The kinetics of bisulphite modification of reactive residues in E.coli tRNA^{Phe}₂

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ABSTRACT

E coli tRNA^{Phe}₂ was modified at 25°C with 3M sodium bisulphite, pH6.0, for periods of up to 48 hours. Three cytidine residues, at positions 17, 74 and 75 from the 5' end were each deaminated to uridine. The 2-methylthio-N⁶-isopentenyl adenosine at position 37 formed a 1:1 bi-sulphite addition product which was stable to alkali. No other residues were permanently modified. The rate of modification of each residue was first order with respect to remaining unmodified nucleotide, the time of half reaction, $t_{1/2}$, being different for each residue. C17 reaction reacted at twice the rate of cytidines in PolyC, indicating that it occupied a very exposed position in the tRNA.

INTRODUCTION

The 3-dimensional structure of Yeast tRNA^{Phe} has recently been determined by X-ray crystallography at 3.0Å¹⁻³ and 2.5Å⁴ resolution. The structure of other tRNA species is thought to be similar. Chemical modification studies on Yeast tRNA^{Phe} have shown that, as was suspected, the bases involved in tertiary structure base pairs in the crystalline form are not available for chemical modification in solution^{5, 6}.

This has provided evidence that the tertiary structure of Yeast tRNA^{Phe} is the same both in the crystalline form and in solution, as well as confirming chemical modification as an effective technique for the investigation of tRNA tertiary structure.

Bisulphite reacts with cytosine and uracil forming bisulphite adducts⁷⁻⁹. The formation of 5, 6 dihydrouracil-6-sulphonate is optimal at pH7 and of 5, 6, dihydrocytosine-6-sulphonate at pH5 to 6. In high concentrations of bisulphite (> 1M) pH5-6, formation of the 5, 6, dihydrocytosine-6-sulphonate facilitates nucleophilic substitution at the exocyclic amino group, allowing the conversion of 5, 6 dihydrocytosine-6-sulphonate to 5, 6 dihydrouracil-6-sulphonate. The bisulphite moiety may then be removed from 5, 6 dihydrouracil-6-sulphonate by treatment with mild alkali¹⁰.

No other major bases in tRNA react with bisulphite under these conditions, but modification of N⁶-isopentenyladenosine¹¹ and 5-methylcytosine⁷ has been observed. The reaction of cytidine residues has been¹² shown to be strongly inhibited by secondary structure in polynucleotides. Consequently this reaction has been used as a probe of cytidine residues in single-stranded regions of tRNA¹¹⁻¹⁶. In these earlier studies, no attempt was made to measure the rates of conversion of the individual reactive cytidine residues to uridine, although such kinetic studies have been made for cytosine,^{12, 17} cytidine and polycytidylate.

Here we present such a kinetic study for the three reactive cytidine residues in E coli tRNA^{Phe}₂. A new reaction of bisulphite with 2-methylthio-N⁶-isopentenyl adenosine (ms^{2,6} A) adjacent to the anticodon of E coli tRNA^{Phe} is also reported.

MATERIALS AND METHODS

Materials

Soluble (³²P) ribonucleic acid from E coli K12 CA265 and all other radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks. Mixed transfer RNA from E coli K12 CA265 was supplied by the Microbiological Research Establishment, Porton, Salisbury, Wilts. Purified tRNA^{Phe} (E coli M.R.E.600) and benzoylated DEAE cellulose were obtained from the Boehringer Corporation.

E coli phenylalanyl - tRNA synthetase was prepared from E coli MRE 600 cells, obtained from the Microbiological Research Establishment, by the method of Stulberg¹⁸, the hydroxylapatite chromatography stages being omitted.

Pancreatic ribonuclease was purchased from the Worthington Biochemical Corporation and ribonuclease T₁ from Calbiochem Ltd. Sodium bisulphite was from Sigma Biochemical Co. Ltd. and RPC-5 was from Miles Laboratories.

Electrophoresis was carried out on cellulose acetate strips, obtained from Oxoid Ltd., and DEAE - cellulose paper (DE 81) from Whatman Biochemicals Ltd. Kodirex X-ray film was obtained from Kodak Ltd.

Aminoacylation of tRNA^{Phe}

The reaction mixture (0.15ml) contained 100mM Tris-HCl pH7.5, 10mM MgCl₂, 10mM KCl, 10mM NH₄Cl, 4mM reduced glutathione, 2mM ATP, 6.7μM L-Phenyl (2, 3-³H) alanine (1-5Ci/mmol), 20μg purified phenylalanyl-tRNA ligase and tRNA containing up to 300pmoles of tRNA^{Phe}. The mixture was incubated at 37°C for 20 minutes. Aliquots were pipetted onto 2.5cm Whatman 3MM filter discs, added to 10% cold trichloroacetic acid, washed

three times in 5% trichloroacetic acid, once in ethanol and dried. The ^3H -phenylalanine was solubilised in 0.5ml 10% hyamine hydroxide (60°C, 20 minutes) and counted in 0.5% (w/v) PPO/0.03% (w/v) POPOP/toluene scintillation fluid.

Purification of *E. coli* tRNA^{Phe}₂

E. coli (^{32}P) tRNA^{Phe}₂ was obtained by chromatography of *E. coli* (^{32}P) mixed tRNA on benzoylated DEAE-cellulose¹⁹ and RPC-5²⁰ (Pearson *et al.*, 1971). *E. coli* tRNA^{Phe}₂ was the second species of tRNA^{Phe} eluted from a benzoylated-DEAE cellulose column on application of a series of gradients of increasing sodium chloride concentration. The purified tRNA^{Phe}₂ was found to be capable of accepting 1200-1400 pmoles per A₂₆₀ unit of tRNA.

Unless otherwise stated, an equimolar amount of unlabelled mixed tRNA carrier was added prior to ethanol precipitation of the purified (^{32}P) tRNA^{Phe}₂. This prevented the loss of phenylalanine accepting activity observed on storage of purified *E. coli* tRNA^{Phe}₂, while increasing total Phe acceptance by only 1-2%.

Modification of tRNA^{Phe} with bisulphite

^{32}P -labelled tRNA^{Phe} (5-20µg, 0.1-0.2µCi per ml) was incubated at 25°C/3M-sodium bisulphite pH6.0 containing 10mM-MgCl₂²¹ for the time indicated and then treated as previously described.

Specific Modification of 2-methylthio-N⁶-isopentenyl adenosine in tRNA^{Phe}

Carrier-free ^{32}P -labelled tRNA^{Phe} (2-10µg; 0.1-0.2µCi), mixed with 40µg of unlabelled tRNA^{Phe} (1.2nmol Phe accepted per A₂₆₀) was incubated at 37°C with 200µl ^{35}S -labelled 1M-sodium bisulphite pH7.0 (1.5 mCi/mmol) for 24h and then treated as described above. The amount of ^{35}S in T₁ oligonucleotide spots was calculated from the ratio of ^{35}S : ^{32}P counts after correction for 11% ^{32}P flowover into ^{35}S , decay and counting efficiency of ^{35}S and ^{32}P . Only one T₁ spot was labelled giving a ratio of ^{35}S : ^{32}P counts of approximately 1:3-suitable for reasonably accurate measurement.

Measurement of position and extent of modification with bisulphite

Pancreatic and T₁ ribonuclease fingerprints were prepared by the method of Sanger *et al.*²² and their nomenclature for presentation of nucleoside 3' monophosphate and nucleotide sequences is used. Each oligonucleotide was characterised by alkaline hydrolysis and electrophoresis on Whatmann 52 paper at pH3.5 and, when necessary, by exonuclease or endonuclease digestion and electrophoresis.

The relative molar yield (y) of each oligonucleotide was determined from its ^{32}P content (x) relative to the total ^{32}P content of the fingerprint (Σx). Thus for an oligonucleotide of chainlength, n, in tRNA^{Phe}

(76 nucleotides) the relative molar yield $y = \frac{76x}{nZx}$.

RESULTS

(a) Sequence analysis of untreated tRNA^{Phe}

In order to establish the chemical purity of the ³²P-tRNA^{Phe} and provide a reference for investigation of the sites and extents of modification by bisulphite, unmodified ³²P-tRNA^{Phe} was fingerprinted, the oligonucleotides analysed and their molar yields determined. The results obtained (Fig. 1; Fig. 2; Table 2, column 1) are consistent with the sequence determined by Barrell and Sanger²³ but cannot be reconciled with that originally proposed by Uziel and Gassen²⁴. On no occasion did we detect GGAs⁴U or As⁴UAG in the fingerprints but only the corresponding GGAU and AUAG.

Site and extent of modification of E coli tRNA^{Phe}₂ with 3M sodium bisulphite

Purified ³²P-labelled E coli tRNA^{Phe}₂ was modified as described in methods. Aliquots were removed over a period of 48 hours, dialysed and fingerprinted. Ribonuclease T₁ fingerprints of tRNA^{Phe}₂ samples removed before and 8^{hours} and 48 hours after addition of bisulphite are shown in Fig. 1.

On comparing these fingerprints, it can be seen that over a period of 48 hours of bisulphite modification three oligonucleotide spots (DCG, CACCA_{OH} and AAm^{2,6}s i AA\CCCCG) were lost from the T₁ RNase fingerprints, indicating modification of one or more bases in these three oligonucleotides. Four new oligonucleotide spots appeared on T₁ fingerprints on bisulphite modification (spots 19, 20, 21, 22), one of which (spot 19) was lost as bisulphite modification proceeded.

Inspection of the pancreatic RNase fingerprints shown in Fig. 2 show that, after bisulphite modification, two oligonucleotide spots (AC and GAAm^{2,6}s i AA\ψ) disappeared and two new spots (Nos. 19 and 20) appeared.

The nucleotide composition of the new oligonucleotides (Table 1) show that spots 19 and 20 do not contain G. This indicated that they had been derived from the 3' terminal T₁ oligonucleotide C₇₂ACCA_{OH} by bisulphite modification of one or two cytidine residues. The oligonucleotide GGGC₇₂ remained undiminished in pancreatic RNase fingerprints of tRNA^{Phe} modified for 48 hours. Therefore the two modified residues at the 3' terminus were C₇₄ and C₇₅, not C₇₂. Modification of C₇₄ was confirmed by loss of AC and production of AU (spot 19) in pancreatic RNase digests of the modified tRNA. Finally, pancreatic RNase digestion of T₁ spot 19 from

Figure 1. T_1 RNase fingerprints of *E. coli* tRNA^{Phe}, unmodified (top left) or bisulphite modified. Key (bottom right) identifies sequences unchanged (closed circles) or lost (broken circles) on modification. Spots which appeared on modification (numbered, shaded circles) are identified in the text.

Figure 2. Pancreatic RNase fingerprints of *E. coli* tRNA^{Phe} unmodified (top left) and bisulphite modified. Key (bottom right) identifies sequences unchanged (closed circles) or lost (broken circles) on modification. Spots which appeared on modification (numbered, shaded circles) are identified in the text.

Table 1

New oligonucleotide		Nucleotide composition				
RNase T ₁ Spot No.		A ⁺	C	G	U*	N
19		1.00	2.35	-	1.28	-
20		1.00	0.91	-	2.00	-
21		-	-	1.00	1.81	-
22		2.74	3.93	1.00	1.17	0.75
Pancreatic RNase Spot No.						
19		3.22	-	1.20	1.00	0.70
20		1.33	-	-	1.00	-

Composition of new RNase T₁ and pancreatic RNase oligonucleotides produced on bisulphite modification of E coli tRNA^{Phe}₂. Oligonucleotides produced by RNase T₁ digestion were assumed to contain one C or a 3' terminal sequence and those produced by pancreatic RNase digestion, one pyrimidine. Dashes indicated nucleotides were present in relative amounts of less than 0.25. N is the nucleotide produced by bisulphite modification of ms^{2,6}iA. The results are mean values of at least three determinations. *Including Ψ, rT, D; †excludes 3' terminal A appearing as unlabelled nucleoside.

an 8 hour modified sample produced a mixture of Cp, A-Cp, A-Up and Up which indicated this spot contained 65% CACUA_{OH} and 35% CAUCA_{OH} arising from modification of C₇₅ and C₇₄ respectively.

Loss of T₁ spot 17 (A_{ms}^{2,6}iAA^{2,6}CCCCG) and the pancreatic RNase spot 15 (GA_{ms}^{2,6}iAA^{2,6}) suggested that ms^{2,6}iA was modified. This was confirmed by analysis of the new T₁ spot (22), which was identical to that found for T₁ spot 17 except that a new nucleotide, X, of Ru²⁵ = 1.14 was present and the spot corresponding to ms^{2,6}iA (Ru = 0.42) was absent. Similar results were obtained on analysis of spots 20 and 15 from the pancreatic ribonuclease fingerprints.

(b) Extent of bisulphite modification with time

From the T₁ fingerprints obtained after modification of tRNA^{Phe} for 8 different times, the molar yield of each oligonucleotide was determined (Table 2). Within experimental error all residues except DCG, CACCA_{OH} and A_{ms}^{2,6}iAA^{2,6}CCCCG remained unaffected by the reagent. The rates of loss of these three residues and appearance of their reaction products is shown in Fig.3. It may be seen that loss of C₁₇ follows pseudo-first order kinetics with a half-reaction time of 2.3 hours (inset Fig.3(b)). Similar kinetics are observed for the reaction of ms^{2,6}iA (inset Fig.3(c)) 9.5 hours modification causing half reaction.

From Fig.3(a) it is clear that the reaction of CACCA_{OH} is of the

Figure 3. Loss and appearance of oligonucleotides in RNase T₁ digests of *E. coli* tRNA^{Phe}₂ on bisulphite modification. Numbering of spots is as for Fig.1. Insets show semi-log plots for disappearance of spots, the time of half reaction indicated by arrows. In (a), loss of both CACCA_{OH} (O-O) and (CACCA_{OH} + CA(C,U)A_{OH}) (Δ-Δ) are shown.

Table 2

Oligonucleotide	Percentage Yield of Oligonucleotide								
	Before add- ition of bisulphite	Time after addition of bisulphite (hours)							
		0	$\frac{1}{2}$	$1\frac{1}{2}$	$3\frac{1}{2}$	$5\frac{1}{2}$	8	24	48
G + G!	102	110	115	96	112	107	93	90	117
AG (2)	128	133	133	121	120	117	115	127	123
CAG	97	109	87	99	88	96	102	86	105
CCCG	86	94	85	99	86	96	102	87	101
pG + pG!	90	86	76	92	96	85	82	77	88
DAG	101	119	115	113	117	120	116	107	104
UCCG	105	109	110	106	104	104	110	99	112
CUCAG	83	87	79	81	93	82	92	80	90
AUAG	52	60	53	50	59	54	60	50	62
T ¹ CG	131	136	128	128	128	123	125	112	130
A ¹ UG	123	132	122	120	130	115	125	127	130
AU ¹ CCG	117	110	105	100	117	111	107	113	114
Um ⁷ GXCCUUG	87	90	76	80	83	80	80	85	87
CACCAOH	96	93	93	48	39	45	24	0	0
19	0	0	20	-	22	-	18	13	0
20	0	0	0	9	15	27	-	78	89
DCG	124	120	94	78	54	25	20	14	2
21	0	0	17	39	65	97	86	126	126
AAms ^{2,6} i AA CCCCCG	86	88	72	74	66	50	42	18	11
22	0	0	16	16	26	45	50	67	90

Percentage yields of oligonucleotides produced by RNase T₁ digestion of *E. coli* tRNA^{Phe}₂ at various stages of bisulphite treatment. - indicates not measured. The results are the mean of two separate bisulphite modification experiments.

consecutive type

The T₁ spot 19 containing the isomeric sequences CA(C,U)A_{OH} generally occurred in too low a yield to accurately determine the degree of individual reaction of C₇₄ and C₇₅ with time of reaction. The preponderance of CACUA_{OH} over CAUCA_{OH} in the 8 hour sample may have arisen either by faster reaction of C₇₅ at the unmodified 3' terminus (K₁ > K₂) or by preferential modification of C₇₄ over C₇₅ in the isomeric CA(C,U)A_{OH} (K₄ > K₃). From our data we were unable to distinguish between these alternatives. However half the 3' terminus is at least partially deaminated in 3.25 ($\pm$ 0.5) hours (Fig. 3 inset) which corresponds to the fastest possible reaction of C₇₄ or C₇₅. Since half the molecules are fully modified after 7 hours (Fig. 3(a) inset), the slowest rate of reaction of C₇₄ or C₇₅ is t_{1/2} = 7 hours.

(c) Selective modification of $ms^{2,6}iA$ by bisulphite

It has been observed by earlier workers that the specificity of the bisulphite reaction is markedly pH-dependent. At pH7.0 there is no deamination of accessible cytidine residues although the alkali-labile 5, 6 dihydrouridine-6-sulphonate adducts are formed as well as a more stable addition product with N^6 -isopentenyl adenosine. To investigate bisulphite reaction with the chemically similar $ms^{2,6}iA$, E coli tRNA^{Phe} was modified with LM-sodium ^{35}S -bisulphite (pH7.0; 37°C) as described in methods.

From these ribonuclease T_1 fingerprints (Fig.4(a), (b)) it may be seen that only spot (17) was diminished and only one new spot (No.22) appeared,

Figure 4. RNase T_1 fingerprint of E coli tRNA^{Phe} modified for 24 hours in LM-bisulphite pH7.0 at 37°C (left). The key (right) identifies sequences, the spot lost (broken circle) and appearing (shaded circle) on modification.

no reaction of C_{17} , C_{74} and C_{75} occurring under these conditions. Similarly in the pancreatic ribonuclease fingerprint (not shown) the only change was loss of $GAms^{2,6}iAAψ$ with concomitant appearance of one new spot. Analysis of these two new spots showed them to be the same as the corresponding oligonucleotides T_{122} and $Panc19$ in tRNA^{Phe} modified with 3M bisulphite pH6.0 at 25°C (Figs.1 and 2). The only ^{35}S -labelled T_1 spot was No.22 ($AAANAψCCCCG$). The calculated 0.76 moles $[^{35}S] HSO_3^-$ was added to $ms^{2,6}iA$

during modification.

DISCUSSION

The only cytidine residues in *E. coli* tRNA^{Phe} which undergo deamination on reaction with bisulphite at 25°C are C₁₇, C₇₄ and C₇₅. These findings, summarised in Fig.5, are in complete agreement with the reactivities one would predict for *E. coli* tRNA^{Phe} of a tertiary structure based on that

Figure 5. The sites and rates of bisulphite modification of residues in *E. coli* tRNA^{Phe}.²³ The tertiary interactions known for Yeast tRNA^{Phe} are included. Evidence for reverse Hoogsteen pairing of S⁴U8-A14 in *E. coli* tRNA^{Phe} has been reported²⁶.

determined for yeast tRNA^{Phe}. Each of the reactive cytidine residues are exposed in such a structure. The remaining cytidine residues are in helical stems or are hydrogen-bonded to other (semi-) invariant bases. Thus C₄₈ in forming a reverse Watson-Crick base pair with G₁₅ is not reactive even though its exocyclic amino group is not directly involved in bonding to G₁₅. The 5, 6 double bond of C₅₆ is very exposed and subject to partial electrophilic iodination²⁷. However C₅₆ is hydrogen-bonded to G₁₉ and is not deaminated by bisulphite.

The reaction of ms²i⁶A though not reported previously, was not totally unexpected since the chemically similar i⁶A, located in a similar position in Yeast tRNA^{Tyr} has been shown to be modified by anti-Markownikoff addition of the sulphite radical to the unsaturated side-chain²⁸. Our findings of a

new product, $R_u = 1.14$, containing one bisulphite molecule per residue, is consistent with a product analogous to that found by Hayatsu et al²⁸, although no attempt was made to establish the free radical nature of the reaction or to exhaustively analyse the product.

It is establishing the pseudo first order nature of the kinetics of the bisulphite reaction with accessible residues in tRNA that our results are perhaps most significant. Although chemical reactivity data is generally in good agreement with the established tRNA structure, its interpretation in structural terms has many potential pitfalls. If a suitably mild reagent is used and its inherent reactivity to monomeric residues is known, it must still be established that modification of one residue does not change the macromolecular conformation, so affecting the reactivity of other residue of the reagent. Such a slow conformational change would show itself in anomalous kinetics of modification. Kinetic studies have been made of the reaction of carbodiimide with E coli tRNA^{Tyr}²⁹ and tRNA^{fMet}³⁰ where rates of reaction were generally first order with respect to remaining unmodified uridine and different for each residue. More unusual kinetics were observed for reactive guanosine residues.

In the work reported here, all reactive cytidine residues were modified at rates proportional to the remaining unmodified cytidine. Under the conditions used the time of half reaction of PolyC was 4.3 hours¹². The analysis of rates show that C₇₄ and C₇₅ react at rates within the range $t_{1/2} = 3.25$ to $t_{1/2} = 7.0$ h i.e. similar to PolyC.

Residue C₁₇ in E coli tRNA^{Phe} reacts considerably more rapidly than residues in PolyC. In yeast tRNA^{Phe} residue 17 is unstacked but since this is a dihydrouridine it is not possible to extrapolate this finding to the sequentially equivalent residue in E coli tRNA^{Phe} although both tRNAs have the same size loop II ($D4V5$; $\alpha = 2$; $\beta = 1$ of Kim et al³¹). Indeed it is possible to stack residues 17 and 60 by changing the position of only residue 17. However since hydrophobic stacking occurs in PolyC^{32, 33} it is tempting to speculate that the higher reactivity of C₁₇ in E coli tRNA^{Phe} is because it is not stacked, having a position similar to that of D17 in Yeast tRNA^{Phe}.

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