al.*, 1976; Quigley & Rich, 1976) and is not deaminated by bisulphite reaction (Lowdon & Goddard, 1976), would be deaminated to uridine in bisulphite-modified phenylalanyl-tRNA^{Phe}.

In spite of their proven sensitivity and reliability as a tRNA conformational probe, chemical modification of aminoacyl-tRNA has not been reported. This is most probably because the amino acid is hydrolysed from the aminoacyl-tRNA under the chemical-modification conditions normally used.

In the studies of modification of *E. coli* phenylalanyl-tRNA by bisulphite reported here, deacylation of the phenylalanyl-tRNA^{Phe} was also observed. However, ambiguities in interpretation were avoided by separation of modified phenylalanyl-tRNA^{Phe} from modified and deacylated tRNA^{Phe} after a period of modification and before structural analysis.

Experimental

Materials

U-³²P-labelled tRNA from *E. coli* K12 CA265 and all other radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Mixed tRNA from *E. coli* K12 CA265 was supplied by the Microbiological Research Establishment, Porton, Salisbury, Wilts., U.K. Purified tRNA^{Phe} (*E. coli* M.R.E. 600) and benzoylelated DEAE-cellulose were obtained from Boehringer, Lewes, East Sussex, U.K.

E. coli phenylalanine-tRNA ligase was prepared from *E. coli* M.R.E. 600 cells, obtained from the Microbiological Research Establishment, by the method of Stulberg (1967), the hydroxyapatite-chromatography stages being omitted.

Pancreatic ribonuclease (Worthington) was purchased from Cambrian Chemicals, Croydon, Surrey, U.K., and ribonuclease T₁ from Calbiochem, London W.1, U.K. NaHSO₃ was from Sigma, Kingston upon Thames, Surrey, U.K., and RPC-5 (reversed-phase-chromatography system 5) was from Miles Laboratories, Stoke Poges, Bucks., U.K.

Electrophoresis was carried out on cellulose acetate strips obtained from Oxoid, London E.C.4, U.K., or

on DEAE-cellulose paper (DE 81) from Whatman Biochemicals, Maidstone, Kent, U.K. Kodirex X-ray film was obtained from Kodak, Wythenshawe, Manchester, U.K.

Methods

U-³²P-labelled *E. coli* tRNA^{Phe} was purified from crude U-³²P-labelled tRNA by fractionation on benzoylelated DEAE-cellulose followed by chromatography on RPC-5, and the phenylalanine-accepting ability of purified tRNA^{Phe} was measured as described previously (Lowdon & Goddard, 1976). The purified U-³²P-labelled tRNA^{Phe} (125–250 nCi/μg; 30–40 pmol of phenylalanine accepted/μg), was pooled, mixed with an equimolar amount of unlabelled tRNA and precipitated by addition of 2.1 vol. of ethanol.

[2,3-³H]Phenylalanyl-[U-³²P]tRNA^{Phe} was prepared by incubation of 80 μg of [U-³²P]tRNA^{Phe} with 1.0 ml of 100 mM-Tris adjusted to pH 7.5 with HCl, 10 mM-MgCl₂, 10 mM-KCl, 10 mM-NH₄Cl, 4 mM-reduced glutathione, 2 mM-ATP, 6.7 μM-[2,3-³H]-phenylalanine (1 Ci/mmol) and 200 μg of purified phenylalanine-tRNA ligase, for 20 min at 37°C. The mixture was then chilled on ice and 0.1 ml of 1 M-sodium acetate (adjusted to pH 5.0 with acetic acid) was added. The phenylalanyl-tRNA^{Phe} was isolated by adsorption on a column (0.7 cm × 3.0 cm) of DEAE-cellulose equilibrated with 50 mM-sodium acetate, pH 5.0, containing 0.1 M-NaCl. The phenylalanine-tRNA ligase and excess phenylalanine were eluted from the column by application of 50 mM-sodium acetate, pH 5.0, containing 0.35 M-NaCl. When no further radioactivity or u.v.-absorbing material was detected in the eluate, the phenylalanyl-tRNA was eluted with a solution containing 0.1 M-sodium acetate, pH 5.0, 2.0 M-NaCl and 30% (v/v) ethanol, and was precipitated by addition of ethanol. The charged tRNA was suspended in 3 M-NaHSO₃/10 mM-MgCl₂, pH 6.0, at 25°C for 8 h. At the end of this time, the modified tRNA was dialysed to remove excess bisulphite, first against 0.1 M-sodium acetate/10 mM-MgCl₂, pH 5.0, and then twice against 10 mM-sodium acetate/10 mM-MgCl₂, pH 5.0, each time for 1 h at 4°C, before fractionation on benzoylelated DEAE-cellulose (Litt, 1968). The radioactivity in a portion (10 μl) of each 1 ml fraction was measured by application to a 2.5 cm Whatman 3MM filter disc, solubilization in 0.5 ml 10% (v/v) Hyamine hydroxide at 60°C for 20 min and counting in 10 ml of scintillation fluid {0.5% (w/v) PPO (2,5-diphenyloxazole) and 0.03% (w/v) POPOP [1,4-bis-(5-phenyloxazol-2-yl)-benzene] in toluene} (see Fig. 1 and the Results section). The separated discharged and charged tRNA^{Phe} were individually pooled, and dialysed against 0.1 M-Tris/HCl, pH 9.0 at 37°C, for 9 h at 37°C to remove bisulphite adducts and to deacylate the Phe-tRNA^{Phe}. The solutions were neutralized by

further dialysis against 10mM-Tris/HCl (pH 7.0)/10mM-MgCl₂, desalted by exhaustive dialysis against water and freeze-dried. The freeze-dried tRNA was digested with pancreatic ribonuclease or T₁ ribonuclease [enzyme/tRNA ratio of 1:10 (w/w)] and the produced oligoribonucleotides were separated and analysed by the methods of Sanger *et al.* (1965). The relative molar yield (y) of each oligonucleotide was determined from its ³²P content (x) relative to

the total ³²P content of the 'fingerprint' (Σx). For an oligonucleotide of chain length n in tRNA^{Phe} (76 nucleotides) the relative molar yield $y = 76x/n\Sigma x$.

Results

Deacylation of phenylalanyl-tRNA during its modification with NaHSO₃

The rate and extent of deacylation of *E. coli* [2,3-³H]phenylalanyl-tRNA^{Phe} under the usual con-

Fig. 1. Separation of bisulphite-modified phenylalanyl-tRNA^{Phe} from bisulphite-modified and deacylated phenylalanyl-tRNA^{Phe}

The NaCl concentration of the elution buffer, containing also 10mM-MgCl₂ and 10mM-sodium acetate, pH 5.0, is shown (·····). The [2,3-³H]phenylalanyl-[U-³²P]tRNA^{Phe} containing both [³H]phenylalanine (----) and [U-³²P]tRNA^{Phe} was eluted only on application of a 0–10% (v/v) ethanol gradient (—) in 2M-NaCl/10mM-MgCl₂/10mM-sodium acetate, pH 5.0.

Table 1. Percentage of molar yields of oligoribonucleotides in T₁-ribonuclease digests of bisulphite-modified phenylalanyl-tRNA^{Phe}, modified and deacylated phenylalanyl-tRNA^{Phe}, modified tRNA^{Phe} and unmodified tRNA^{Phe}

The percentage molar yields were determined as described in the Experimental section. The new oligonucleotides are numbered as in the key of Fig. 2, and sequences were determined as described previously (Lowdon & Goddard, 1976).

Sequence of oligoribonucleotide	Percentage molar yield			
	Unmodified	Modified tRNA ^{Phe}	Modified Phe-tRNA ^{Phe}	Deacylated modified Phe-tRNA ^{Phe}
Gp+2':3'-cyclic Gp	102	103	104	108
A-Gp	128	132	135	135
C-A-Gp	97	104	98	96
C-C-C-Gp	86	95	95	96
C-A-C-C-A	96	31	20	38
pGp	90	105	107	102
D-A-Gp	101	112	102	95
D-C-Gp	124	20	17	16
U-C-C-Gp	105	106	104	96
C-U-C-A-Gp	83	85	86	92
A-U-A-Gp	52	62	55	45
T-Ψ-C-Gp	131	127	133	95
A-Ψ-U-Gp	123	101	103	119
A-U-U-C-C-Gp	117	101	109	108
A-A-ms ² i ⁶ A-A-Ψ-C-C-C-C-Gp	86	31	30	21
U-m ⁷ G-X-C-C-U-U-Gp	87	88	78	77
C-A(C,U)A (spot 19)	0	23	15	17
C-A-U-U-A (spot 20)	0	45	64	43
D-U-Gp (spot 21)	0	86	82	88
A-A-N-A-Ψ-C-C-C-C-Gp (spot 22)	0	48	30	42

ditions of modification by bisulphite was measured as trichloroacetic acid - insoluble $[2,3\text{-}^3\text{H}]$ phenylalanyl-tRNA^{Phe} remaining after incubation in 3M-NaHSO₃ (pH6)/10mM-MgCl₂. These results showed the reaction to be first-order with respect to the phenylalanyl-tRNA that remained aminoacylated. The rate of deacylation at pH6.0 ($t_{1/2} = 3.5$ h) was comparable with the rates of modification of exposed cytidine residues to uridine in uncharged tRNA^{Phe}, where the time of half-reaction, $t_{1/2}$, was in the range 2.25–7h (Lowdon & Goddard, 1976).

Separation of modified phenylalanyl-tRNA and modified deacylated tRNA^{Phe}

Modification of $[2,3\text{-}^3\text{H}]$ phenylalanyl-[U-³²P]-

tRNA^{Phe} in 3M-NaHSO₃ (pH6.0)/10mM-MgCl₂ for 8h resulted in deacylation of 70–80% of the charged tRNA. To separate the charged and discharged forms of the modified tRNA, the reaction mixture was dialysed as described above and fractionated on a column (1.0cm×5.0cm) of benzoylated DEAE-cellulose equilibrated with 0.3M-NaCl/10mM-MgCl₂/10mM-sodium acetate, pH5.0, at 4°C. Increasing the salt concentration released the deacylated tRNA, but the more hydrophobic phenylalanyl-tRNA required an ethanolic solution for its elution (Fig. 1). (A small amount of [³H]phenylalanine, which was hydrolysed from the $[2,3\text{-}^3\text{H}]$ phenylalanyl-tRNA during the chromatography, was eluted by high-salt no-ethanol buffer).

Fig. 2. 'Fingerprints' of ribonuclease-T₁ digests of bisulphite-modified *E. coli* phenylalanyl-tRNA^{Phe} (a) and bisulphite modified *E. coli* tRNA^{Phe} (b)

These 'fingerprints' (a and b) may be compared with that of unmodified *E. coli* tRNA^{Phe} (c). The key (d) identifies sequences that remained unchanged (closed circles) or were lost (broken circles) on bisulphite modification of either phenylalanyl-tRNA^{Phe} or tRNA^{Phe}. New spots that appeared on modification (shaded circles) are numbered according to Table 1, which shows the percentage molar yields of each spot in the 'fingerprints' shown here and in that of deacylated bisulphite-modified phenylalanyl-tRNA^{Phe} (not shown).

Fig. 3. Structure of *E. coli* tRNA^{Phe} determined by Barrell & Sanger (1969)

The tertiary-structure hydrogen-bonding between bases known for yeast tRNA^{Phe} and the sites of C→U modification are shown.

'Fingerprint' analysis of modified phenylalanyl-tRNA^{Phe}, modified and deacylated phenylalanyl-tRNA^{Phe} and modified uncharged tRNA^{Phe}

Fig. 2 shows 'fingerprints' of ribonuclease T₁ digests of charged modified phenylalanyl-tRNA^{Phe}, of uncharged tRNA^{Phe} modified and treated in a manner identical with that for phenylalanyl-tRNA^{Phe} and of untreated tRNA^{Phe}. The 'fingerprints' of the bisulphite-modified tRNA species show several differences from that of untreated tRNA, the structure of which is shown in Fig. 3; modification of the 3'-terminus, C-A-C-C-A, resulted in two new spots, C-A(C,U)A (spot 19 in key to Fig. 2) and C-A-U-U-A (spot 20). The spot corresponding to D-C-Gp is greatly diminished, and a new spot 21 (D-U-Gp) has appeared. Modification of ms²i⁶A at position 37 resulted in partial loss of the oligonucleotide A-A-ms²i⁶A-A-Ψ-C-C-C-C-Gp produced on digestion with ribonuclease T₁ and the appearance of A-A-N-A-Ψ-C-C-C-C-Gp (spot 22), where N most probably is an alkali-stable bisulphite adduct of ms²i⁶A. The location and identification of these new products was aided by previous studies (Lowdon & Goddard, 1976) of uncharged tRNA^{Phe} modification by bisulphite.

The 'fingerprints' of charged modified phenylalanyl tRNA^{Phe} and modified uncharged tRNA^{Phe} (Fig. 2) are qualitatively identical with each other and with that of modified phenylalanyl-tRNA^{Phe}, which was deacylated during the modification ('fingerprint' not shown). This indicates that no new

residues in tRNA^{Phe} become available for bisulphite modification, nor are any cytidine residues protected from modification as a result of aminoacylation. The extents of modification of the reactive residues, shown in Table 1, reveal no great differences in the extent of modification of the reactive residues.

Discussion

We have found no differences in the sites of reaction of NaHSO₃ with *E. coli* tRNA^{Phe} and phenylalanyl-tRNA^{Phe}. The only reactive residues are C₍₁₇₎, ms²i⁶A₍₃₇₎, C₍₇₄₎ and C₍₇₅₎, which are located in three sequentially and spatially distant regions of the molecule. The differences in extent of modification of these residues in the different fractions are small and probably not significant. For example, the apparent slightly higher reactivity of residues C₍₇₄₎ and C₍₇₅₎ of the 3'-terminus C-A-C-C-A₍₇₆₎ in the phenylalanyl-tRNA^{Phe} may be accounted for by a stabilization of phenylalanyl-tRNA^{Phe} when C₍₇₄₎ and C₍₇₅₎ are modified. (Only 60% of the deacylated modified phenylalanyl-tRNA molecules are modified at C₍₇₄₎ or C₍₇₅₎ compared with 70–80% in other fractions.) Similarly, although the relative molar yield of T-Ψ-C-Gp in modified deacylated phenylalanyl-tRNA^{Phe} is not so high as in other modified tRNA fractions, no new product corresponding to T-Ψ-U-Gp could be detected. We therefore conclude that no gross conformational change, such as breakage of tertiary-structure hydrogen-bonding to expose previously 'buried' cytidine residues, occurs on aminoacylation of *E. coli* tRNA^{Phe}.

This conclusion differs from that of studies by Caron *et al.* (1976) on the same tRNA, in which attachment of spin labels indicated that phenylalanyl-tRNA^{Phe} differs from tRNA^{Phe} at other regions of the molecule, namely in the environment of X₍₄₇₎ and s⁴U₍₈₎. In the chemical-modification technique reported by us, 5–10% reaction of any residue would be detected. Thus loosening of the tertiary-structure hydrogen bonds of G₍₁₉₎-C₍₅₆₎ and G₍₁₅₎-C₍₄₈₎, if it occurs at all on aminoacylation, is not sufficient to increase the reactivity of C₍₄₈₎ and C₍₅₆₎ to within 10% of the reactivity of the non-bonded residues C₍₁₇₎, C₍₇₄₎ and C₍₇₅₎. (Our results, however, do not exclude the possibility of some subtle change in the conformation not detectable by our techniques.)

If the tRNA does not undergo a major conformational change on aminoacylation, this does not rule out such a change at a later stage in translation, that of codon-dependent binding of the EF-Tu-GTP-aminoacyl-tRNA complex to the ribosome. Experimental evidence to support such a conformational change has been reported (Schwartz *et al.*, 1974, 1976; Sprinzl *et al.*, 1976; Robertson *et al.*, 1977).

The use of benzoylated DEAE-cellulose to separate modified phenylalanyl-tRNA^{Phe} and modified deacylated phenylalanyl-tRNA^{Phe} may limit direct application of our methods to study of tRNA species to which aromatic amino acids are attached. However, if dihydroxyboryl-substituted cellulose is used to effect the separation (McCutchan *et al.*, 1975) the method should prove applicable to chemical-modification studies of any aminoacyl-tRNA.

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